

**ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ**  
**Institut des neurosciences cellulaires et intégratives (INCI) UPR3212**

**THÈSE** présentée par :

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soutenue le : **13 septembre 2024**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline : Neurosciences

**A molecular toolbox to study the  
pleiotropic functions of phosphatidic  
acid in neurosecretion**

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# Acknowledgements

First and foremost, I would like to thank the members of my thesis jury: **Sylvie Friant**, **Maité Montero-Hadjadje**, **Gérard Lambeau** and **Ludger Johannes** who have gracefully agreed to take the time to evaluate my work.

Having performed my thesis at the Institute of Cellular and Integrative Neuroscience (INCI UPR3212), I would like to thank the institute's director **Michel Barrot**, together with, **Pascal Darbon**, director of the UAR3156, for enabling me to perform this work in their institute.

Additionally, I would like to thank members of my thesis follow-up comity: **Yannick Goumon** and **Hubert Schaller** for the interesting discussions and guidance they have provided during my thesis.

I would like to offer my most sincere thanks to the people who accompanied me every day during these last four years. All of them are not only great scientists without whom I would certainly never have been able to perform all this work, but I am also especially grateful for the human interaction I was able to have with all those amazing people:

**Nicolas Vitale**, my thesis director, who guided me during my first steps in the world of science. I was especially struck by your open mindedness in approaching science, you never were reluctant in listening to my weird ideas, and always encouraged me to push them further. Additionally, you enabled me to perform my thesis in an environment where I was just restrained by my imagination and could explore everything I wanted. I am conscious that such a luxury can be rare in the scientific world, and I hope that I will be able to continue working with such freedom. To me, you were the perfect thesis director, thank you!

**Stéphane Gasman**, co-director of the membrane traffic in the nervous and neuroendocrine system research group, I am especially grateful to you for providing guidance in my future career. Moreover, without your critical and down-to-earth advice, I would certainly still be performing experiments by now.

**Sylvette Chasserot-Golaz**, who initiated me to the beautiful world of optical microscopy. I am also especially grateful for the numerous interesting discussions we had on membrane contact sites and cell biology in general.

**Sebahat Ozkan**, who taught me how to perform molecular cloning at the start of my thesis and as always available to answer all my molecular biology related questions.

**Stéphane Ory**, for his original initiation in image analysis and interesting discussions on biophysical aspects of cellular biology.

**Michael Okafor**, my old desk neighbor, for his introduction in cell culture and maintenance, but also for the numerous discussion we had along the way. I am sure that you will become a great scientist someday and I hope that we will be able to work together again!

**Marie-France Bader**, for her pertinent advice in chromaffin cell culture. Without them, I would have certainly never been able to perform my work on such an original and relevant model.

**Micol Vicari**, whom I was lucky enough to supervise during her master's internship. You were always motivated and involved in your work. I am sure that you will find great success in your future career and life.

**Laura Streit**, for teaching me how to perform carbon fiber amperometry and spike analysis. I wish you the best in your future start-up adventures.

**Emeline Tanguy**, for showing me initially how to perform western blots and the interesting discussions we had about phosphatidic acid. I am sure that your early career as a professor will continue to flourish in the years to come.

**Frédéric Doussau**, for showing me how to perform organotypic cerebellum slices, and the interesting discussion about synaptic transmission.

**Bérengère Joffre**, even though you started your thesis a year after me, we were able to evolve together for three years. I am certain that if you continue like that, everything will work out in the end.

**Claudine Boissier**, for her helpfulness and providing support for shipments and ordering material.



I also thank all the other current and old members of the membrane traffic in the nervous and neuroendocrine system research group: **Petra Tóth, Olga Gubar, Marguerita Caputo, Alice Basile, Jason Ecard** and **Anne-Marie Haeberlé**.

**Julie Quiri, Yaëlle Guedj** and **Cédric Klipfel** for their administrative support, I am sure that you helped me even more than I know.

**Dennis Wagner**, for his informatic assistance, I wish you the best for your retirement.

I would also like to thank all the people who accompanied me during these eight years of studies: **Robin, Mélanie, Valod, Amira, Florian, Etienne, Baptiste**, and **Fred**. I am lucky to have shared those years with all of you!

Of, course, I also thank all the friends I made in Strasbourg, **Oussama, Pedro, Ziyad, Karina, Meltem, Taptap, Etienne, Jean-Yves** and **Anna**. I will always remember all the game nights and moments spent together.

I would also like to give my most sincere thanks to **Mélanie, Meryll, Flo** and **Ben**. I am grateful to be able to count you among my closest friends and I hope that we will be able to spend more time together in the years to come.

**Schnido**, thank you for all your moral and fluffy support.

I also thank **Daravann, Vutha, Paul, Coco, Surya, Liny, Alice, Léonie** and **Clémence** for their presence and care during the past nine years.

My most sincere thanks also go to my mother, **Annegret Wolf** and her companion **Roland Boehm**, an also my father **Gerhard Wolf** and his wife **Annie Piovan-Gaudenzio**, who enabled me to perform those eight years of study, but also supported me along the way.

I also thank my brother, **Patrick Wolf**. I am especially grateful to have a someone who always encourages me. Sharing those four years of thesis with you was just amazing and I hope that we will be able to exchange scientific ideas. I wish you all the luck for your post-doc and I am sure that you will become an excellent researcher.

Finally, I would like to thank my fiancée, **Ammra**, for being with me since the beginning of my thesis and even long before that. You supported me all along the way, never doubted me and did all you could for me to succeed. I am lucky to have met you and without you I would never have been able to perform all this. **Thank you!**

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# Abbreviations

**ACBD5:** acyl-CoA binding domain 5

**ALPS:** Arf lipid packing sensor

**AP:** Addaptor protein

**Arf:** ADP-ribosylation factor

**ARNO:** ADP-ribosylation factor  
nucleotide-binding-site opener

**BAR:** Bin/Amphiphysin/Rvs domain

**BDNF:** Brain derived neurotrophic factor

**CATCHR:** Complexes associated with  
tethering containing helical rods

**CDP:** Cytidine diphosphate

**CDS:** Cytidine diphosphate  
diacylglycerol synthase

**CEPT:** Choline/ethanolamine  
phosphotransferase

**CERT:** Ceramide transport protein

**CgA:** Chromogranin A

**CGN:** *Cis*-Golgi network

**CIBN:** Cryptochrome interacting basic  
helix loop 1 N-terminal domain

**CK:** Choline-kinase

**CMP:** Cytidine monophosphate

**CoA:** Coenzyme A

**COP:** Cytoplasmic coat proteins

**CPT:** Choline phosphotransferase

**CRY2:** Cryptochrome 2

**CTP:** Cytidine triphosphate

**CuAAC:** Copper(I) catalyzed alkyne-  
azide cycloaddition

**DAG:** Diacylglycerol

**DGAT:** Diacylglycerol-acyl transferase

**DGK:** Diacylglycerol kinases

**DHA:** Docosaheptaenoic acids

**DHAP:** Dihydroxyacetone phosphate

**D $\beta$ H:** Dopamine- $\beta$ -hydrolase

**EE:** Early endosome

**EEA1:** Early endosome antigen 1

**EK:** Ethanolamine-kinase

**ELOVL:** Elongation of very long chain  
fatty acids

**EPA:** Eicosapentaenoic acid

**ER:** Endoplasmic reticulum

**ERGIC:** Endoplasmic reticulum-Golgi  
intermediate compartment

**ESCRT:** Endosomal sorting complex  
required for transport

**E-Syt:** Extended synaptotagmin

**FIPI:** Fluoro-indolyl  
deschlorohalopemide

**FLIM:** fluorescence lifetime imaging

**FRAP:** Fluorescence recovery after  
photobleaching

**FRET:** Förster resonance energy transfer

**F-MMM:** Fluid-mosaic membrane model

**GAP:** GTPase activating proteins

**GDI:** Guanine nucleotide dissociation inhibitors

**GEF:** Guanine nucleotide exchange factors

**GGA:** Golgi-localizing,  $\gamma$ -adaptin ear homology domain, Arf-binding protein

**GPI:** Glycophosphatidylinositol

**GPAT:** Glycerol-3-phosphate acyl transferases

**GUV:** Giant unilamellar vesicles

**G3P:** Glycerol-3-phosphate

**Hrs:** Hepatocyte growth factor-regulated tyrosine kinase substrate

**Hsp70:** 70kDa heat shock protein

**LE:** Late endosome

**LDL:** Low-density lipoprotein

**LPA:** Lysophosphatidic acid

**LPAAT:** Lysophosphatidic acid acyltransferase

**LTP:** Lipid transfer protein

**SM:** Sphingomyelin

**MAM:** Mitochondria associated membranes

**MARCKS:** Myristoylated alanine rich protein kinase C substrate

**MCS:** Membrane contact site

**MTC:** Multisubunit tethering complex

**mTOR:** Mammalian target of rapamycin

**MVB:** Multivesicular body

**M $\beta$ CD:** methyl- $\beta$ -cyclodextrin

**NADPH:** Nicotinamide adenine dinucleotide phosphate hydrogen

**NBD:** Nitrobenzodiazole

**NGF:** Nerve growth factor

**NSF:** N-ethylmaleimide-sensitive factor

**N-WASP:** Neural Wiskott-Aldrich syndrome protein

**ORP:** Oxysterol binding related protein

**OSBP:** oxysterol binding protein

**PA:** Phosphatidic acid

**PABD:** Phosphatidic acid binding domain

**PACAP:** Pituitary adenylate cyclase activating polypeptide

**PAP:** Phosphatidic acid phosphatase

**PC:** Phosphatidylcholine

**PDE4A1:** Phosphodiesterase-4A1

**PE:** Phosphatidylethanolamine

**PEMT:** Phosphatidylethanolamine N-methyltransferase

**PG:** Phosphatidylglycerol

**PH:** Pleckstrin homology domain

**PI:** Phosphatidylinositol

**PIK:** Phosphatidylinositol kinase

**PIP:** Phosphatidylinositol phosphate

**PIS:** Phosphatidylinositol synthase

**PKC:** Protein kinase C

**PLA1:** Phospholipase A1

**PLA2:** Phospholipase A2

**PLC:** Phospholipase C

**PLD:** Phospholipase D

**PM:** Plasma membrane

**PNMT:** Phenylethanolamine N-methyl transferase

**PS:** Phosphatidylserine

**PSS:** Phosphatidylserine synthase

**PTPIP51:** Protein tyrosine phosphatase interacting protein 51

**PX:** Phox homology domain

**RE:** Recycling endosome

**RIM:** Rab interacting molecules

**RIPL:** Rab7-interacting lysosomal protein

**RSK2:** Ribosomal S6 kinase 2

**SCAMP:** Secretory carrier membrane protein

**SCG:** secretogranin

**SG:** Secretory granule

**SPG:** Spastic paraplegia gene

**SM protein:** Sec1/Munc18-like protein

**SNAP:** Soluble N-ethylmaleimide-sensitive factor attachment proteins

**SNAP25:** 25kDa synaptosome associated protein

**SNARE:** Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

**SPAAC:** Strain-promoted alkyne-azide cycloaddition

**StARD3:** StAR related lipid transfer domain 3

**STIM1:** Stromal interaction molecule 1

**STED:** Stimulated-emission-depletion

**TAMM41:** Mitochondrial translocator assembly and maintenance homolog 41

**TGN:** *Trans*-Golgi network

**TRAPP:** Transport protein particle

**t-SNARE:** Transmembrane SNARE

**VAMP:** Vesicular-associated membrane protein

**VAP:** VAMP-associated protein

**VMAT:** Vesicular monoamine transporter

**Vps:** Vacuolar protein sorting

**V-ATPase:** Vacuolar ATPase

**v-SNARE:** Vesicular SNARE





# Introduction

## I. Lipids

Alongside proteins, nucleic acids and carbohydrates, lipids are a fundamental class of molecules that constitute the bulk of living cells. These amphipathic molecules have first been noticed for their inability to mix with water, due to their nonpolar features. They were primarily considered as substrates for energy storage. But, as time goes on, the diversity of lipids together with a better understanding of their properties has prompted researchers to reconsider the functions of lipids within cells. Today, they are not only considered as simple fuel for life, but also as dynamic membrane components, major precursors for fundamental molecules essential for life and crucial signaling molecules. This is reflected by the complex metabolic processes occurring within cells to maintain their lipid composition, or to disrupt it, allowing the proper execution of specific functions.

### A. Definition and classification

Although the different fat molecules known at that time had not been grouped as single class of molecule, Michel Eugène Chevreul, the father of lipid chemistry, defined fats in his famous work entitled: *“Chemical research on fatty substances of animal origin”* as *“substances which burn with a large flame and deposit carbon black, which are soluble in alcohol, and which are not, or very little, in water”*. He also noted that the concept of fats remained very vague and that it was impossible to provide a precise scientific definition of those compounds with his actual understanding ([Chevreul, 1823](#)). In his work, Chevreul also proposed one of the first classification of fats based on their reactions to various treatments, such as temperature variations and acids.

The word *“lipide”* as a general term for *“designating the group of substances that include fats and ether salts (esters) sharing analogous properties (lecithins, phosphatides, etc.)”* was introduced nearly one century later by the French pharmacologist Gabriel Bertrand in his project of chemical biology nomenclature reform ([Bertrand, 1923](#)). The French society of chemical biology voted unanimously this proposition on July 3<sup>rd</sup>, 1923 ([“Bulletin de la Société de chimie biologique,” 1923](#)).

Soon later, Walter Ray Bloor, in his paper *Biochemistry of the fats*, defined lipids as:

- i) Insoluble in water but soluble in fat solvent such as ether or chloroform
- ii) Having an actual or potential relationship to fatty acids when esterified
- iii) Utilized by living organisms

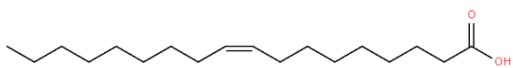
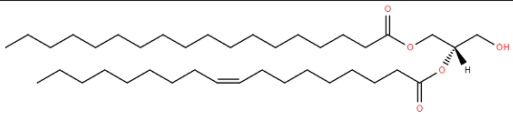
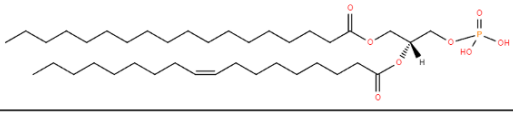
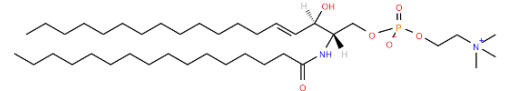
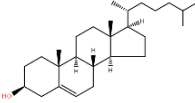
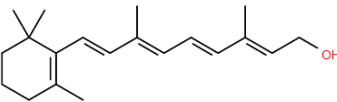
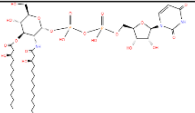
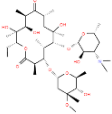
In his introductory paper, Bloor also suggested a revised classification of lipids in three primary categories: simple lipids, involving fatty acid esters with glycerol and other alcohols, compound lipids, featuring fatty acid esters with an additional chemical moiety, like phosphate for phospholipids or nitrogen for glycolipids, and derived lipids comprising compounds derived from the other two classes, such as fatty acids and sterols (Bloor, 1925).

Throughout the 20<sup>th</sup> century, advancements in analytical techniques like column chromatography (Kuhn et al., 1931) and novel extraction methods (Thannhauser and Setz, 1936) enabled the identification of numerous previously unknown lipids. As our understanding of these molecules deepened over time, the term lipid gradually came to encompass a wide and diverse array of compounds. Today, it is still broadly defined as: “Any of a group of naturally occurring fats or fat-like substances characterized by being insoluble in water but soluble in solvents such as chloroform or alcohol” (Cammack et al., 2006).

At the dawn of the 21<sup>st</sup> century, the widespread use of mass spectrometry to study lipids led to an exponential increase in the discovery of lipidic compounds (Murphy et al., 2001). To facilitate international collaboration, the LIPID MAPS consortium proposed a universal method for lipid classification into eight categories (Tab. 1), which is now widely accepted (Fahy et al., 2005):

- **Fatty acids** are fundamental building blocks of other lipids and consist of a polycarbonate chain that confers hydrophobicity.
- **Glycerolipids** include any lipid with a glycerol backbone except for glycerophospholipids. They are composed of a glycerol backbone with one or more acyl chains attached in positions *sn*-1, *sn*-2, or *sn*-3 position. Glycerolipids are mainly found in lipid droplets and primarily known for energy storage within cells. They can also form complexes with carbohydrate groups, creating glyceroglycolipids.

- **Glycerophospholipids** often called **phospholipids**, are the most common and ubiquitous lipids found in living organisms and are the main components of biological membranes. They feature a glycerol backbone and a phosphate headgroup at the *sn*-3 position, to which various molecules can be attached.
- **Sphingolipids**, unlike glycerolipids are defined by a sphingoid backbone with various fatty acids attached in *sn*-1 or *sn*-2 position. The most abundant sphingolipid in mammalian cells is sphingomyelin (SM). Sphingolipids can also associate with carbohydrates, forming sphingoglycolipids such as cerebrosides or gangliosides.
- **Sterol lipids** are defined by four fused carbon rings. Cholesterol is the most common sterol in mammalian cells and, due to its lack of hydrophobic properties, is mostly found embedded in biological membranes or bound to protein carriers.
- **Prenol lipids** are derived from isoprene units and are known to be the precursor of important vitamins such as A, E, and K ([Dam, 1935](#); [Moore, 1930](#); [Sure, 1924](#))
- **Saccharolipids** consist of a sugar backbone linked to fatty acids. They are mostly produced by bacteria and can be converted into components of the lipopolysaccharide barrier of gram-negative bacteria ([Raetz and Whitfield, 2002](#)).
- **Polyketides** are a diverse group of secondary metabolites with lipidic properties, derived from a precursor molecule consisting of a chain of alternating ketone. Produced by bacteria, fungi, plants, and some animals, they have numerous applications such as antibiotics (erythromycins) or anticancer drugs (rapamycin) ([Kibwage et al., 1985](#); [Schwecke et al., 1995](#)).

| Category             | Common name            | Systematic name                                          | Structure                                                                             |
|----------------------|------------------------|----------------------------------------------------------|---------------------------------------------------------------------------------------|
| Fatty acyls          | Oleic acid             | 9-cis-octadecenoic acid                                  |     |
| Glycerolipids        | Diacylglycerol (DAG)   | 1-octadecenyl-2-(9Z-octadecenyl)-sn-glycerol             |     |
| Glycerophospholipids | Phosphatidic acid (PA) | 1-octadecenyl-2-(9Z-octadecenyl)-sn-glycerol-3-phosphate |     |
| Sphingolipids        | Sphingomyelin (SM)     | N-(hexadecanoyl)-sphing-4-enine-1-phosphocholine         |     |
| Sterol lipids        | Cholesterol            | Cholest-5-en-3β-ol                                       |    |
| Prenol lipids        | Vitamin A              | Retinol                                                  |     |
| Sacharolipids        | UDP-2,3-diacyl-GlcN    | UDP-2,3-bis-(3R-hydroxy-tetradecanoyl)-αD-glucosamine    |   |
| Polyketides          | Erythromycin           | N/A                                                      |  |

**Table 1:** The LIPID MAPS classification

Table recapitulating the eight categories of the LIPID MAPS classification with examples. Chemical structures of the different lipids were obtained on [lipidmaps.org](http://lipidmaps.org). Adapted from [Fahy et al., 2005](#).

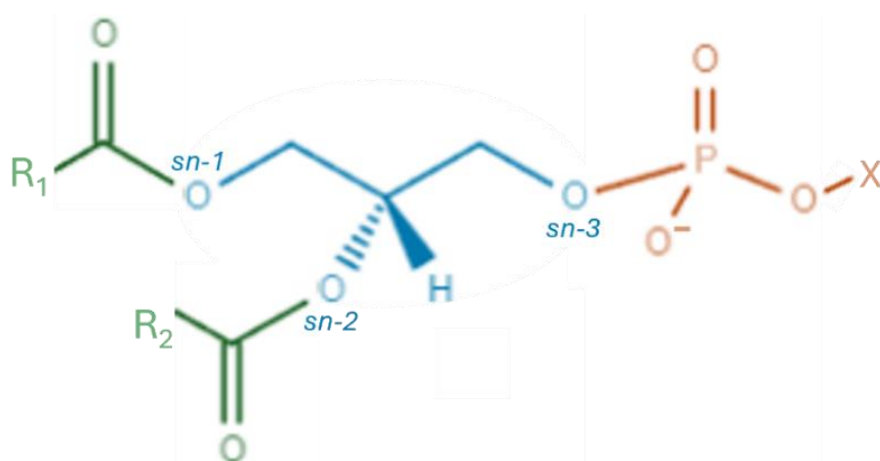
Proteins can also undergo covalent bonding with lipids. These secondary modifications can anchor them into biological membranes or influence their location within the cells. To date, four types of such modifications have been identified:

- Acylation:** involving the addition of an acyl chain to specific amino acids of proteins. Depending on the fatty acid used, this process can be termed myristoylation for myristic acid addition (14:0) or palmitoylation for palmitic acid addition (16:0). Myristoylation targets the N-terminal glycine of a protein, while palmitoylation targets the sulfur of cysteine (S-palmitoylation) or nitrogen residues of lysins and serins (N-palmitoylation) ([Linder and Deschenes, 2007](#)). Additionally, other fatty acids, such as oleic acid (18:1,  $\omega$ -9), can be attached to proteins, as seen with ghrelin ([Kojima et al., 1999](#)).

- **Prenylation:** This modification consists of the addition of an isoprenoid group (farnesyl or geranylgeranyl) to a cysteine, typically located on the CaaX box of the protein's C-terminus ([Wang and Casey, 2016](#)).
- **Lipidation:** This is a general term for the conjugation of a phospholipid to a protein. The most common example is the addition of a glycosylphosphatidylinositol (GPI) anchor to the C-terminus of proteins ([Fujita and Kinoshita, 2012](#)). Other forms of lipidation include the addition of phosphatidylethanolamine (PE) to Atg8 or its mammalian counterpart LC3, which is crucial to initiate autophagy ([Kaufmann et al., 2014](#)).
- **Cholesterol addition:** Although rare, the most notable example is cholesterol attachment to hedgehog proteins, which are involved in embryonic development ([Porter et al., 1996](#)).

## B. Phospholipids

Phospholipids are the primary structural lipids in eukaryotic biological membranes. They are built around a glycerol backbone to which a saturated or cis-unsaturated acyl chain is attached in *sn*-1 and *sn*-2 position through esterification. Additionally, these lipids are characterized by a phosphate headgroup in *sn*-3 position, to which various molecules can attach, resulting in different phospholipids (Fig. 1). To date, 1 769 phospholipids have been curated, and 8 328 have been computationally generated, bringing the total potential phospholipid species to over 10 000 ([Conroy et al., 2024](#)). This vast diversity arises because phospholipids can harbor various side chains and different headgroups.

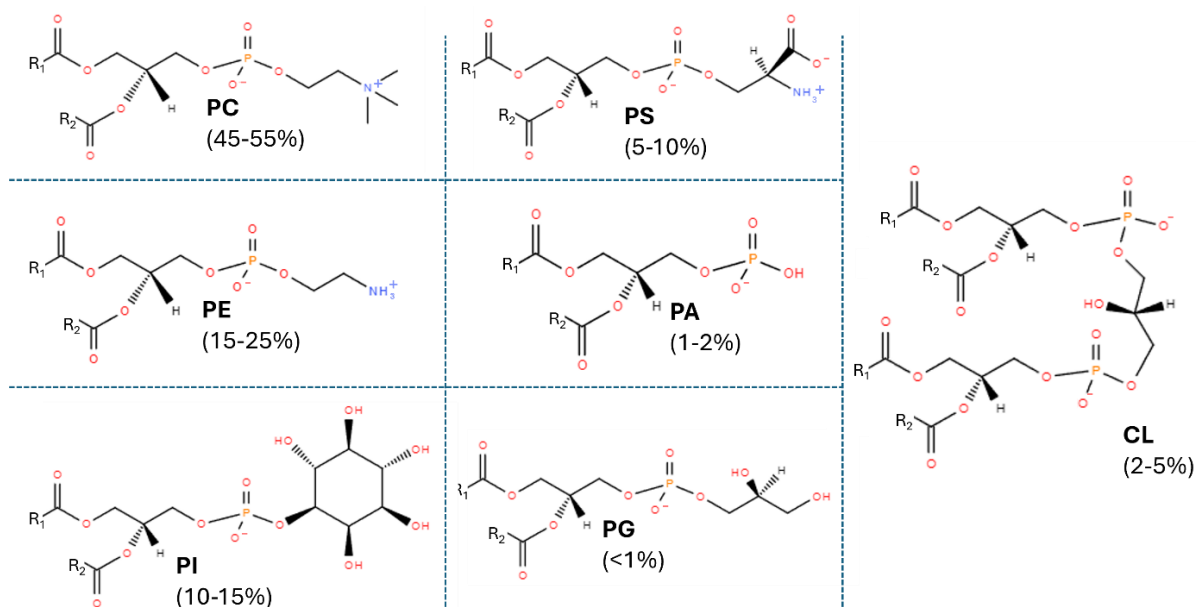


**Figure 1:** Structure of a phospholipid

Structure of a phospholipid orientated around a glycerol backbone (blue) with two acyl chains in *sn*-1 and *sn*-2 position (green), and a phosphate headgroup in *sn*-3 position (red) which can be linked to various molecules. Created with [lipidmaps.org](https://lipidmaps.org)

Seven phospholipids with distinct headgroups are generally described in mammalian cells membranes (ordered by abundance) (Vance, 2015) (Fig. 2):

- Phosphatidylcholine (**PC**) with a phosphocholine headgroup (45-55% of total lipids), which has also been called lecithin in the past.
- Phosphatidylethanolamine (**PE**) with a phosphoethanolamine headgroup (15-25%)
- Phosphatidylinositol (**PI**) with a phosphoinositol headgroup (10-15%)
- Phosphatidylserine (**PS**) with a phosphoserine headgroup (5-10%)
- Cardiolipin (**CL**) with a PG head group (2-5%)
- **PA** with a unique phosphate headgroup (1-2%)
- Phosphatidylglycerol (**PG**) with a phosphoglycerol headgroup (<1%).



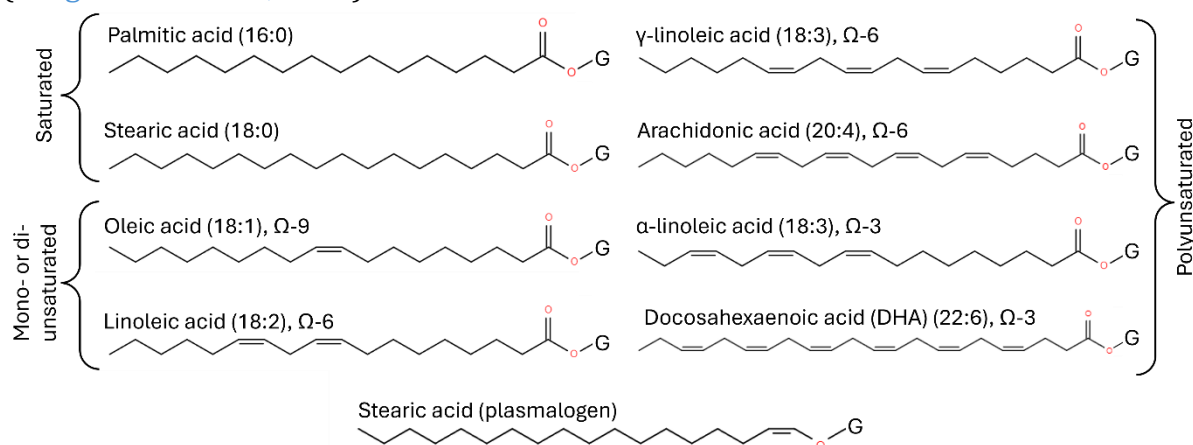
**Figure 2:** Most common phospholipids in mammalian cells

Chemical representations of common phospholipids of mammalian cells at a pH of 7.5. Created on [lipidmap.org](http://lipidmap.org)

In addition to having multiple possible headgroups, phospholipids can also harbor various side chains. Those side chains consist of fatty acids, which can vary in length (typically ranging from 12 to 26 carbons for mammalian cells), in levels of hydrogen unsaturation (generally, from 0 and up to 6 unsaturations, generating *cis*, or Z double bonds in the polycarbonate chain), and in the position of these unsaturations ( $\omega$ -3, -6 or -9 depending on whether the first double bond is situated on the 3<sup>rd</sup>, 6<sup>th</sup> or 9<sup>th</sup> carbon of the chain counting from the distal methyl group) (Rustan and Drevon, 2001) (Fig. 3). Generally, the side chain in *sn*-1 position is saturated, and when phospholipids have an

unsaturated side chain, it is usually found on the *sn*-2 position ([Anderson and Sperling, 1971](#)). However, PE, PS and PC with two docosahexaenoic acids (DHA) side chains (22:6,  $\omega$ -3) have been reported in retinal cells ([Miljanich et al., 1979](#)).

Moreover, in addition to differences in chain length and saturations, the acyl chains of a phospholipid can form an ether bond instead of the typical ester bond with the glycerol core. Depending on the type of bond, two different types of ether lipids can be formed: plasmalogs (Alkyl-ether bond) and plasmalogens (Vinyl-ether bond) ([Braverman and Moser, 2012](#)). This modification typically occurs on the saturated side chain in *sn*-1 position of phospholipids with a polyunsaturated chain in *sn*-2 position. Most of ether lipids found in mammalian cells are plasmalogens and are particularly enriched in certain tissues. For example, they represent more than 20% of the total phospholipids in the brain ([Panganamala et al., 1971](#)).



**Figure 3:** Example of acyl chains that commonly constitute phospholipids

Chemical representations of typical side chains that can be found on phospholipids in mammalian cells (G: Glycerol backbone). Created with [lipidmaps.org](#)

### C. Phospholipid metabolism

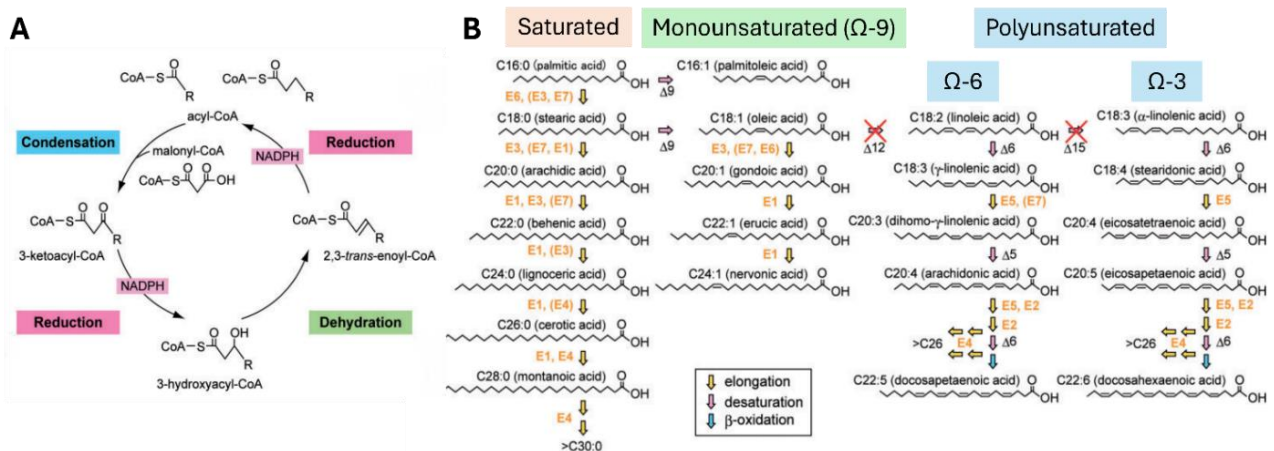
To maintain homeostasis, cells continuously produce new phospholipids, primarily in the endoplasmic reticulum (ER), mitochondria and peroxisomes. This process also consumes fatty acids, which can be synthesized *de novo*, generated from phospholipid metabolism, or provided from food. Additionally, signaling phospholipids can also be produced in various other cellular compartments in response to specific signaling pathways. These signaling lipids can alter the intrinsic properties of membranes, recruit or activate proteins to fulfill their signaling functions.



### 1. *De novo* synthesis of fatty acids

The initial elongation of fatty acid begins in the cytoplasm with a sequence of four successive chemical reactions catalyzed by fatty acid synthetase, a protein complex identical in all mammals (Maier et al., 2008). Starting with acetyl-coenzyme A (CoA), malonyl-CoA is added to the carbon chain, then reduced, dehydrated and reduced again, by consuming two nicotinamide adenine dinucleotide phosphate hydrogens (NADPH). This process adds two carbons to the chain. Through successive addition of malonyl-CoA to the acyl-CoA, palmitic acid- (16:0) or stearic acid- (18:0) CoA is produced (Fig. 4A). Further elongation enabling elongation of the chain with additional carbons can occur in the ER, facilitated by specific elongation of very long chain fatty acids (ELOVL) enzymes (Kihara, 2012) (Fig. 4B).

Un saturations are introduced by desaturase enzymes, which create a *cis* double bond between specific carbons on the chain. A typical unsaturation reaction is for example the formation of oleic acid (18:1,  $\omega$ -9), from stearic acid (18:0), catalyzed by  $\Delta$ -9 desaturase (where  $\Delta$ X indicates the position of the unsaturation counting from the proximal carboxyl functional group). Humans do not express  $\Delta$ -12 and -15 desaturase, therefore, two essential fatty acids (linoleic acid (18:2,  $\omega$ -6) and  $\alpha$ -linolenic acid (18:3,  $\omega$ -3)) must be provided through diet to produce polyunsaturated fatty acids such as arachidonic acid (20:4,  $\omega$ -6), eicosapentaenoic acid (EPA) (20:5,  $\omega$ -3) and DHA (22:6,  $\omega$ -3) after the actions of  $\Delta$ -6 desaturase,  $\Delta$ -5 desaturase and ELOVL2, 5 and 7 enzymes (Kihara, 2012) (Fig. 4B). Additionally, *trans*- or E fatty acids from the alimentation have been reported to inhibit those desaturases, thus affecting the endogenous production of polyunsaturated fatty acids (Choi et al., 2002).



**Figure 4:** *De novo* fatty acids synthesis

(A) Initial elongation of fatty acids by fatty acid synthetase. (B) Further elongation and desaturation of fatty acids, E: ELOVL,  $\Delta$ : desaturase. Adapted from Kihara, 2012.

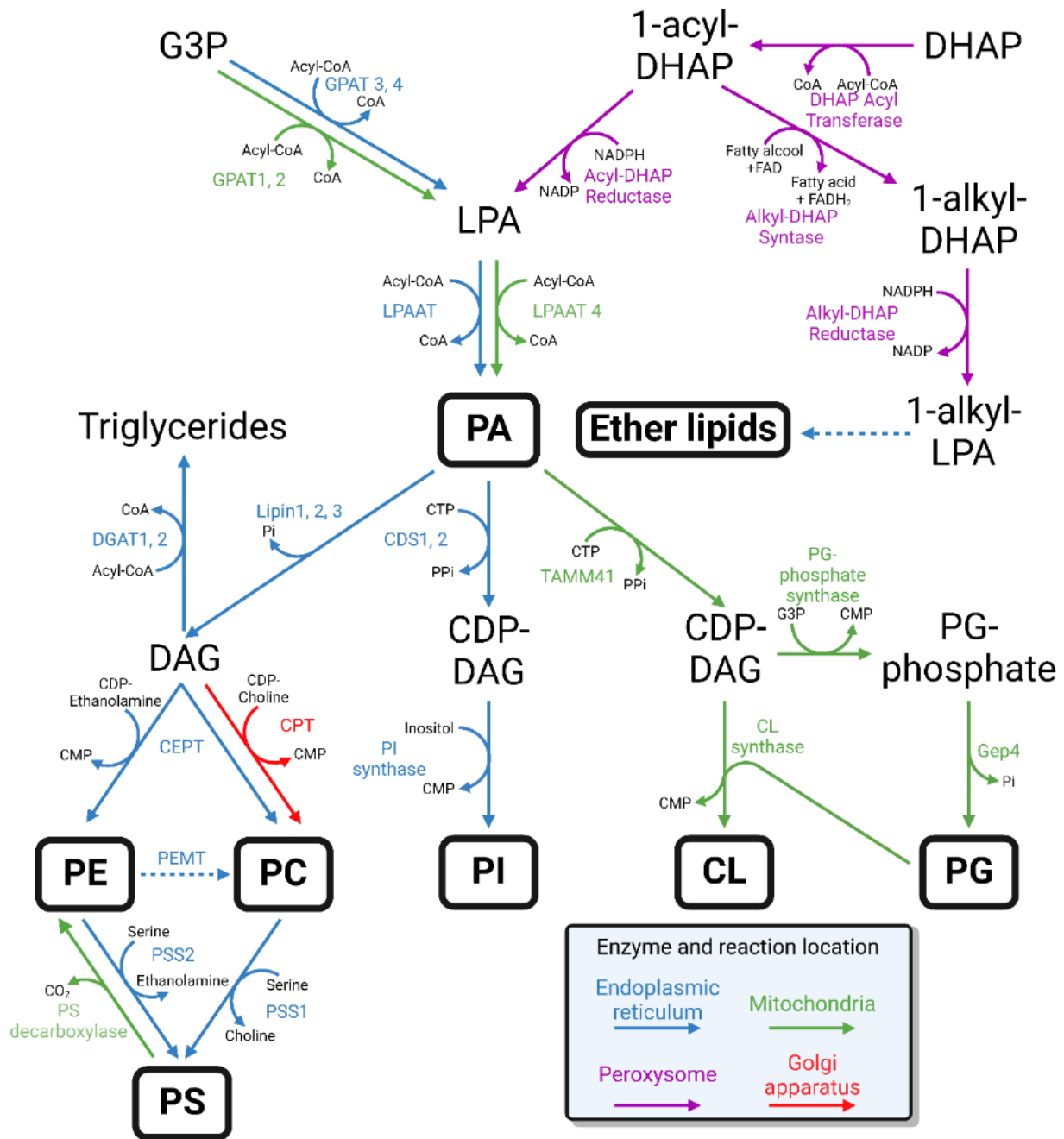
## 2. *De novo* synthesis of glycerolipids

Historically, *de novo* synthesis of phospholipids has primarily been reported to occur in the ER. However, more recent studies revealed that this process also involves other organelles, such as mitochondria or peroxisomes. The first step in producing all glycolipids is the formation of PA. It begins with the acylation of a glycerol-3-phosphate (G3P) backbone at the *sn*-1 position by G3P acyl transferases (GPAT), consuming an acyl-CoA to produce lysophosphatidic acid (LPA). To date, four GPATs have been identified: GPAT3 and 4, which reside in the ER (Cao et al., 2006; Nagle et al., 2008) and GPAT1 and 2, located in mitochondria (Lewin et al., 2004; Yet et al., 2002). GPAT1 preferentially promotes the addition of saturated fatty acids (Kornberg and Pricer, 1953), and no specific acyl chain preference has been noted for the three other GPATs. In peroxisomes, LPA can also be produced by the acylation of dihydroxyacetone phosphate (DHAP) by DHAP acyltransferase (Thai et al., 1997), which is then converted into LPA by acyl-DHAP reductases (Schlossman and Bell, 1976). Additionally, acyl-DHAP can be converted into alkyl-DHAP and subsequently into alkyl-LPA by alkyl-DHAP synthase and alkyl-DHAP reductase, respectively, to produce ether lipids (Bishop et al., 1982). Regardless of its origin, LPA can be converted into PA by the addition of a second acyl chain via LPA acyltransferases (LPAAT). This process primarily happens in the ER, where most LPAATs are expressed, however, LPAATs can also produce PA in other organelles such as LPAAT4 in mitochondria (Bradley et al., 2015) (Fig. 5). Some LPAAT isoforms show preferences for specific fatty acids: LPAAT1 tends to add saturated or monounsaturated fatty acids, while LPAAT2 prefers polyunsaturated ones (Shindou et al., 2009). This newly formed PA is then used in two pathways to produce the other six structural phospholipids: the DAG pathway and the cytidine diphosphate (CDP)-DAG pathway.

PC and PE are produced from PA through the DAG pathway (Kennedy and Weiss, 1956). Their synthesis begins in the cytoplasm with the phosphorylation of choline or ethanolamine by choline/ethanolamine kinase (Aoyama et al., 1998). Phosphocholine and phosphoethanolamine are then activated by cytidyl transferase enzymes consuming cytidine triphosphate (CTP) to replace the phosphate with CDP. On the cytosolic leaflet of the ER, PA is converted into DAG by the three isoforms of lipin (Donkor et al., 2007), which possess PA phosphohydrolase activity. CDP-choline and CDP-ethanolamine are finally added to DAG by choline/ethanolamine phosphotransferase (CEPT) mainly present in the ER. Additionally, a choline phosphotransferase (CPT) enzyme which can produce PC

directly in the Golgi apparatus has also been reported ([Henneberry et al., 2002](#)). PE can also be converted into PC by PE N-methyltransferase (PEMT) in a rapid two-step reaction in hepatocytes ([Ridgway and Vance, 1988](#)). PS is produced by PS synthase (PSS) from either PC or PE, replacing choline (PSS1) ([Kuge et al., 1985](#)) or ethanolamine (PSS2) ([Kuge et al., 1997](#)) headgroup with serine. Interestingly, while this reaction takes place in the ER, in yeast, these enzymes are located at specific ER sites, associated with mitochondrial membranes (MAM) ([Gaigg et al., 1995](#)). Additionally, PS can be converted back to PE in the outer mitochondrial membrane by PS decarboxylase ([Kuge et al., 1991](#)). Triglycerides can also be synthesized from DAG in the ER for energy storage in lipid droplets, a reaction catalyzed by DAG-acyl transferases (DGAT), of which two isoforms have been identified: DGAT1 and DGAT2 ([Cases et al., 2001, 1998](#)) (Fig. 5).

The CDP-DAG pathway is essential for the synthesis of PI, PG and CL. CDP-DAG is formed from PA and CTP by CDP-DAG synthase (CDS). Initially, it was thought that this process occurred exclusively in the ER, where CDS1 and 2 are expressed ([Inglis-Broadgate et al., 2005](#)). Interestingly, CDS1 does not show substrate specificity for PA subtypes, while CDS2 prefers PA containing a stearic acid (18:0) at the *sn*-1 position and an arachidonic acid (20:4,  $\omega$ -6) at the *sn*-2 position ([D'Souza et al., 2014](#)). In the ER, PI is synthesized by PI synthase, which adds inositol to CDP-DAG, releasing cytidine monophosphate (CMP) ([Paulus and Kennedy, 1960](#)). In yeast, PI synthase has been reported to be associated with MAMs ([Fischl and Carman, 1983](#)). Due to the specificity of CDS2, PI often has a typical acyl chain composition with arachidonic acid in *sn*-2 position (18:0-20:4,  $\omega$ -6), this is especially notable in the central nervous system, where most PIs have this composition ([Traynor-Kaplan et al., 2017](#)). PG biosynthesis occurs in mitochondria. CDP-DAG is produced directly from PA on the mitochondrial inner membrane by the mitochondrial translocator assembly and maintenance homolog 41 (TAMM41), which also has CDS activity ([Blunsom et al., 2018](#)). PG is then formed through two steps: CMP is exchanged with G3P by PG-phosphate synthase to produce PG-phosphate, which is then dephosphorylated by the phosphatase Gep4 ([Osman et al., 2010](#)). Finally, CL is synthesized by combining PG with another CDP-DAG molecule, a reaction catalyzed by CL synthase ([Houtkooper et al., 2006](#)) (Fig. 5).



**Figure 5:** *De novo* glycerolipid and ether lipid synthesis

Schematic representation of the pathways of phospholipids synthesis in mammalian cells with the supposed organelle where these reactions take place. Ether lipids are produced from 1-Alkyl-LPA and is very resembling to typical phospholipid synthesis produced from LPA. Created on [Biorender.com](https://www.biorender.com)

Therefore, *de novo* lipid synthesis is a complex interplay between various enzymes and organelles with PC, PE, PS, triglycerides and PI being mostly produced in the reticulum and PG and CL exclusively produced in mitochondria. PA represents a major branching point in this process and determines which phospholipid will be produced. This is dependent on two factors: 1) its conversion to either DAG or CDP-DAG and 2) the organelle where this conversion takes place. However, the question on how exactly the fate of a

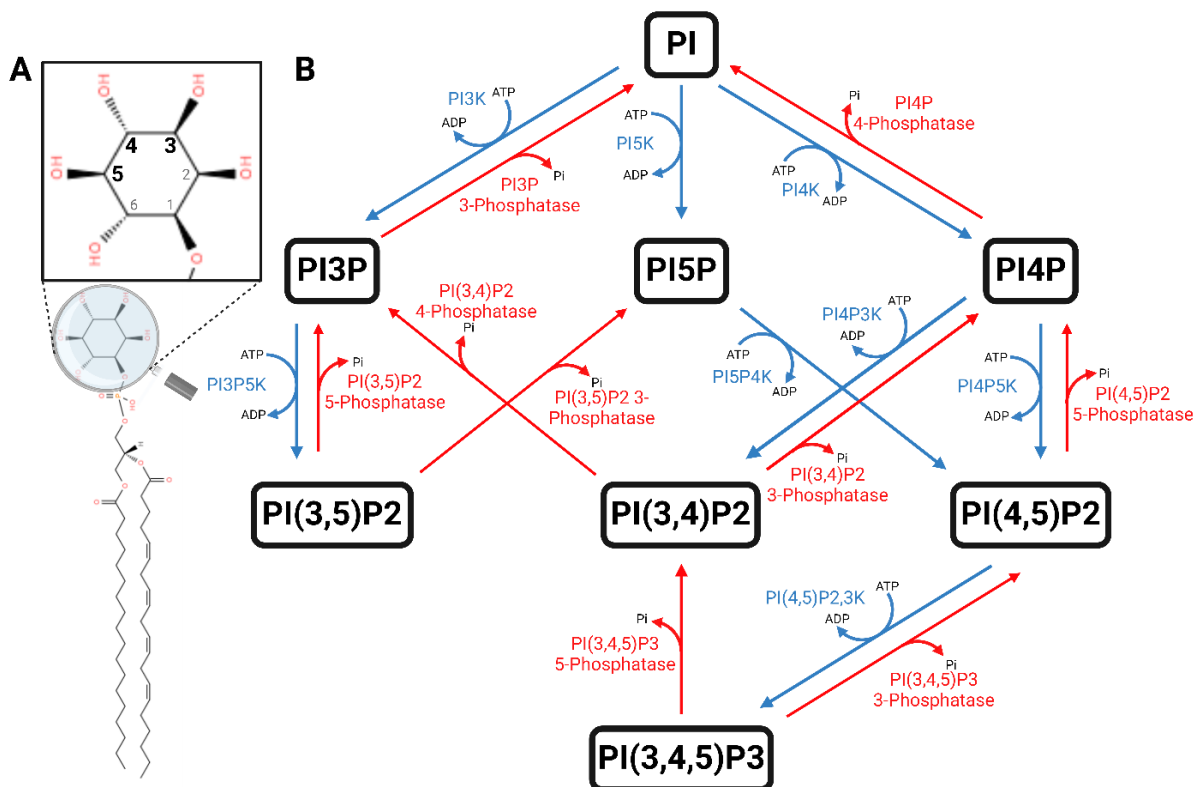
phospholipid is controlled by going down one or the other path directly within cells remains challenging to answer and still needs further investigation.

### 3. Signaling lipids metabolism

Besides being crucial structural components of biological membranes and serving as energy storage, lipids also play a key role in signaling within organisms. These signaling lipids operate at various levels. For instance, steroid hormones, derived from cholesterol in the adrenal cortex, gonads and placenta, send signals throughout the whole body (endocrine signaling) (Holst et al., 2004). Eicosanoids, derived from polyunsaturated fatty acids like arachidonic acid (20:4,  $\omega$ -6), eicosapentaenoic acid (20:5,  $\omega$ -3) or docosahexaenoic acid (22:6,  $\omega$ -3) act on neighboring cells to modulate inflammatory responses (paracrine/autocrine signaling) (Dennis and Norris, 2015). Endocannabinoids, derived from arachidonic acid (20:4,  $\omega$ -6) containing PE, PI or DAG act on presynaptic neurons to regulate their activity (paracrine signaling) (Wang and Ueda, 2009). Additionally signaling lipids can have direct intracellular effects.

PIs are a notable example of intracellular signaling lipids. PI is synthesized in the ER and dispatched to specific organelles. During this process, specific PI kinases (PIKs) can phosphorylate the inositol headgroup of PIs at position 3, 4 and 5 (Fig. 6A), producing seven additional PI-phosphate (PIP) derivatives. PIP phosphatases can also cleave these phosphoester bonds on specific positions creating a complex metabolic network to maintain PIP homeostasis (Posor et al., 2022) (Fig. 6B). Numerous enzymes with PIK or PI phosphatase activity have been identified, many of which act on multiple PIPs and possess multiple enzymatic functions. For example, Sac1 can act on PI3P, PI4P and PI(3,4)P<sub>2</sub> exhibiting 3- and 4- phosphatase activity (Nemoto et al., 2000). Thus, the specific PIP subtype produced depends on the availability of their PIP substrate, but also their subcellular distribution, and the signaling pathways regulating them. The signaling functions of PIPs depend largely on the proteins they are interacting with. Numerous of such interactants have been identified, particularly throughout the discovery of specific PIP binding domains such as pleckstrin homology (PH) domains (Harlan et al., 1994), FYVE domains (Patki et al., 1997), and Phox homology (PX) domains (Xu et al., 2001). Currently, nearly 2000 PIP binding domains have been identified and categorized into over than 60 families (Overduin and Kervin, 2021). Despite this diversity, the binding of a protein to a

subtype of PIP can be highly specific. This specificity has been utilized to create cellular PIP biosensors, such as the FYVE domain of Hrs for PI3P (Gillooly et al., 2000), the PH domain of phospholipase C (PLC)  $\delta 1$  for PI(4,5)P<sub>2</sub> (Stauffer et al., 1998), and the PH domain of ADP-ribosylation factor nucleotide-binding-site opener (ARNO) for PI(3,4,5)P<sub>3</sub> (Venkateswarlu et al., 1998). It also is noteworthy to mention that a single mutation can change the PIP they are interacting with. For example, a single codon polymorphism (Gly-Gly versus Gly-Gly-Gly) in the PH domain of ARNO, changes its the specificity from PI(3,4,5)P<sub>3</sub> to PI(4,5)P<sub>2</sub>=PI(3,4,5)P<sub>3</sub>, respectively (Ogasawara et al., 2000).



**Figure 6:** Phosphoinositide signaling metabolism

(A) Depiction of a typical PI where the three positions which can be phosphorylated on the inositol ring are numerated in bold. (B) Schematic representation of the pathways of PIPs metabolism. Blue pathway leads to phosphorylation of the PI(P) and red ones to dephosphorylation. Created on [Biorender.com](https://www.biorender.com), inspired from Posor et al., 2022.

In addition to PIPs, other phospholipids and DAG also function as intracellular signaling molecules. These molecules are modified by specific enzymes activated in response to certain signaling cascades, primarily through the action of phospholipases. The latter have been classified depending on the chemical bond they are targeting (Fig. 7): Phospholipase A1 (PLA1) and phospholipase A2 (PLA2) cleave the acyl chains at the *sn*-1 and *sn*-2 position, respectively. On the one hand, this process produces lysophospholipids,



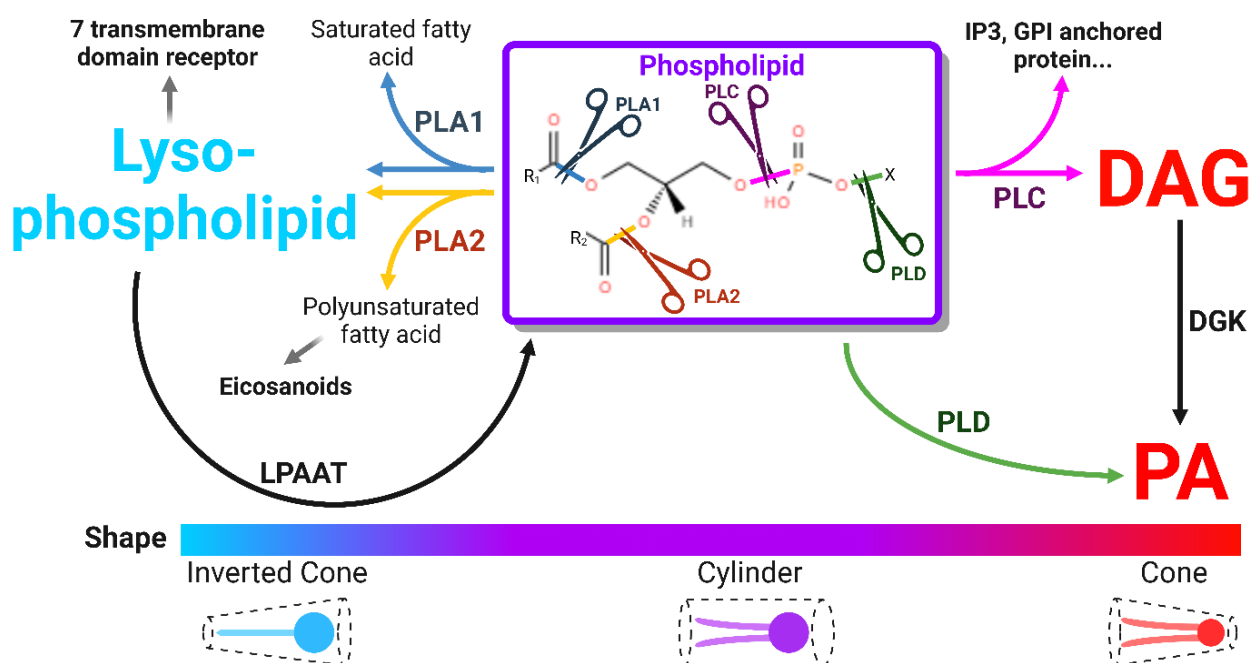
which can activate seven transmembrane domain receptors within the cell ([Pasternack et al., 2008](#); [Sugo et al., 2006](#)). On the other hand, it also releases free fatty acids which can also have signaling functions. For instance, PLA2s are particularly important for generating polyunsaturated fatty acids, which later on can be subsequently converted into eicosanoids to modulate immune responses ([Norris et al., 2014](#)). Interestingly, plasmalogen lipids, which have an ether bond at the *sn*-1 position instead of an ester bond, are resistant to PLA1 activity ([Mawatari et al., 2018](#)). Finally, phospholipase B can cleave both acyl chains successively, combining the activity of PLA1 and PLA2.

PLC cleaves the *sn*-3 bond of the glycerol backbone, resulting in the production of DAG. DAG can then recruit specific proteins with a C1 domain, initially identified in protein kinase C ([Parker et al., 1986](#)). However, the released molecule also has signaling functions, as an example, PLC $\delta$ 1 cleaves PI(4,5)P2 to produce DAG and inositol-3-phosphate, the latter can bind specific channel receptors on the ER to release intracellular calcium stores ([Berridge, 1987](#)). Additionally, PLCs are also known to release GPI-anchored proteins into the extracellular medium at a defined moment ([Müller et al., 2012](#)).

Phospholipase D (PLD) produces PA by cleaving the bond linking the phosphate to a specific head group. Among the 6 PLD isoforms identified by homology of their catalytic domains (His-x-Lys-xxxx-Asp), only the isoform 1, 2, and 6 have been shown to display phospholipase D activity. In mammalian cells, PLD1/2 selectivity use PC as substrate, whereas PLD6 uses CL. PLD1 and PLD2 are the most studied PLD isoforms and produce PA at the plasma membrane (PM) and in membrane compartments of the secretory and endolysosomal pathways ([Tanguy et al., 2021](#)). Like PIPs and DAG, PA can recruit and activate specific proteins expressing a PA binding domain (PABD) such as the mammalian target of rapamycin (mTOR) ([Fang et al., 2001](#)), phosphodiesterase-4A1 (PDE4A1) ([Baillie et al., 2002](#)), and Opi1p or Spo20p in yeast ([Liu et al., 2007](#); [Loewen et al., 2004](#)). These PABDs have also been used more recently as PA biosensors ([Kassas et al., 2017](#)). However, a general PA binding motif has not yet been unveiled, making it difficult to predict which proteins this signaling lipid might interact with to fulfill its functions. Additionally, other metabolic pathways, such as phosphorylation of DAG by DAG kinases (DGK) ([Topham, 2006](#)), or acylation of PLA by LPAATs, like the RIBEYE protein in ribbon synapses ([Schwarz et al., 2011](#)) can also produce signaling PA. Therefore, given the wide range of metabolic substrates these enzymes utilize, different PA subspecies with specific

functions can be produced depending on their acyl chain composition (Tanguy et al., 2018b, 2020b). However, the impact of the acyl chain on the signaling of a given phospholipid has only recently begun to be explored.

In addition to this impact on cell signaling through protein recruitment and by providing key metabolic intermediates, phospholipases can also alter the overall shape of phospholipids and membrane topology. Typically, the most abundant structural phospholipids within membranes, such as PC, PI or PS have a cylindrical shape. However, when these phospholipids are converted into lysophospholipids by PLAs, they adopt an inverted cone shape. Similarly, PA and DAG, produced by PLD and PLC have a conical shape (Lee et al., 1993) (Fig. 7). These changes in lipid geometry induced by the activation of different phospholipases have been proposed to be one of the primary drivers of their cellular actions (Suetsugu et al., 2014), especially in vesicular trafficking (Tanguy et al., 2016).



**Figure 7:** Signaling phospholipid metabolism

Representation of the impact of phospholipases and phospholipid metabolism on lipid shape and signaling. Four types of enzymes can target different areas of phospholipids: PLA1 and 2 respectively cleave the ester bond in *sn*-1 and *sn*-2 position to produce lysophospholipids and free fatty acids, PLC cleaves the phosphodiester bond in *sn*-3 position producing DAG and releasing the headgroup and PLDs cleave the phosphodiester bond linking a specific headgroup to the phosphate producing PA. All those reactions do not only produce many important signaling molecules but also intrinsically change the physiochemical properties of phospholipids altering the properties of biological membranes. Created on [Biorender.com](https://www.biorender.com)

In summary, lipids constitute a broad category of molecules, primarily defined by their hydrophobic properties. Among the various lipids discovered, fatty acid, cholesterol



and phospholipids form the majority of those found in mammalian cells. These many molecules accomplish three essential cellular functions: energy storage, cellular membrane formation, and signaling. Hence, to maintain lipid homeostasis, cells have developed complex metabolic pathways to produce and degrade these molecules as needed. Phospholipids exemplify this diversity and complexity, as they harbor multiple headgroups and various fatty acids. Cells tightly regulate these aspects during lipid biosynthesis by segregating the process within specific organelles (PC, PE, PS and PI are mainly produced in the ER while PG and CL in mitochondria), and though the substrate specificity of lipid metabolism enzymes (some GPATs and LPAATs prefer certain fatty acids).

Although PA represents only a minor fraction of cellular lipids, it serves as a critical branching point in the biosynthesis of all other glycerophospholipids and acts as a major signaling lipid. The cellular location of PA during its biosynthesis appears to determine the specific phospholipid which will be produced later. This complicates the understanding of PA's signaling functions as the cellular effects of interfering with its biosynthesis could be attributed to a loss of PA, or a down product of its metabolism. Hence, to properly unravel PA's signaling functions, given its complex metabolic fates, one would need to be able to control its levels with subcellular precision and refine timing. Additionally, cells heavily emphasize controlling the composition of acyl chains of phospholipids, highlighting the significance of the hydrophobic part of phospholipids in their signaling functions. On the one hand, this can be explained by the biological relevance of polyunsaturated free fatty acids, produced by PLA2s. But on the other hand, the typical acyl chain composition of the most studied signaling lipid, PI, with an arachidonic acid (20:4,  $\omega$ -6) in *sn*-2 position, the enrichment of polyunsaturated phospholipids in specific organs (central nervous system, heart...) and the consequences of a diet lacking  $\omega$ -3 fatty acids (cognitive defects, heart failure...) hint at the importance of the fatty acid composition of signaling phospholipids. Unfortunately, this aspect has been very poorly explored at the cellular and molecular level, until recently, due to a lack of relevant tools.

## II. Biological membranes

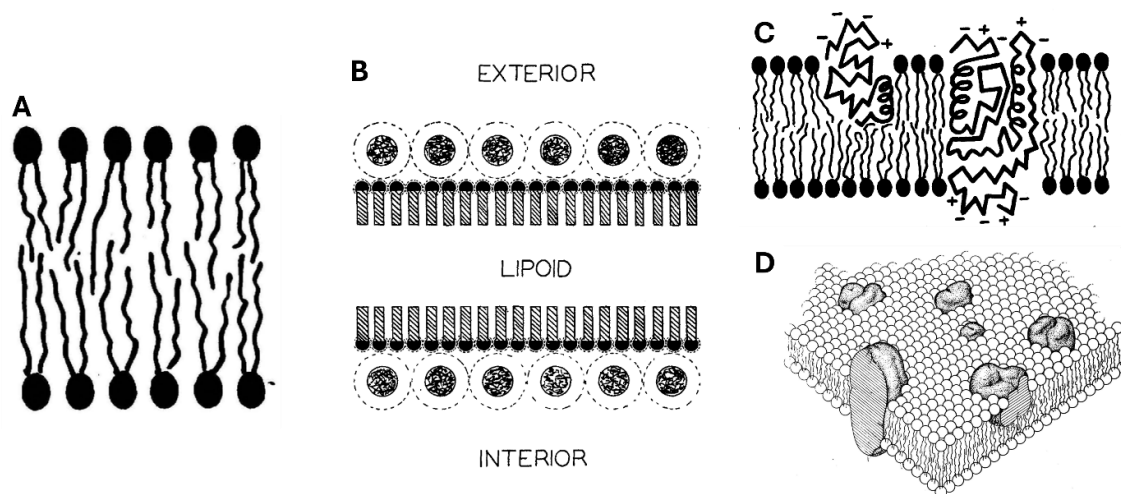
While it has always been considered obvious that cells, the basic unit of living beings, were isolated from the environment by a barrier, their exact nature has been discovered quite late. Lipids, thanks to their hydrophobic properties, have come up as the main building block of those cellular membranes. However, biological membranes are way more than just a barrier, they are dynamic entities, changing over time and linking our understanding of lipid biochemistry with principles of cellular dynamics. During the last century, the evolution of membrane models has been deeply connected to breakthroughs in lipid research. Even nowadays, these models continue to be refined as new insights into lipid- and protein-lipid interactions emerge. Thus, understanding lipid biophysical properties, is essential to fully appreciate their role in biological processes.

### A. Nature of biological membranes

The concept that biological membranes are composed of lipids has been nucleated by Charles Ernest Overton's work in 1895, when he studied the abilities of various compounds to enter cells. He noted that liposoluble molecules can enter cells as fast as ethanol ([Overton, 1895](#)). However, it was only a few years later that Irving Langmuir established the importance of the amphipathic nature of lipids in membrane formation. He studied the surface tension of different oil monolayers on water and observed that all tested lipids occupied the same surface areas, despite significant differences in molecular weights. He postulated that *"organic liquids arrange themselves in such a way that their active portion are drawn inwards, leaving the least active portion of the molecule to form the surface layer"*. This would explain those similarities, as the surface layer, in all experiments, is formed by the same methyl group ([Langmuir, 1917](#)). The fact that biological membranes are composed of a lipid bilayer has been demonstrated by pioneer work of Gorter and Grendel in the 1920's. By working with blood samples from multiple animals, they purified erythrocytes, composed of a unique lipid bilayer and determined the total surface of those cells. Then by extracting lipids of those samples and, like Langmuir's a few years prior, measuring the surface area occupied by those lipids; they noticed that the ratio between the surface of the lipid extract and the surface of the cells is always close to two. They therefore proposed that *"every chromocyte (erythrocyte) is*

surrounded by a layer of lipoids (lipids), of which the polar groups are directed to the inside and to the outside” (Gorter and Grendel, 1925) (Fig. 8A).

Ten years later, Danielli and Davson proposed the first model of lipid bilayers with proteins embedded to explain the selective permeability of cellular membranes to specific ions. They suggested that proteins were adsorbed on both polar sides of the lipid bilayers, imparting specific charges at peculiar regions of the membrane. This would explain the selective permeability of membranes to certain ions based on their charge and size (Danielli and Davson, 1935) (Fig. 8B). This model of cellular membranes has been accepted by the community for more than 30 years until the fluid-mosaic membrane model (F-MMM) was proposed by Singer and Nicolson in 1972 (Fig. 8C and D). Both those scientists postulated a potential relationship between the established structure of biological membranes and “*integrated proteins*” associated with them. Those proteins harbor non-polar domains making them partially embedded in/across cellular membranes. Those amphipathic proteins are therefore able to float and diffuse across the lipid bilayer and are distributed randomly across the surface of a two-dimensional liquid (Singer and Nicolson, 1972).



**Figure 8:** The evolution of biological membrane models

(A) Representation of the basic structure of a lipid bilayer, proposed by Gorter and Grendel (Singer and Nicolson, 1972). (B) Model of biological membranes with proteins adsorbed on both surface of the lipid bilayer proposed by Danielli and Davson (Danielli and Davson, 1935). (C) and (D) cross section and three-dimensional view of the F-MMM proposed by Singer and Nicolson (Singer and Nicolson, 1972).

The F-MMM has since been widely accepted as a base model to describe biological membranes. However, as early as 1976, scientists started to notice that this model was not entirely conform with experimental data. For example, movements of proteins on membranes did not always follow the random patterns initially predicted by the F-MMM

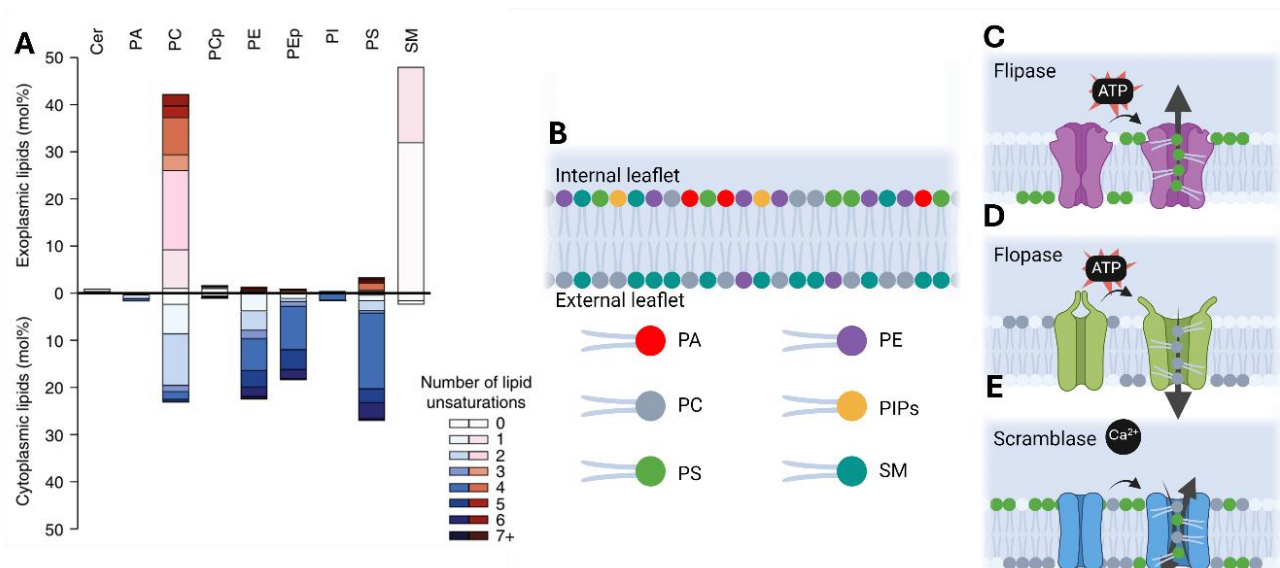
([Nicolson, 1976](#)). It became evident that other features of biological membranes needed to be considered further. Some of those properties were added to the F-MMM model, which has been updated multiple times since its original publication ([Nicolson, 2013](#)).

## **B. Lipid bilayers topological asymmetries**

The concept of lipids being unevenly distributed across each leaflet of the lipid bilayer has first been introduced by Mark S. Bretscher in 1972. He studied the lipid bilayers of red blood cells by using chemical reactions to selectively target specific amino groups of phospholipids (PS and PE). He observed that the staining was low when using chemical reagents which could not diffuse through the membrane. He postulated that PE and PS were predominantly present on the inner leaflet ([Bretscher, 1972](#)). Nowadays, by using various types of additional approaches, such as phospholipid selective enzymes ([Bütikofer et al., 1990](#); [Verkleij et al., 1973](#)), annexin V binding assays ([Dachary-Prigent et al., 1993](#)), and fluorescent lipid probes ([Martin and Pagano, 1987](#)), a more comprehensive view of the phospholipid asymmetrical distribution across the lipid bilayer of most eukaryotic cells has been established. PC and SM are mostly present on the outer leaflet, whereas PA, PE, PS and PIPs are predominant on the inner leaflet of membranes ([Daleke, 2008](#); [Quinn, 2002](#)) (Fig. 9A and B). More recent analyses of red blood cell membrane showed that those asymmetries do not only concern the head groups of lipids, but also their side chains with polyunsaturated phospholipid enriched in the cytoplasmic leaflet ([Lorent et al., 2020](#)). Other types of lipids are also unevenly distributed across the lipid bilayer but have been less studied. This for example is the case for cholesterol which is predominantly found in the inner leaflet of membranes ([Wood et al., 2011](#)).

This asymmetry was initially believed to be intrinsic to biological membranes through a process called "*lipid topogenesis*". Enzymes responsible for phospholipid production are present on one side of the membrane, causing the produced phospholipid to be naturally predominant on that side ([Bell et al., 1981](#)). However, the fact that structural phospholipids are mostly produced on the cytoplasmic leaflet of the ER is in contradiction with this model as it would suggest that most phospholipids remain on this leaflet. In fact, many phospholipids can be exchanged passively from one leaflet to another by a mechanism known as flip-flop, allowing them to spontaneously translocate to the other layer. This process occurs at a relatively slow rate, typically taking several hours for a single lipid ([McConnell and Kornberg, 1971](#)). This translocation speed is dependent on

multiple factors such as the intrinsic charge of the headgroup, and the length and saturation of the side chains ([Middelkoop et al., 1986](#)). For example, it has been reported for example that PE-NBD (18:0-22:6) has a flip-flop half-life of 0.29h against 11.5h for PE-NBD (18:0-18:1) ([Armstrong et al., 2003](#)). Furthermore, it has also been suggested that the binding of a specific protein to a lipid can impede flip-flop. Spectrin, for example, can sequester PS at the inner leaflet of red blood cells membranes thereby contributing to PS asymmetry across the two leaflets ([Mommers et al., 1979](#)). However, this does not explain how saturated phospholipids, like PC, are predominantly present on the opposite leaflet. Consequently, the concept of active lipid transport across membranes has been hypothesized since the 70's ([Bretscher, 1972](#)). This process is energy/ATP dependent, as it implies the transport of specific lipids against their gradient ([Seigneuret and Devaux, 1984](#)). The proteins involved in this transport across membrane leaflets have been named flippases and floppases. They can translocate phospholipids respectively, on the inner leaflet to the outer leaflet of a membrane and from the outer to the inner leaflets ([Faddeel and Xue, 2009](#)) (Fig 9C and D). Finally, a last type of translocation proteins has been described: scramblases, which do not have any described phospholipid head group specificity scramble lipids randomly across the bilayer (Fig. 9E). Scrambling does not consume ATP and is generally induced by an increase in cytoplasmic calcium concentrations in response to specific signaling pathways ([Sims and Wiedmer, 2001](#)).



**Figure 9:** The asymmetric distribution of phospholipids across the bilayer

(A) Prevalence of certain lipids across the bilayer of red blood cells ([Lorent et al., 2020](#)). (B) Schematic representation of phospholipids and sphingomyelin across the lipid bilayer. Created on [Biorender.com](#). (C), (D) and (E) lipid translocation mechanisms across the lipid bilayer representing respectively a flipase (C), floppase (D) and scramblase (E) actions. Created on [Biorender.com](#).

### C. Lateral asymmetries of biological membranes

Alongside topological asymmetries, biological membranes are also subject to lateral asymmetries, meaning that lipids are not distributed equally along the membrane plane. This effect can be attributed to phase transitions within the lipid bilayer. At physiological temperatures, membranes are in a disordered phase, allowing their components to move relatively freely and maintaining high fluidity. However, various factors can locally affect membranes, inducing a transition into an ordered phase, where movements are restricted, and fluidity is low. This local decrease in fluidity induces segregation of specific domains within membranes (Quinn, 2012). Due to the intrinsic lateral movement of lipids across membranes, those domains can form and dissipate quickly, varying in sizes ranging from a few lipids (nanodomains) to large clusters consisting of tens of thousands of lipids (microdomains). Cholesterol is crucial on regulating membrane fluidity. Its effects were first observed in the 1920's by Neil Kensington Adam, who noted that a monolayer mixture of cholesterol and PC or various fatty acids occupied less surface area than anticipated (Leathes, 1925). Nowadays, cholesterol is viewed as a lipid which, at physiological temperatures, increases lipid order and decreases fluidity (Mouritsen and Zuckermann, 2004). Therefore, cholesterol is frequently crucial for inducing phase separation of lipid domains and therefore promoting lateral asymmetries in biological membranes.

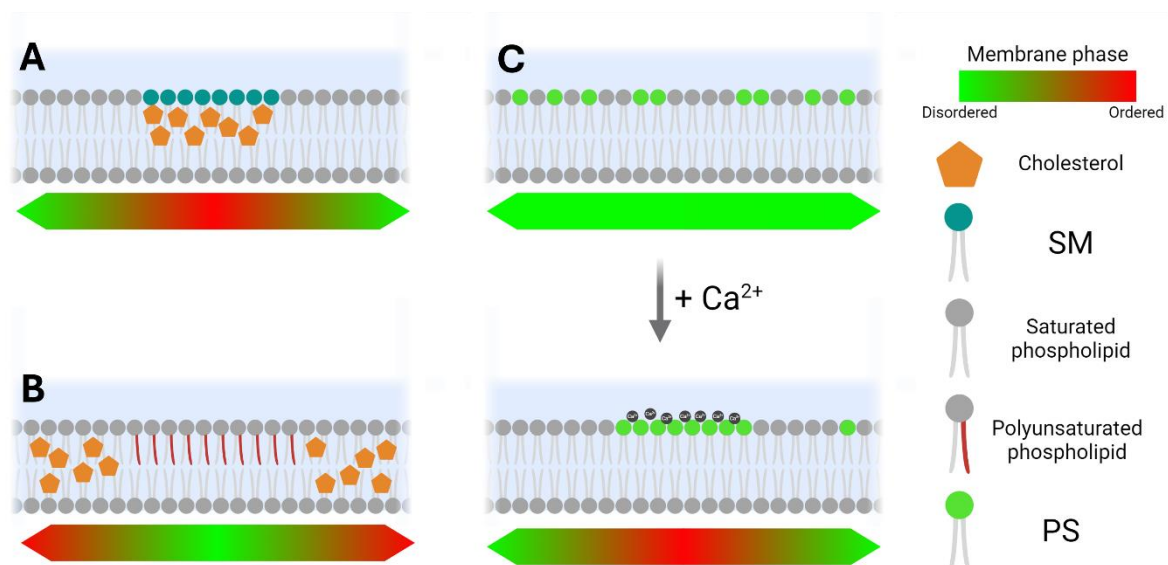
Some of these domains can also form due to intrinsic affinity between certain lipids. A well-known example are membrane rafts, which were proposed by Kai Simons and Gerrit van Meers to explain the polarization of epithelial cells (Simons and Van Meer, 1988). They have been defined as: *“small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes”* during the keystone symposium on lipid rafts and cell function, in 2006 (Pike, 2006). They result from the phase separation of sphingolipids and cholesterol, facilitated by specific hydrogen bonds (Fig. 10A). These rafts serve as important platforms for protein recruitment providing, for example, anchoring sites for glycosylphosphatidylinositol (GPI) anchored proteins (Simons and Ikonen, 1997).

Another example of cholesterol induced lipid domain formation is the phase separation of polyunsaturated lipids. This phenomenon was proposed by Demel et al., who noticed that the surface area of polyunsaturated and monounsaturated PC monolayers, when mixed with cholesterol, increased with the degree of unsaturation



(Demel et al., 1972). This effect is primarily due to steric constraints and packing defects imposed by multiple *cis* double bonds in the acyl chain of polyunsaturated phospholipids (Brzustowicz et al., 2002). Consequently, cholesterol preferentially localizes in regions with saturated phospholipids, inducing phase separation of disordered polyunsaturated lipid domains (Fig. 10B). Interestingly those disorder lipid domains composed of unflexible polyunsaturated chains also induce lipid packing defects, especially when formed by non-cylindrical lipids. Those imperfections in lipid arrangement can ultimately favor insertion of proteins into the membrane (van den Brink-van der Laan et al., 2004).

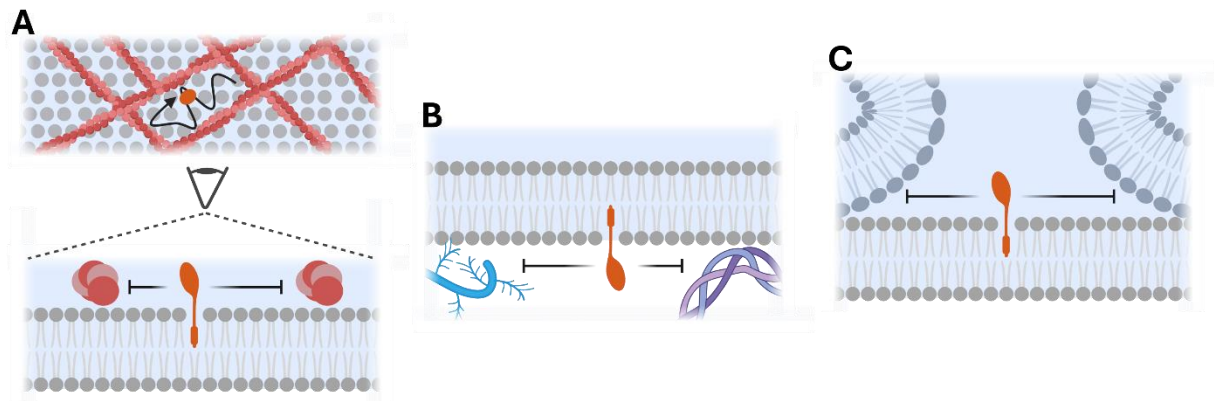
Other lipid-lipid interactions can trigger phase separation thanks to their headgroups. Calcium-induced phase separation, first reported by Onishi and Ito, exemplifies this phenomenon. By using spin labeled PS and PC, they were able to demonstrate phase separation of PS on membranes after calcium addition. This effect occurs rapidly and is reversible upon the addition of EDTA, a calcium chelator (Onishi and Ito, 1974). Similar effects were quickly observed for other anionic lipids such as PA (Galla and Sackmann, 1975). This phenomenon was primarily attributed to the crosslinking of the negatively charged headgroups of phospholipids with divalent positively charged ions, resulting in a local decrease in fluidity (Fig. 10C). Furthermore, this effect can also manifest when a protein with a specific affinity for certain phospholipids approaches the membrane, as reported for spectrin and PS (Mommers et al., 1979).



**Figure 10:** Example of lipid domains segregation caused by lipid-lipid interactions (A) Membrane raft formed by a local accumulation of SM and cholesterol. (B) Phase separation of polyunsaturated phospholipids due to their decreased binding affinity for cholesterol. (C) Calcium induced phase separation generated by an accumulation of negatively charged PS. Created on Biorender.com.

Finally, another supporting factor for lateral asymmetries in biological membranes is the sequestration of integrated proteins into specific regions facilitated by membrane matrix proteins. This phenomenon is exemplified by the subcortical actin mesh present in most mammalian cells. Employing fluorescence recovery after photobleaching (FRAP) to compare protein diffusion on regular and spheroid red blood cells, Sheetz et al., reported an elevation in lateral mobility of those proteins. As these spheroid cells lack crucial molecules of the membrane matrix, such as actin, they suggested that subcortical actin filaments can entrap membrane proteins, confining them to specific areas of the membrane (Fig. 11A) (Sheetz et al., 1980). Later, by using gold particles attached to E-cadherin on cultured mouse keratinocytes, Kusumi et al., conducted single particle tracking experiments to explore the effect of calcium on cell differentiation. They directly measured that in high calcium medium, protein diffusion across membrane increased. Additionally, they established that diffusion was confined to an area 300-600 nm wide, with a surface of 0.04-0.24  $\mu\text{m}^2$ , and upon calcium induced differentiation, E-cadherins were more prone to exit these confined areas. Thus, they suggested that cells could dynamically regulate the diffusion of molecules by acting on the cortical actin cytoskeleton (Kusumi et al., 1993). It turns out that these regulations can also be governed by signaling lipids such as PIPs (Janmey and Lindberg, 2004). More recent single particle tracking experiments indicated that in addition to proteins, lipids are also sequestered in a similar manner by these membrane matrix proteins (Fujiwara et al., 2016). Moreover, the actin cytoskeleton is not the sole matrix protein capable of confining specific molecules along the lipid bilayer. For example, extracellular matrix proteins regulate the movement of specific neurotransmitter receptors in postsynaptic sites (Fig. 11B) (Frischknecht et al., 2009). Contact sites between different membrane compartments have also been proposed to act as diffusion barriers for lipids and proteins, creating segregated domains (Fig. 11C) (Shcheprova et al., 2008).





**Figure 11:** Example of lateral diffusion restrictions

(A) Movement restriction induced by confinement with the cortical actin meshwork (red). (B) Movement restriction induced by confinement with extracellular matrix proteins such as glycosaminoglycans (blue) or collagen (purple). (C) Movement restriction induced by contact sites between membrane compartments. Created on [Biorender.com](https://www.biorender.com).

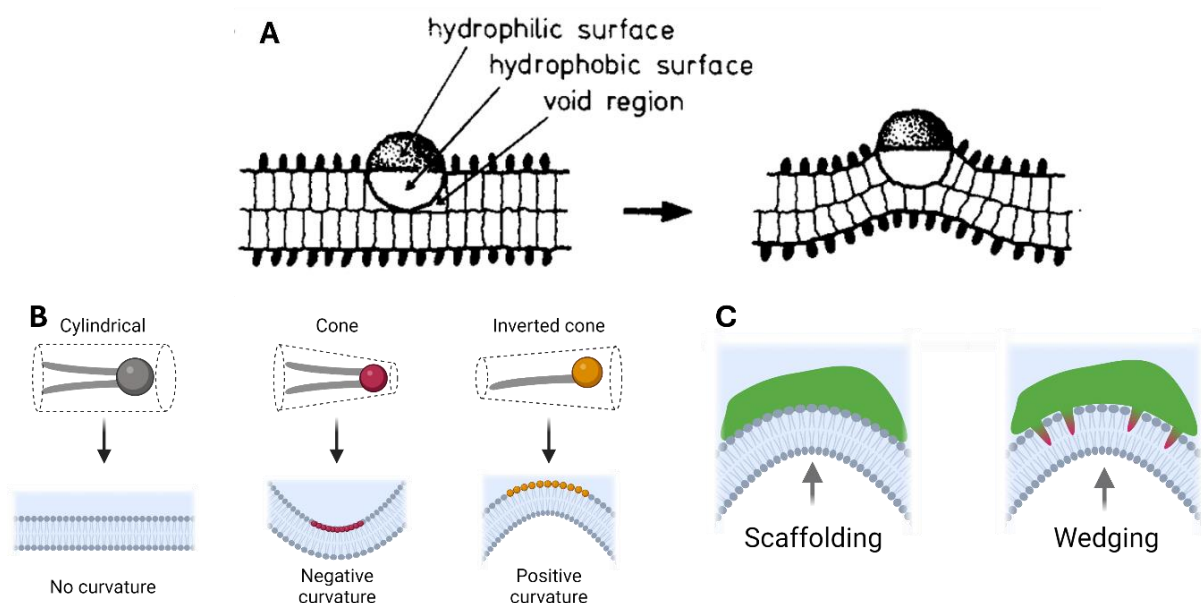
#### D. The mattress model of lipid-protein interactions in membranes

It is also worth noting that there are alternative models for biological membranes. For example, in 1977, Jacob Israelachvili, proposed the mattress hypothesis ([Israelachvili, 1977](#)). He noticed that by considering biological membranes as a two-dimensional fluid, insertion of certain proteins could create void regions within the lipid bilayer. This is incompatible with our current understanding of thermodynamics (Fig. 12A). Israelachvili therefore suggested that lipids could adopt complex shapes to fill these voids, leading to the development of the mattress model of lipid-protein interactions in membranes a few year latter ([Mouritsen and Bloom, 1984](#)). This model emphasizes hydrophobic pairing between membrane components to satisfy the law of entropy, rather than membrane fluidity and dynamics. Consequently, it provided the theoretical framework to consider biological membranes as elastic entities, who can curve and bend. Both models are useful in their own regard, to describe different features of biological membranes. In the following two paragraphs, I will discuss two additional properties of lipid bilayers that align more closely with the mattress model.

The first property is the ability of biological membranes to curve, which is essential for various cellular mechanisms including vesicular trafficking as I will develop later. This ability can be attributed to the intrinsic properties of lipids in the bilayer or their interactions with specific curvature-inducing proteins.

As seen previously, phospholipids, with specific headgroups and side chains, adopt different geometries in bilayers. For example, PC (cylindrical shaped), PA (cone shaped) and LPA (inverted cone shaped) can generate flat, negative, or positive membrane curvature, respectively, when they accumulate locally ([Kooijman et al., 2005b](#)) (Fig. 12B). Additionally, lipid packing defects generated by unsaturation in phospholipid side chain can also accentuate their conical shape, as double bonds reduce flexibility and increase the volume of the non-polar part of the phospholipid ([Pinot et al., 2014](#)) which can ultimately promote membrane deformation. It has also been suggested that an increase in the amount of lipids on one side of the bilayer through the action of flippases can also induce curvature ([Devaux et al., 2008](#)).

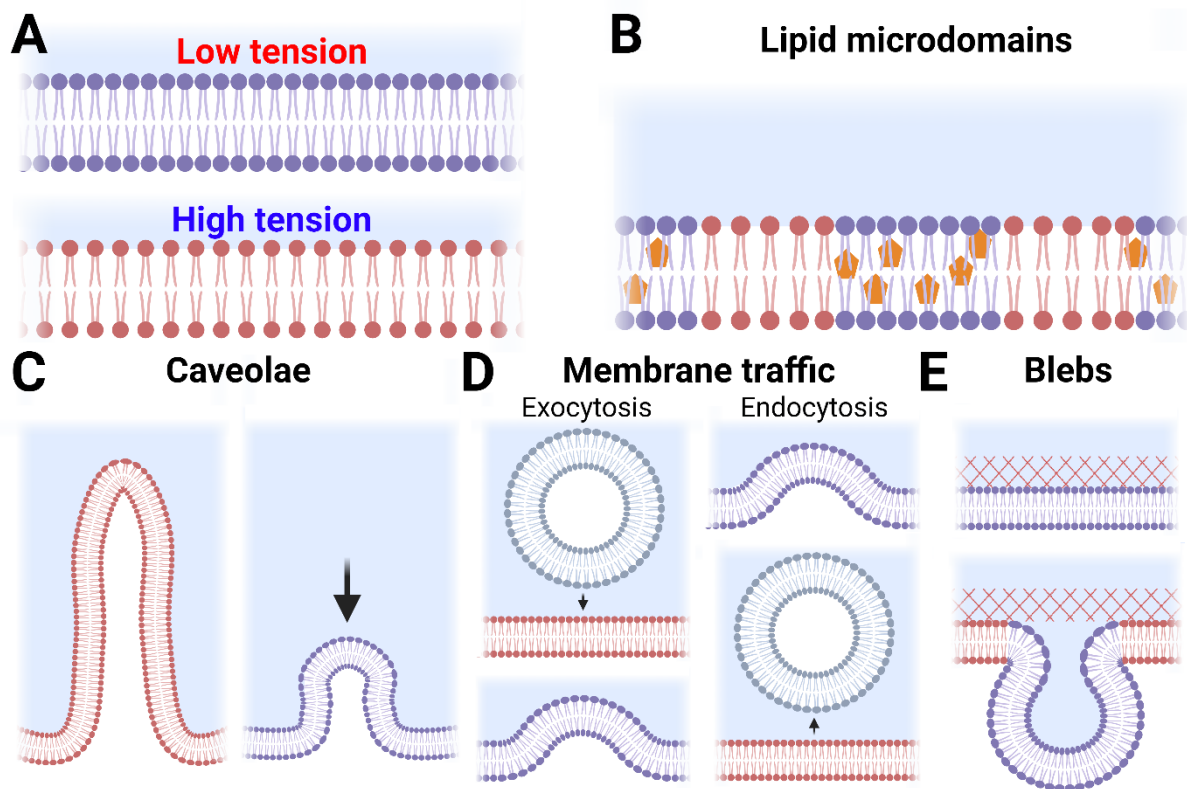
The lipid bilayer can also adopt specific curvatures through interactions with proteins and/or recruit specific proteins to curved areas ([McMahon and Boucrot, 2015](#)). Two types of protein domains have been reported to perform such tasks: 1) the Bin/Amphiphysin/Rvs (BAR) domain, acting as a rigid scaffold and by binding specific lipids in membrane favors curvature ([Gallop et al., 2006](#)), and 2) the Arf lipid packing sensor (ALPS) domain, whose hydrophobic regions insert into the bilayer and act as wedges to promote curvature ([Drin et al., 2007](#)) (Fig. 12C).



**Figure 12:** The mattress model of biological membranes and curvature

(A) Illustration picturing a globular amphipathic protein inserted in a bilayer. By considering membranes as a two-dimensional fluid, void regions are created. Therefore, lipids must accommodate the shape of the protein ([Israelachvili, 1977](#)). (B) Example of how different lipid shapes can alter the bilayer; cylindrical lipids do not induce any curvature while cone and inverted cone shaped ones induce respectively negative and positive membrane curvature. Created on [Biorender.com](#). (C) Example of protein (green with hydrophobic part in red) induced curvature of the lipid bilayer through scaffolding or wedging. Created on [Biorender.com](#).

Additionally, biological membranes exhibit elastic properties enabling them to resist an increase in membrane area (Fig. 13A). This membrane tension primarily arises from the osmotic pressure created by differences in ions concentrations in the cytoplasm on opposite sides of cellular membranes and from the interaction of cells with extracellular substrates (Sens and Plastino, 2015). Membrane tension also depends on the intrinsic properties of membranes. For instance, generating microdomains with cholesterol in saturated and unsaturated lipids membranes can differentially affect tension laterally (Fig. 13B). In other words, the force exerted on membranes is not homogeneous, and stiffer areas of the membrane are less prone to deformation than the non-segregated parts (Jacobs et al., 2021). This biophysical parameter is crucial for the function of transmembrane channels such as the nicotinic receptor (Pan et al., 2012), influencing curvature generation by BAR domain proteins (Simunovic and Voth, 2015), and ultimately altering membrane trafficking (Wu et al., 2017). Finally, when tension increases excessively, membrane rupture can lead to cell damage and eventually death. Therefore, cells have developed specific mechanisms to tightly control this membrane tension. One such mechanism is “storing” excess membrane in invaginations called caveolae, which can be utilized to relief tension upon reaching a certain threshold (Sinha et al., 2011) (Fig. 13C). These structure are formed by stress fibers of the actin cytoskeleton, induced by cavin and caveolin proteins recruited by negatively charged lipids such as PS and PI(4,5)P2 (Parton et al., 2020). Additionally, membrane trafficking also helps to regulate membrane tension, as exocytosis events can be induced by an increase in tension (Gautier et al., 2011) and a decrease in membrane tension is often associated to endocytosis (Raucher and Sheetz, 1999) (Fig. 13D). Moreover, the cortical actin cytoskeleton can also regulate elastic properties of membranes by forming a rigid scaffold associated with transmembrane proteins or proteins linked with specific lipids of the membrane (Lieber et al., 2013). An increase in membrane surface can act on those molecular linkers, generating blebs: a detachment of the membrane from the cortical cytoskeleton, inducing outward budding of the membrane (Fig. 13E) (Norman et al., 2010).



**Figure 13:** Membrane tension in lipid bilayers

(A) Illustration a membrane under high (red) and low (purple) tension. (B) Influence of lipid microdomain formation on membrane tension. (C) Regulation of membrane tension by the retraction of caveolae. (D) Regulation of membrane tension by membrane trafficking. (E) Regulation of membrane tension by the cortical actin cytoskeleton, generating blebs. Created on [Biorender.com](https://www.biorender.com)

In summary, phospholipids are essential for forming biological membranes as they self-organize into a semipermeable bilayer. The thousands of different lipids that constitute membranes have various physicochemical properties, allowing for lateral asymmetries and packing defects. Additionally, the distribution of lipids across the bilayer is asymmetrical, with negatively charged lipids generally facing the cytosolic side. Proteins can also be integrated to this barrier, interacting with specific lipids within membranes, undergoing secondary lipid modification, or possessing hydrophobic transmembrane domains. Consequently, biological membranes can be viewed as a two-dimensional fluid in which proteins float or as a mattress subject to hydrophobic interactions within the bilayer. Cells have evolved to leverage on these physiochemical properties, influencing the behavior of lipids within membranes to maintain homeostasis, target proteins, and perform specific cellular functions.

### **III. Membrane compartments**

Biological membranes are crucial for eukaryotic cell functions, by constituting a barrier between the extracellular environment, and the intracellular environment, composed of the cytoplasm and divers organelles. Additionally, they form a complex and intricate intracellular network composed of multiple membrane compartments allowing individualization of various organelles, each specialized in distinct cellular functions (Fig. 14). Various membrane compartments are present in most mammalian cells, including:

The **nucleus** is delimited by a double lipid bilayer called the nuclear envelope. It is specialized in storing, maintaining, expressing, and regulating genetic information. The inner and outer nuclear membrane can join to form nuclear pores which are the main sites of entry and exit from the nucleus ([Lammerding, 2011](#)).

The **ER** is continuous with the outer layer of the nuclear envelope and is composed of a single lipid bilayer that extends throughout the cell forming protrusions to contact other organelles. It is specialized in the synthesis, folding, modification, transport of proteins and structural lipids. It also operates as an intracellular calcium store. The ER is often differentiated into the rough ER, where ribosomes carry out RNA transcription, and smooth ER, where lipid biosynthesis occurs ([Voeltz et al., 2002](#)).

The **Golgi apparatus** is formed by multiple stacks of Golgi cisternae, each composed of a lipid bilayer. It mainly functions as a sorting organelle, directing various cargos to different cellular areas. This compartment is also the place where tertiary proteins modifications, such as glycosylation, methylation... take place. The Golgi apparatus is subdivided in several regions, who are often considered as distinct membrane compartments: the ER-Golgi intermediate compartment (ERGIC), the *cis*-Golgi network (CGN), the medial Golgi, and the *trans*-Golgi network (TGN) ([Nakano, 2022](#)).

The **PM** composed of a single lipid bilayer and various proteins, acts as the semipermeable barrier forming the outer membrane of all cells. It also serves as a major signaling platform by representing the link between the outer medium and the interior of cells.

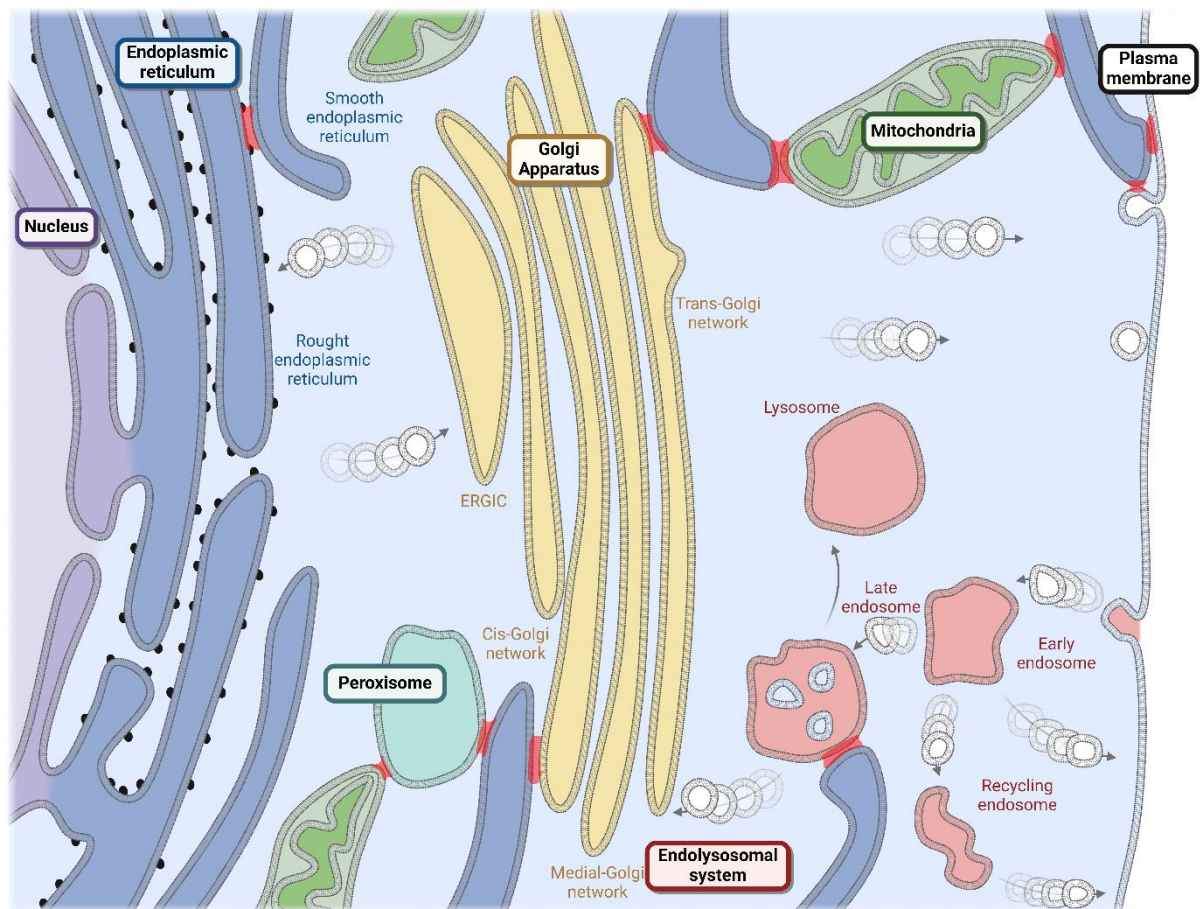
The **endo-lysosomal network** is composed of different vesicular structures that primarily derived from the PM. It is responsible for sorting molecules that have been endocytosed by the cell, directing them to other membrane compartments, or degrade them. Multiple different endosomes coexist within cells depending on their processing state and fate: early endosomes (EE), recycling endosomes (RE), late endosomes (LE),



lysosomes and multivesicular bodies (MVB) are some of them (Cullen and Steinberg, 2018).

**Peroxisomes** are small organelles involved in many metabolic processes enabling initial steps of phospholipid and ether lipid biosynthesis, catabolism of long chain fatty acids and detoxification of reactive oxygen species.

**Mitochondria** is a complex network composed of two lipid bilayers that can dynamically fuse and split. They are primarily known for being the main source of ATP production in cells and synthesis of lipids (PG and CL), but they also play roles in apoptosis and calcium homeostasis (McBride et al., 2006).



**Figure 14:** Membrane compartments of eucaryotic cells

Scheme depicting the major membrane compartments found in most eucaryotic cells along with different processes enabling molecular exchanges between those compartments (transport vesicles are colored in white and MCS are highlighted in red). Created on [Biorender.com](https://www.biorender.com).

Membrane compartmentalization is an essential organizing principle of eukaryotic cells. By preventing interference between various molecular actors, it enables faster and more consistent execution of specific functions that are performed in those organelles. However, for this organization principle to function appropriately, processes enable to

exchange molecules from one compartment to another. Two major and complementary exchange mechanisms have been unveiled so far: vesicular trafficking and membrane contact sites (MCS) (Fig. 14).

## **A. Vesicular trafficking**

Vesicular trafficking is the process of transporting materials within cells by using small membrane bound vesicles. Various types of vesicles are present in cells, differing based on their origin, composition, and destination. Transport vesicles move materials between organelles, while secretory vesicles, originating from the TGN, transport molecules to the PM for release in a regulated or constitutive manner. Finally, endosomes handle the trafficking of different molecules internalized from the PM. Despite of their differences, all vesicles follow a similar fundamental process in eukaryotic cells: i) Vesicles form from a donor compartment, incorporating various types of cargo in their lumen, ii) Vesicles are transported to their acceptor compartment where they are tethered and iii) Vesicles dock before eventually fusing with the acceptor compartment to release their cargo. This general paradigm has extensively been described by James Rothman, Randy Schekman and Thomas Südhof, winners of the 2013 Nobel prize in physiology and medicine ([Bonifacino, 2014](#)).

### **1. Vesicle biogenesis**

The general mechanism of vesicle biogenesis begins at specific sites within donor compartments where different cargos accumulate. On the cytoplasmic side of the bilayer, specific PIPs and GTPases from the ADP-ribosylation factor (Arf) family accumulate to recruit coat adaptor proteins (AP) triggering vesicle formation ([Bremser et al., 1999](#); [Serafini et al., 1991](#)). During this process, the assembling coatomer can also recruit various transmembrane export receptors to incorporate different soluble cargos from the donor compartment's lumen into the forming vesicle ([Powers and Barlowe, 2002](#)) (Fig. 15).

The next step is characterized by an outward budding of the donor membrane, driven in part by positive curvature induced by scaffolding/wedging mechanisms of the coatomer. Depending on the vesicle type, different cytoplasmic coat proteins (COP) are to

perform this process: COPI and COPII mediates vesicle formation for traffic from the Golgi apparatus to the ER and vice versa respectively (Tab. 2), while clathrin is responsible for vesicle formation on the PM, endosomes and the TGN ([Bonifacino and Glick, 2004](#)) (Fig. 15A). In addition, those different interactants must bind to specific signaling lipids and segregated lipid domains, for instance recruitment of Arf1 and AP only takes place at phase separated polyunsaturated lipids disordered microdomains ([Manneville et al., 2008](#)). This enables precise control of the cargo of the future transport vesicle and the generation of specific specialized membrane subareas on the donor compartment where vesicle biogenesis happens.

For clathrin, the APs involved in vesicle budding vary depending on the origine of the donor membrane, ensuring specific targeting in an organelle-dependent manner. AP-1 is involved in budding from the TGN and RE, promoting bidirectional transport by interacting with Arf GTPases ([Wang et al., 2003](#)). AP-2 primarily functions at the PM, and is recruited by PI(4,5)P2 to regulate clathrin dependent-endocytosis ([Rohde et al., 2002](#)). Although not directly controlled by any known Arf protein, AP-2 is indirectly activated by Arf6 which activates PI4P-5-kinase to produce PI(4,5)P2 ([Krauss et al., 2003](#)). AP-3 is crucial for vesicle biogenesis in the endolysosomal network ([Peden et al., 2004](#)) and plays a major role in synaptic vesicle recycling from endosomes ([Blumstein et al., 2001](#)). Clathrin does not seem indispensable for initiating budding with AP-3 ([Peden et al., 2002](#)). Two other AP isoforms, AP-4 and 5, have been identified but they are expressed at much lower level (300 times) than AP-1, -2 or -3 and do not appear to bind clathrin ([Hirst et al., 2013](#)). AP-4 has redundant transport functions with AP-1 and orchestrates budding of TGN vesicles destined to fuse with endosomes ([Dell'Angelica et al., 1999](#); [Hirst et al., 1999](#)). AP-5, the most recently identified AP, is involved in retrograde traffic from LE toward the TGN ([Hirst et al., 2011](#)). It is Arf-independent and binds membranes through the intermediate of spastic paraplegia genes (SPG) 11 and 15 ([Hirst et al., 2018](#)).

Besides those canonical APs, two other proteins families can recruit clathrin for vesicle biogenesis: 1) Golgi-localizing,  $\gamma$ -adaptin ear homology domain, Arf-binding proteins (GGA), which facilitates the transport from the TGN to LE ([Boman et al., 2000](#)) ; and 2) the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which enables direct vesicular transport from EE to LE ([Raiborg et al., 2001](#)) (Tab. 2).



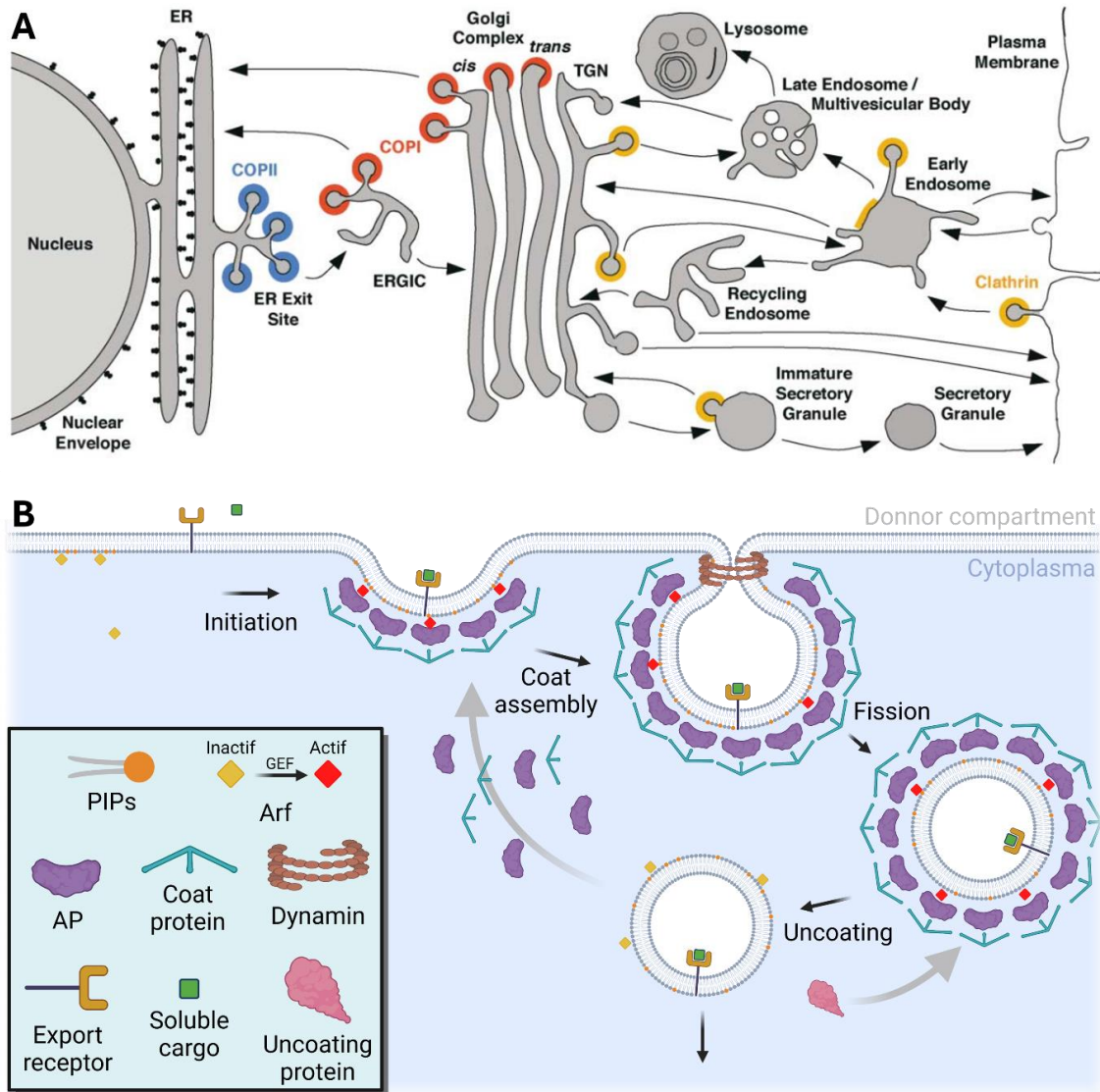
| Coat            | Regulator        | Budding compartment | Target compartment    | Reference                                        |
|-----------------|------------------|---------------------|-----------------------|--------------------------------------------------|
| COPI            | Arf1, Arf4, Arf5 | Golgi, ERGIC        | ER, Golgi, ERGIC      | (Wong-Dilworth et al., 2023)                     |
| COPII           | Sar1 ?           | ER                  | ERGIC                 | (Kasberg et al., 2023)                           |
| AP-1 + Clathrin | Arf1, PI4P       | TGN, RE             | TGN, RE               | (Ren et al., 2013; Wang et al., 2003)            |
| AP-2 + Clathrin | Arf6, PI(4,5)P2  | PM                  | EE, RE                | (Krauss et al., 2003; Rohde et al., 2002)        |
| AP-3            | Arf1             | RE                  | LE, synaptic vesicles | (Faundez and Kelly, 2000; Ooi et al., 1998)      |
| AP-4            | Arf1, Arf3       | TGN, RE             | TGN, RE               | (Boehm et al., 2001; Dell'Angelica et al., 1999) |
| AP-5            | SPG11, 15        | LE                  | TGN                   | (Hirst et al., 2018, 2011)                       |
| GGA + Clathrin  | Arf1, PI4P       | TGN                 | LE                    | (Boman et al., 2000; Wang et al., 2007)          |
| Hrs + Clathrin  | PI(3)P           | EE                  | LE                    | (Raiborg et al., 2001)                           |

**Table 2:** Coat proteins

Table recapitulating the main coat proteins of eukaryotic cells, their adaptors, some of their identified regulators and the vesicular trafficking route they are enabling.

Once the vesicle's overall  $\Omega$  shape is formed, GTPases of the dynamin family sever the vesicle from the donor compartment (Heymann and Hinshaw, 2009). This is achieved by constructing a dynamin ring around the neck of the preformed vesicle upon GTP hydrolysis (Fig. 15B). Several parameters promote this fission, such as negative membrane curvature induced by endophilins (Bai et al., 2010) or amphiphysin (Takei et al., 1999), and dynamin insertion into the lipid bilayer through interaction with negatively charged lipids like PIPs and PA (Burger et al., 2000). After the vesicle has physically separated from the donor membrane, the coatomer disassembles through the specific actions of ATP-dependent uncoating proteins, such as the 70kDa heat shock protein (Hsp70) (Schlossman et al., 1984) (Fig. 15B).

Of note, small GTPases, APs, and coat proteins are not always essential for initial budding of vesicles. For instance, the endosomal sorting complexes required for transport (ESCRT), is able, among many other functions, to induce internal membrane budding to form MVB (Babst et al., 2002). Additionally, ultrafast clathrin-independent endocytosis has been observed at the synapses of hippocampal neurons. Although, not yet fully understood, this process is extremely rapid (down to 200 ms) (Watanabe et al., 2013).



**Figure 15:** Mechanisms of vesicle biogenesis

(A) Schematic representation of the main coat proteins involved in specific vesicular pathways. COPI is in red, COPII in blue, and clathrin in yellow (Bonifacino and Glick, 2004). (B) Representation of the general steps of vesicle biogenesis 1) Cargo accumulates on specific transmembrane receptor; 2) Activation of GTPases, favored by PIPs recruit APs, 3) Coat assembly initiates budding of a vesicle, 4) Dynamin GTPases sever the vesicle from the donor membrane and 5) uncoating proteins disassemble the coatomer. Created on Biorender.com.

## 2. Vesicle transport and tethering

Once vesicles are free in the cytoplasm, they are transported to the appropriate acceptor compartment. There are two main types of vesicular transport, based on the distance that need to be traveled which can be long or short range. These transport processes often overlap, with a vesicle frequently relying on both during its journey across the cell (Brown, 1999).

Most of the times, long range transport relies on microtubules, cytoskeletal elements extending throughout the cell, with a fast-growing plus end oriented toward the periphery and a slow growing minus end facing the microtubule organizing center (Rodionov et al., 1999). Specific molecular motors, capable of hydrolyzing ATP with their heavy chain to take “steps” along these cytoskeletal elements, provide the necessary driving force for vesicle movement (Wirth et al., 2023). On the one hand, kinesins, generally move towards the plus end of the microtubule in an anterograde direction, and, on the other hand dynein-dynactin complexes, move towards the minus end in a retrograde manner (Caviston and Holzbaur, 2006) (Fig. 16). The light chain of these molecular motors can bind different types of vesicles and cargo through various molecular cues on their surface, with Rab GTPases being notable examples. These GTPases are recruited to their corresponding vesicles upon activation to associate with transport proteins (Horgan and McCaffrey, 2011). Additionally, molecular motors can interact with membrane rafts (Rai et al., 2016) or specific signaling lipids such as PA or PIPs (Kumar et al., 2024; Wang et al., 2017).

Short-range transport typically occurs at the cell periphery and depends on actin microfilaments and myosin motor proteins (Fig. 16). Unconventional myosins (Myosin I and V), move toward the plus/barbed end of the microfilament upon ATP hydrolysis, with myosin VI being the only one capable of moving in the opposite direction (Wells et al., 1999). Unlike microtubules, actin microfilaments lack directional organization at the cellular level (Small et al., 1978), so myosin subtypes do not impose any overall movement directionality within the cell. Like microtubule-based motors, myosins bind their cargo through various proteins, with Rab GTPases and PIPs being well-studied examples (Titus, 2018). Furthermore, myosins are regulated by intracellular calcium concentrations. Hence, the calcium-binding protein calmodulin can associate with myosins and enhance their mobility, but high cytoplasmic calcium concentrations lead to the dissociation of calmodulin and a reduction in myosin motility (Krementsov et al., 2004).

Upon reaching their destination, vesicles are attached to the membrane and molecularly associate with their acceptor compartment to prevent diffusion in the cytoplasm. This process, known as tethering, also sets the stage for subsequent fusion with the acceptor compartment by promoting the assembly of the soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) complex. These

tethers are anchored to the acceptor compartments through interactions with specific signaling lipids ([Baek et al., 2010](#)), Rabs ([Stenmark, 2009](#)) or by having transmembrane domains themselves. They extend into the cytoplasm to engage with small GTPases present on different types of vesicles or highly curved membranes ([Drin et al., 2007](#)).

Two main types of tethering complexes have been identified: coiled-coil tethers and multisubunit tethering complexes (MTC) (Fig. 16). The latter are composed of multiple proteins and are subdivided into two groups, based on architectural similarities and trafficking pathways: complexes associated with tethering containing helical rods (CATCHR) and class C Vacuolar protein sorting (Vps) MTCs ([Szentgyörgyi and Spang, 2023](#)):

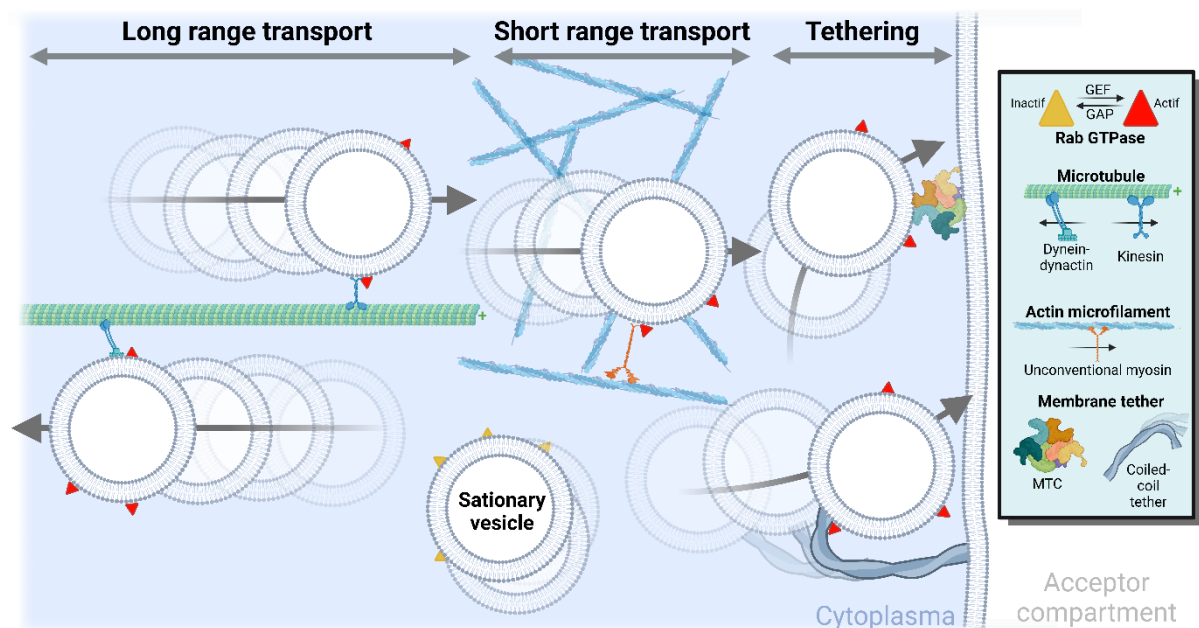
- **Coiled-coil tethers** consist of one dimeric giant protein coiled together. They are primarily represented by the Golgin protein family, which tethers vesicles to the Golgi apparatus ([Munro, 2011](#)), except for early endosome antigen 1 (EEA1), present only on EE ([Simonsen et al., 1998](#)).
- **CATCHR complexes** are characterized by long flexible legs ([Chou et al., 2016](#)). Five CATCHR complexes have been identified, each responsible for tethering different vesicles within the secretory pathway (Tab. 3).
- **Class C Vps MTCs** regulate tethering in the end lysosomal network, with four complexes discovered so far (Tab. 3).

The transport protein particle (TRAPP) complexes have also been referred to as MCT in the past. However, their primary role appears to be acting as guanine nucleotide exchange factors (GEFs) for Rab protein activation rather than serving as direct a physical link between vesicles and acceptor compartment ([Brunet and Sacher, 2014](#)).

| MTC         |         | Pathway      | Subunits                                                 | Recognized small GTPase                       |
|-------------|---------|--------------|----------------------------------------------------------|-----------------------------------------------|
| CATCHR      | DSL     | CGN-ER       | ZW10, RINT1, NAG                                         | Rab8 and 18                                   |
|             | COG     | TGN-CGN      | COG1 to 8                                                | Rab1 and 6                                    |
|             | GARP    | Endosome-TGN | Vps51 to 54                                              | Rab4                                          |
|             | EARP    | EE-RE        | Vps50 to 53                                              | Rab6                                          |
|             | Exocyst | TGN-PM       | Exoc1 to 8                                               | Rab8, Rab11, Cdc42, Rho1, Rho3, RalA and RalB |
| Class C Vps | HOPS    | EE-LE        | Vps11, 16, 18, 33A, 39 and 41                            | Rab7, Arl8 and Rab2                           |
|             | CORVET  | TGN-EE       | Vps8, 11, 16, 18, 33A and TGFBRAP1                       | Rab5                                          |
|             | FERARI  | EE-RE        | Vps45, VIPAS39, EHD1, ANK1, 2, 3, Rabenosyn-5, Rab11FIP5 | Rab5, Rab10 and Rab11                         |
|             | CHEVI   | EE-RE        | Vps33B, VIPAS 39                                         | Rab25, Rab10 and Rab11A                       |

**Table 3:** Multisubunit tethering complexes

Table recapitulating MTCs present in mammalian cells, the pathways they are supporting, their subunits and the small GTPases they can recognize. Adapted from (Szentgyörgyi and Spang, 2023).



**Figure 16:** Molecular mechanisms of vesicle transport and tethering

Schema depicting the main vesicular transport mechanisms within mammalian cells. Long range transport is orchestrated by kinesins (anterograde) or dynein (retrograde) along microtubules. Short range transport is performed by unconventional kinesins (I, V and VI). And finally, coiled-coil tether, or MTCs, tether the vesicle to an acceptor compartment. Created on [Biorender.com](https://www.biorender.com)

### 3. Vesicle docking and fusion

After tethering, vesicles are docked to the acceptor compartment. This docking process involves the association of vesicular SNARE (v-SNARE) proteins on the vesicle and transmembrane SNARE (t-SNARE) proteins on the acceptor compartment, forming SNARE pins (Fig. 17). Additionally, N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAP) can also be part of this complex (Weber et al., 1998). This initial assembly of the SNARE complex is chaperoned by Sec1/Munc18-like (SM) proteins. The exact function of those SM proteins has remained elusive for many years. Recently it has been proposed that they might act as a template for the assembly of the complex. Indeed, Munc18 can rescue the function of an unfolded SNARE complex (Jiao et al., 2018). While there are many different t-SNAREs and v-SNAREs, they cannot all form a complex between themselves. Only specific SNARE pins composed of the right v- and t-SNARE can induce SNARE complex assembly at a given organelle (Hong and Lev, 2014) (Tab. 4). The docking process represents therefore an additional checkpoint to ensure that the right vesicle will fuse with the right compartment. The number of SNARE pins involved can vary depending on the types of vesicles that will fuse. A single SNARE pin has been shown to suffice to induce fusion of two vesicles *in vitro* (van den Bogaart et al., 2010) albeit with a slow kinetics, whereas, up to 12 SNARE pins are required for the docking of synaptic vesicles in neuronal active zones (Bera et al., 2023). One might assume that larger vesicles might need even more SNARE pins.

This docking process is often linked to tethering, as many tethering factors also play a role in this initial SNARE pin assembly (Hong and Lev, 2014), some SM proteins can also directly be part of an MTC. For example, specific Rab GTPases on their corresponding vesicles are involved in this process (Søgaard et al., 1994). The localization of t-SNARE proteins can be regulated by lateral asymmetries in membrane, such as lipid rafts (Chamberlain et al., 2001), which create specific microdomains on the acceptor compartment for membrane fusion. Additionally, specific signaling lipids promote this docking process, such as PS on the acceptor membrane, which attaches to v-SNAREs via  $\alpha$ -synuclein (Lou et al., 2017). Finally, fatty acids, like arachidonic acid and ALA have also been shown to promote the initial assembly of SNARE pins in synaptosomes (Rickman and Davletov, 2005).



| Pathway                  | v-SNARE | t-SNARE              | SM protein |
|--------------------------|---------|----------------------|------------|
| ER-ERGIC                 | Bet1    | Stx5, GS27, Sec22b   | Sly1       |
| ERGIC-CGN                | Bet1    | Stx5, Sec20 and 22b  | Sly1       |
| CGN-ER                   | Slt1    | Stx5, Sec20 and 22b  | N/A        |
| TGN-CGN                  | GS15    | Stx5, GS28, Ykt6     | Sly1       |
| TGN-PM<br>(constitutive) | VAMP8   | Stx4, SNAP23         | N/A        |
| TGN-PM<br>(Regulated)    | VAMP2   | Stx1, SNAP25         | Munc18     |
| PM-EE                    | VAMP2/3 | Stx13, SNAP25 and 29 | N/A        |
| EE-LE                    | VAMP8   | Stx7, Vti1b, Stx8    | Vps33      |
| LE-lysosome              | VAMP7   | Stx7, Vti1b, Stx8    | Vps33      |

**Tabel 4:** SNARE complexes offer specificity in vesicle targeting

Table recapitulating the v- and t-SNAREs composing the SNAREpins enabling membrane fusion for different inter-organelle vesicular transport together with their associated SM protein. Adapted from ([Hong and Lev, 2014](#)).

Once the vesicle is docked to the acceptor compartment, SNARE pins assemble from vesicle's most distal point to its central point, forming a stable four-helix bundle made of the cytoplasmic protuberances of v-SNARE and t-SNAREs proteins ([Matos et al., 2003](#)). This zippering process increases the proximity of vesicles with the target compartment, enabling the fusion of the two lipid bilayers by overcoming the energy barrier keeping them separate (Fig. 17). The initial nature of the fusion pore was a hot topic of debate for quite some time, with two main hypotheses proposed to describe this process ([Jackson and Chapman, 2008](#)).

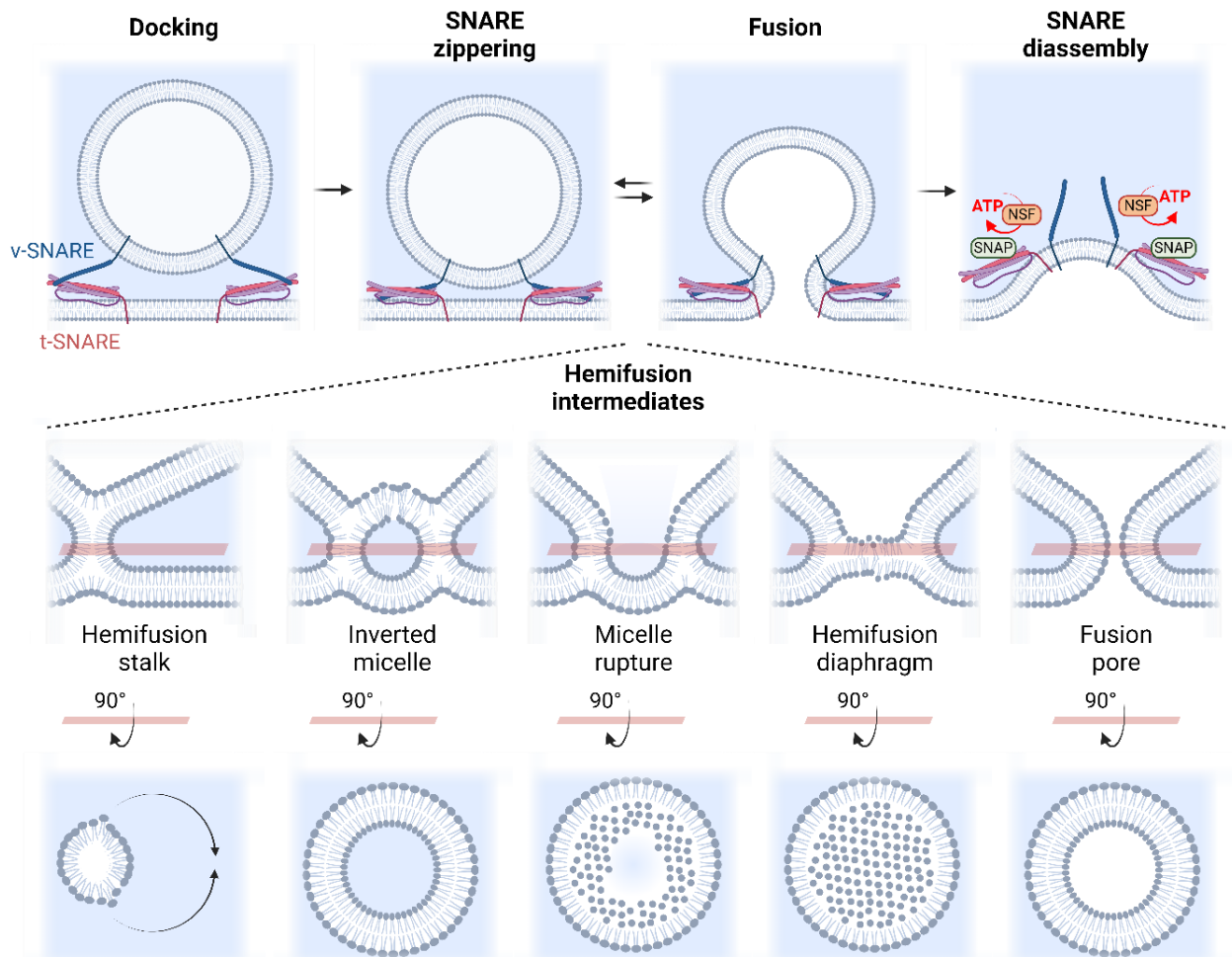
One model suggested that the fusion pore is formed by transmembrane proteins in the vesicle and/or acceptor compartment, with proteins like syntaxin forming a barrel structure composed of seven transmembrane domains from seven individual syntaxin molecules ([Han et al., 2004](#)) or the V0 domain of the vacuolar ATPase (V-ATPase) ([Peters et al., 2001](#)). However, the presence of V0 in the fusion pore has been later refuted both for synaptic vesicles and secretory granules using chromophore assisted light-inactivation ([Poëa-Guyon et al., 2013](#)). Moreover, the diameter of the initial fusion pore can fluctuate at the initial stage of fusion in a process called flickering. This has been observed in protein-free *in vitro* fusion assays with pure lipid bilayers ([Chanturiya et al., 1997](#)). Moreover, fusion assays with various membrane stains have shown that both membranes



mix before the actual fusion occurs ([Melikyan et al., 1995](#)). The latter is incompatible with the idea of an initial fusion pore made solely of proteins as vesicular lipids would not be able to diffuse to the other membrane because of the barrier formed by the proteins forming the pore. This has led to the proposal of lipid-based fusion pore model, involving hemifusion intermediates like the fusion stalk and fusion diaphragm. Specific charged and shaped lipids can influence the formation of these intermediates ([Chernomordik et al., 1995](#)), and such structures have been visualized *in vitro* on giant unilamellar vesicles (GUV) ([Nikolaus et al., 2010](#)), on native membranes with SNARE pins ([Reese et al., 2005](#)) and in the active zones of neurons using electron tomography ([Zampighi et al., 2006](#)).

Initially thought to be incompatible, the proteinic and lipidic models of fusion pore formation may have been reconciled through coarse grain simulations. These simulations show that SNARE pins can guide the bilayer in the formation of those intermediates ([Risselada et al., 2011](#)). First, the zippering process enables stalk formation by altering the orientation of SNARE pin transmembrane region, twisting the side chains of interacting phospholipids. Then, the stalk forms a ring encompassing part of the cytoplasm in an inverted micelle, counteracting the bilayer's tendency to grow in the membrane plan. This inverted micelle ruptures due to tensile stress forming the hemifusion diaphragm. Finally, this rupture exposes the hydrophobic core of the hemifusion diaphragm to the hydrophilic part of the zipped SNARE pin that stands out on the other part of the membrane, this polar mismatch is what changes orientation of lipids and drives fusion pore formation (Fig. 17).

After fusion, SNARE pins must be disassembled. This is achieved through the hydrolysis of ATP by NSF, which disassembles the tightly bound SNARE helixes, preparing for the next round of fusion ([Littleton et al., 2001](#)) (Fig. 17). Remarkably, the membrane fusion process itself does not directly consume energy. Disassembling SNARE pins afterwards enables the delay of ATP consumption needed to generate the huge forces needed to fuse two lipid bilayers. Hence, downpayment of this energetic debt represents a massive advantage for cells. It allows to fuse many vesicles without an immediate energy cost and therefore be able respond to various situations. Moreover, it can also accelerate the speed of the fusion processes itself, by limiting the problem of ATP diffusion within the cytoplasm. Enabling biological marvels such as ultrafast synaptic transmission, where multiple rounds of fusion must occur simultaneously every other nanosecond.



**Figure 17:** Vesicle docking and fusion

Schema representing the final step of vesicle fusion. 1) Loose assembly of SNAREpins enable the docking process, 2) SNARE zippering enables to increase proximity of vesicular and target membranes, 3) The fusion pore is formed and expands, 4) The closed SNAREpin is finally disassembled via the ATPase activity of NSF and SNAP. The formation of the fusion pore itself is mediated by lipid-protein interactions and requires the formation of multiple hemifusion intermediates: the fusion stalk and diaphragm together with an intermediate state of inverted micelle predicted by simulations. The middle row illustrates a point of view from a vertical plane to the membrane while the bottom row a perspective from the horizontal plane. Created on [Biorender.com](https://www.biorender.com).

## B. Membrane contact sites

In addition to vesicular transport MCSs have emerged as an alternative pathway for molecular exchanges between organelles within the cell. The phenomenon of increased proximity between different cellular membranes in cells has been recognized for over 60 years ([Bernhard and Rouiller, 1956](#)) but has not been studied very extensively until the last decade. It has now been established that nearly all membrane compartments can dynamically form and disband these contacts ([Shai et al., 2018](#); [Valm et al., 2017](#)). In recent years, those structures have gained significant attention, and nowadays, there is no doubt that they are fundamental in organizing membrane traffic and cellular homeostasis. As

previously discussed, MCSs can act as a diffusion barriers in membranes, generating laterally segregated domains (Shcheprova et al., 2008). Additionally, MCSs have emerged as crucial platforms for lipid metabolism, particularly MAMs (Kannan et al., 2017; Vance, 1990) (see lipid metabolism). They also facilitate lipid distribution to various organelles through specialized lipid transfer proteins (LTP) (Mesmin et al., 2013). Furthermore, they play vital roles in vesicular trafficking and positioning by linking different types of vesicles for concurrent transport (Salogiannis et al., 2016), participate in mitochondrial fusion and fission (Friedman et al., 2011), and are essential for maintaining calcium homeostasis within the cell (Csordás et al., 2010).

Recently, a consortium of experts defined MCSs as an “*areas of close apposition between the membranes of two organelles*” (Scorrano et al., 2019). However, these sites are much more than just juxtaposition between two membranes. Scorrano and colleagues proposed that MCSs must meet four specific criteria:

- **Tethering:** There must be at least one, often multiple, molecular links between the two membranes, called tether, bringing them in proximity (approximately 30 nm). Due to the diversity of MCSs, no specific distance should be used as a general criterion.
- **No fusion:** This tethering process is distinct from tethering vesicles to acceptor membranes during vesicular trafficking. MCSs are not intended to prepare for an eventual fusion of the linked organelles. For instance, docked vesicles do not qualify as MCSs despite meeting all other criteria.
- **Function:** An MCS must perform one or more specific functions within the cell, such as lipid or calcium transfer, organelle positioning, or acting as a diffusion barrier.
- **Composition:** An MCS must have a precise molecular composition that enables it to perform its functions. This includes not only proteins but also membrane micro/nanodomains and signaling lipids.

Most of the proteins present in an MCS are tethers, whose function is to act like a spacer, maintaining the proximity of the membranes while preventing any potential fusion event. Tethers can be single proteins bridging the gap, such as extended synaptotagmins (E-Syt), which anchor to the ER via a transmembrane domain and bind PM PI(4,5)P2 to establish contact between these two membranes (Giordano et al., 2013). However, tethers

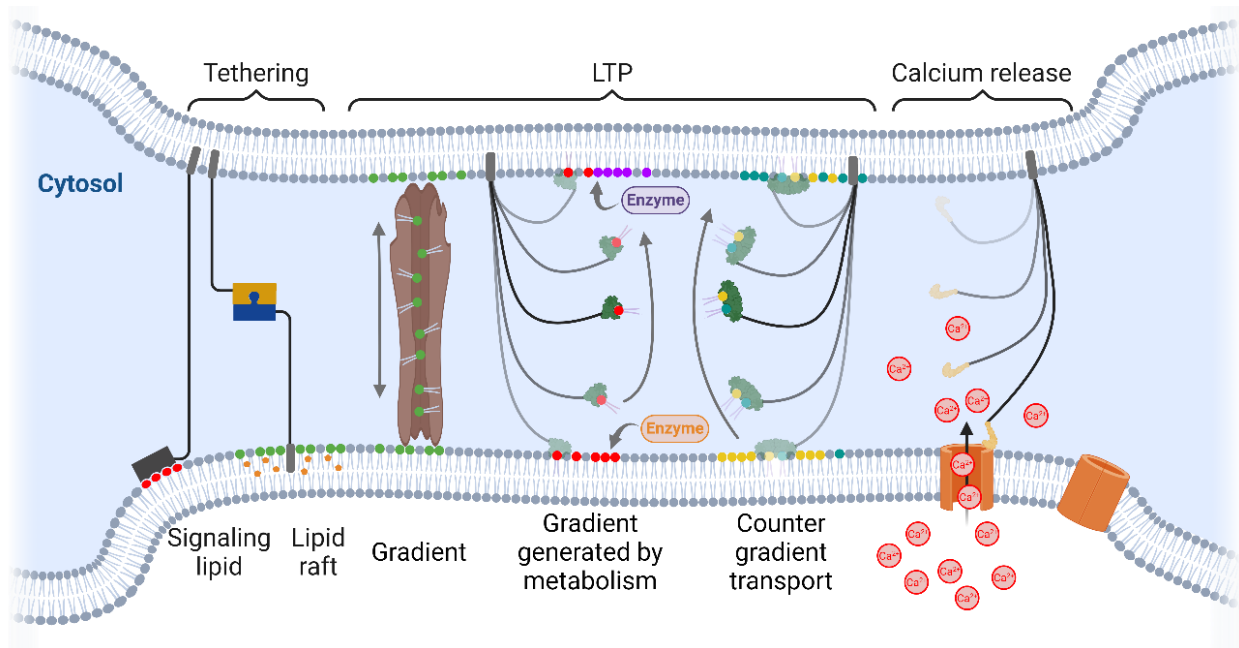
typically work in conjunction with one another and dimerize with another tether located on the opposite membrane, forming a heterotypic dimer to keep the MCS in place (Fig. 18). This arrangement allows a single one of those protein to participate in different types of contacts (Tab. 5). For example, vesicle-associated membrane protein (VAMP)-associated proteins (VAP) on the surface of the ER, can dimerize with various partners: with StAR-related lipid transfer domain-3 (StARD3) for ER-LE contacts (Alpy et al., 2013), with acyl-CoA binding domain 5 (ACBD5) for ER-peroxisome contacts (Hua et al., 2017), with the Kv2.1 potassium channel for ER-PM contacts (Johnson et al., 2018), or with oxysterol binding protein (OSBP) for ER-TGN contacts (de la Mora et al., 2021).

| MCS                   | Tether                                            | Function                                                                                                                                      | Reference                                             |
|-----------------------|---------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|
| ER-PM                 | VAPs                                              | ER resident, associates with many different LTPs                                                                                              | (Murphy and Levine, 2016)                             |
|                       | E-Syt1, 2 and 3                                   | LTP (nonspecific, phospholipids, glycolipids and DAG transport)                                                                               | (Saheki et al., 2016)                                 |
|                       | Oxysterol binding related proteins 5 (ORP5), ORP8 | LTP (exchanges PM PI4P with ER PS)                                                                                                            | (Chung et al., 2015)                                  |
|                       | Nir2                                              | LTP (exchanges PM PA with ER PI(4,5)P2)                                                                                                       | (Kim et al., 2015)                                    |
|                       | Junctophilin1, 2, 3 and 4                         | Extracellular calcium entry to store operated calcium release form the ER coupling                                                            | (Takeshima et al., 2015)                              |
|                       | DHPR RyR                                          | Extracellular calcium entry to store operated calcium release form the ER coupling                                                            | (Cheng et al., 2005)                                  |
|                       | STIM1 ORAI                                        | Calcium entry into the cytoplasm                                                                                                              | (Park et al., 2009)                                   |
|                       | Kv2.1                                             | Control neuronal excitability by clustering channels                                                                                          | (Johnson et al., 2018)                                |
| ER-mitochondria (MAM) | PTPIP51                                           | Regulates mitochondria calcium homeostasis by inducing calcium import from the ER LTP (PA transport to mitochondria by associating with VAPb) | (De Vos et al., 2012), (Giordano and Prodrumou, 2021) |
|                       | IP3R VDAC Grp75                                   | Regulates mitochondria calcium homeostasis by inducing calcium import from the ER                                                             | (Szabadkai et al., 2006)                              |
|                       | Fis1 BAP31                                        | Induction of apoptosis                                                                                                                        | (Iwasawa et al., 2011)                                |
|                       | MFN1/2                                            | Control of mitochondria fusion and fission upon ER contact                                                                                    | (de Brito and Scorrano, 2008)                         |
|                       | VAPs                                              | ER resident, associates with many different LTPs                                                                                              | (Murphy and Levine, 2016)                             |
| ER-endosome           | StARD3                                            | LTP (imports cholesterol from the ER to endosomes)                                                                                            | (Wilhelm et al., 2017)                                |
|                       | ORP1L ORP5                                        | LTP (exports cholesterol from endosomes to the ER)                                                                                            | (Du et al., 2011)                                     |
|                       | VAPs                                              | ER, resident associates with many different LTPs                                                                                              | (Murphy and Levine, 2016)                             |
| ER-Golgi              | OSBP                                              | LTP (exchanges ER cholesterol with Golgi PI4P)                                                                                                | (Mesmin et al., 2013)                                 |
|                       | CERT                                              | LTP (imports ceramides from the ER to the Golgi)                                                                                              | (Kumagai et al., 2007)                                |
|                       | FAPP2                                             | LTP (imports glucosylceramide from the ER to the Golgi)                                                                                       | (D'Angelo et al., 2007)                               |
|                       | PxdA                                              | Pairs transport of EE with peroxisomes                                                                                                        | (Mesmin et al., 2013)                                 |

**Table 5:** Examples of MCS, their tethers and proposed functions

Table illustrating some tether found between specific inter-organelle MCS. Adapted from (Eisenberg-Bord et al., 2016).

Tethering is often just one of the many functions of these proteins, which can also play various other roles (Tab. 5). As previously mentioned, they can act as LTPs to facilitate the non-vesicular transport of lipids between organelles. For example, E-Syts, can translocate different types of lipids depending on their concentration gradient ([Saheki et al., 2016](#)). This relies on lipid concentration gradients across membranes, which drive the specific lipids to the right organelle. For example, ceramides, produced in the ER, must travel to the Golgi apparatus to be converted in SM. This transport, mediated by ceramide transport proteins (CERT) and VAPs, depends on the conversion of ceramide to SM to maintain low ceramide levels in the Golgi apparatus ([Kumagai et al., 2007](#)) (Fig. 18). When lipid translocation goes against its concentration gradient, it pairs with the transport of another lipid. An example is the PI4P/cholesterol exchange between the TGN and the ER, mediated by the VAPa/OSBP pair. PI4P is transported to the TGN, and following its hydrolysis by Sac1, multiple cholesterol molecules are transferred to the ER against their gradient ([Mesmin et al., 2013](#)). Another famous example is the PA/PI(4,5)P<sub>2</sub> exchange by the Nir2-VAPb pair during the phosphoinositide cycle, where high level of PA at the PM triggers the exchange with ER PIPs, replenishing the PM with signaling lipid after PLC activation ([Kim et al., 2015](#)). This mechanism resembles the function of a sodium ion pump at the PM, and PI4P gradient has since been proposed to couple the translocation of many other lipids against their gradient ([Ghai et al., 2017](#); [Moser von Filseck et al., 2015](#)) (Fig. 18). Besides lipid transport, tethers can perform other functions. For instance, the stromal interaction molecule 1 (STIM1) present on the ER which activates ORAI on the PM induces calcium influx from the extracellular medium ([Park et al., 2009](#)) (Fig. 18). Moreover, since MCSs are usually maintained by multiple tethers, a single contact can support multiple functions ([Besprozvannaya et al., 2018](#)) (Tab. 5).



**Figure 18:** Membrane contact sites and their functions

Schema depicting a typical MCS, tether spanned across two membrane compartments and can have multiple, not exclusive functions, for example: maintaining the MCS in place, transporting lipids (different transport modes are illustrated in a hydrophobic tunnel formed between both membranes, or by exchanging lipid using gradients) or inducing calcium release. These tethers are often associated to membranes and regulated by signaling lipids. Created on [Biorender.com](https://www.biorender.com).

MCSs are also characterized by their distinct lipid composition, which often differs significantly from the surrounding regions of the contacted organelles. Specific signaling lipids are essential in maintaining or disbanding these structures, as they provide contact points for tethers, such as the interaction between E-Syt and PI(4,5)P<sub>2</sub> ([Giordano et al., 2013](#)). Lipid microdomains can serve as anchoring points for palmitoylated or GPI-associated tethers. For instance, ER-mitochondria MCSs often form at lipid raft locations ([Hayashi and Fujimoto, 2010](#)). Moreover, these contact sites are hubs for signaling lipid metabolism, with specific signaling lipids produced directly at the site by enzymes located near MCSs ([Vance, 1990](#)). For example, the Sac1 GTPase, described previously, can convert PI4P into PI at TGN-ER contact sites ([Stefan et al., 2011](#)). Similarly, enzymes involved in the synthesis of other phospholipids, such as those producing PS (PSS1 and PSS2), are enriched at MAMs ([Kannan et al., 2017](#)). Additionally, MAMs are also equipped with various LPTs, enabling mitochondria to fulfill their role in *de novo* synthesis of phospholipids, such as the VAPb/protein tyrosine phosphatase interacting protein 51 (PTPIP51) for PA import into mitochondria ([Giordano and Prodromou, 2021](#)).



## C. Membrane identity

Altogether, vesicular trafficking and MCS are the two essential complementary processes maintaining the integrity of all membrane compartments in a temporally and spatially sensitive manner. There are multiple checkpoints established by the intrinsic nature of effector protein assemblies. Some AP and their respective coats can only assemble on a given donor compartment (Tab. 2), vesicular tethering complexes enables discrimination of one type of transport vesicle (Tab. 3), SNARE complex assembly by itself and subsequent membrane fusion is very dependent on the involved v- and t-SNARE (Tab. 4), and finally, tethers between MCS are also specifically expressed in certain organelles (Tab. 5). However, this does not explain how those effectors always get recruited at the right time and the right place to accomplish their given function. Cells have therefore multiple ways to differentiate the identity of certain membrane compartments and organize membrane trafficking, among which the most well-studied are small GTPases, signaling lipids and the inherent differences in membrane properties of organelles.

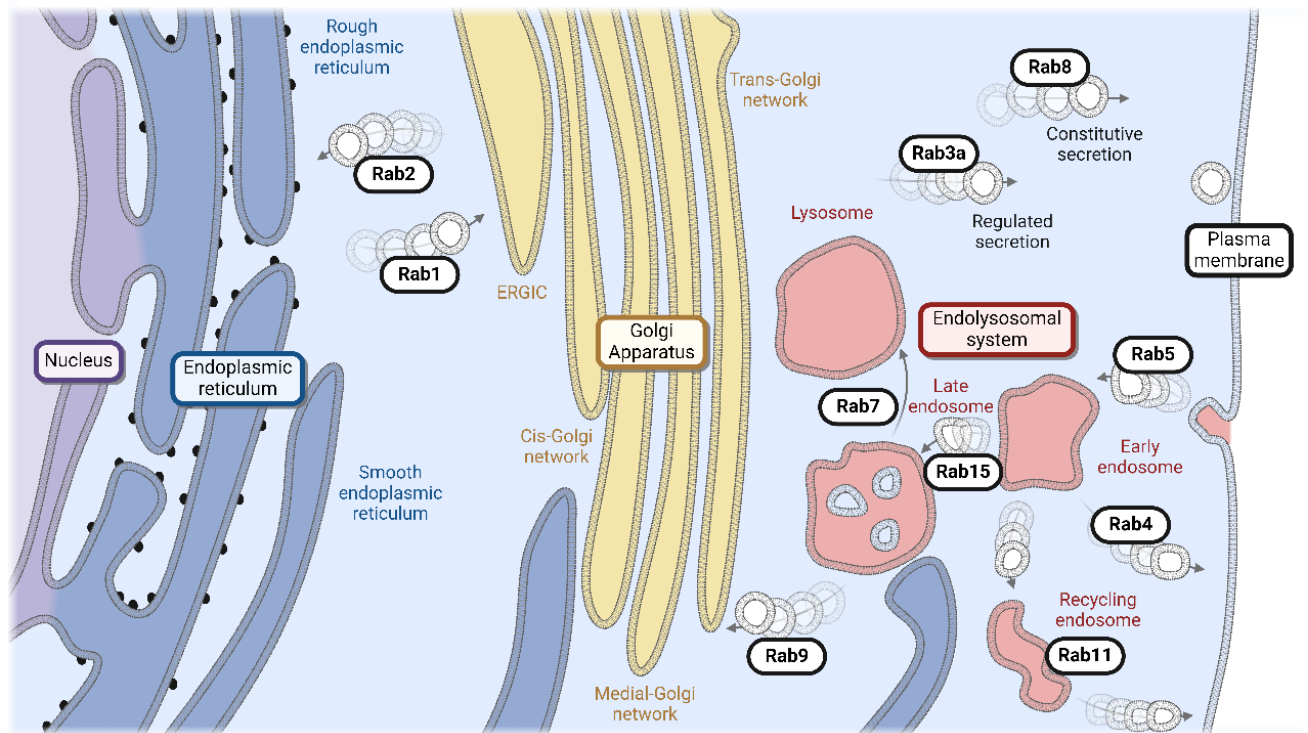
### 1. Small GTPases

Small GTPases of the Ras superfamily are a group of enzymes that can recruit and activate multiple effectors. They can enter an active state after the replacement of a GDP for a GTP by a GEF and become inactivated by hydrolyzing this GTP into GDP, through the activation by a GTPase activating proteins (GAP). Guanine nucleotide dissociation inhibitors (GDI) can also prevent activation of those enzymes by locking them in an inactivated state. The Ras superfamily is composed of four families according to sequence homology and cellular functions: i) the Ras family is involved in cell growth, survival and differentiation, ii) the Rho family is involved cytoskeleton regulation, iii) the Rab family regulates membrane trafficking (with Ran involved in nuclear transport) and, iv) the Arf family can induce vesicle biogenesis and regulates lipid metabolism ([Wennerberg et al., 2005](#)). Of course, many of these families play fundamental roles in vesicular traffic and MCS function and dynamics. Given that they can be activated at specific sites and moments, they represent ideal candidates for regulating those complex cellular processes. Rho GTPases influencing actin cytoskeleton organization ([Millard et al., 2004](#)) can also affect vesicles transported by unconventional myosins, utilizing those cytoskeletal structures for movement. But they can also have a more direct effect, for example Cdc42 is able to



bind the coat of retrograde vesicle originating from the Golgi and inhibits the recruitment of dynein (Chen et al., 2005). Additionally, an increase of cortical actin density by manipulating Rho GTPases can affect the exocytic process (Gasman et al., 1997) and many different Rho protein influence endocytic pathways (Qualmann and Mellor, 2003). Moreover, Rho GTPases can also affect the tethering process of vesicle. For instance, Rho3 and Cdc42 are also upstream regulators of the Exo70 subunit of the exocyst MTC (Wu et al., 2010). As described in the chapter on vesicle biogenesis, APs are the downstream effectors of many Arf GTPases. Moreover, Arf6 is a known regulator of signaling lipids by acting on their metabolic enzymes. It can therefore indirectly affect membrane fusion by activating PLD1 to produce PA (Vitale et al., 2002) or PI4P-5-kinase to produce PI(4,5)P2 (Honda et al., 1999).

However, the most essential GTPases in membrane trafficking are undoubtedly members of the Rab family. They were identified early on as regulators of membrane trafficking and proposed to act as membrane organizer (Chavrier et al., 1990; Zerial and McBride, 2001). Although Rabs by themselves are rarely directly involved in the molecular mechanisms of vesicular trafficking, many key proteins to traffic can be found among Rab effectors. Thus, Rab GTPases can be considered as cellular zip codes for specific organelles or transport vesicles, capable of sequentially recruiting appropriate proteins at the right time upon activation. Over sixty Rabs have been identified based on sequence homology (Stenmark and Olkkonen, 2001), with most being exclusively expressed on one pathway (donor compartment, transport vesicle, and acceptor compartment) (Fig. 19A). However, a single Rab can have multiple effectors involved in different steps of vesicular trafficking. For example, Rab5, the most extensively studied Rab is implicated in transport of EE from the PM and interacts with GDI anchored proteins to orchestrate cargo selection (McLauchlan et al., 1998), hRME-6 for vesicle uncoating (Semerdjieva et al., 2008), Kif16B for transport (Hoepfner et al., 2005), and EEA1 for tethering and subsequent homotypic fusion with EE (Simonsen et al., 1998) (Fig. 19B). Additionally, it is worth mentioning that Rabs are not limited to vesicular trafficking, as some of their effectors are tether proteins responsible for establishing MCS. For instance, Rab7 can recruit Rab7-interacting lysosomal protein (RIPL) associated with the cholesterol LTP ORP1L to control LE positioning and cholesterol homeostasis (Rocha et al., 2009).



| Rab    | Pathway       | Effector                               | Function                                                                                                                                | Reference                                      |
|--------|---------------|----------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|
| Rab1   | ER → Golgi    | Coiled-coil tether of the Golgi        | Tethering and fusion of COPII vesicle with the CGN                                                                                      | (Moyer et al., 2001)                           |
| Rab2   | Golgi → ER    | PKC $\alpha$ and DHAP                  | Recruit dynein for retrograde transport                                                                                                 | (Tisdale et al., 2009)                         |
|        |               | PKC $\beta$                            | Recruitment of COPI coat                                                                                                                | (Tisdale, 2000)                                |
| Rab3a  | TGN → PM      | Rim                                    | Secretory granule and synaptic vesicle docking                                                                                          | (Wang et al., 1997)                            |
|        |               | Synapsin                               | Recruitment of synaptic vesicles                                                                                                        | (Giovetti et al., 2004)                        |
|        |               | Granuphilin                            | Docking of secretory granule in pancreatic cells                                                                                        | (Coppola et al., 2002)                         |
| Rab4   | EE → PM       | Rabip4                                 | Sorting from EE for subsequent release at the PM                                                                                        | (Cormont et al., 2001)                         |
| Rab5   | PM → EE       | Kif16B                                 | Transport from PM to EE                                                                                                                 | (Hoepfner et al., 2005)                        |
|        |               | GDI                                    | Cargo selection at the PM                                                                                                               | (McLauchlan et al., 1998)                      |
|        |               | hRME-6                                 | Clathrin uncoating                                                                                                                      | (Semerdjieva et al., 2008)                     |
|        |               | EEA1                                   | Tethering and homotopical fusion with EE                                                                                                | (Simonsen et al., 1998)                        |
| Rab7   | LE → Lysosome | Rab7 interacting lysosomal protein     | Recruits Dynein/dynactin for retrograde transport from LE to lysosomes<br>Binds to ORP1L in low cholesterol condition for repositioning | (Jordens et al., 2001)<br>(Rocha et al., 2009) |
| Rab8   | TGN → PM      | Myosin V                               | Transport from the TGN to the PM                                                                                                        | (Roland et al., 2007)                          |
| Rab9   | LE → Golgi    | 47-kD tail-interacting protein (TIP47) | Cargo selection, recruitment of mannose 6 phosphate receptor                                                                            | (Carroll et al., 2001)                         |
| Rab11  | RE → PM       | Myosin V                               | Transport of ER back to the PM                                                                                                          | (Hales et al., 2002)                           |
| Rab 15 | EE → LE       | Rab15 effector protein                 | ?                                                                                                                                       | (Strick and Elferink, 2005)                    |

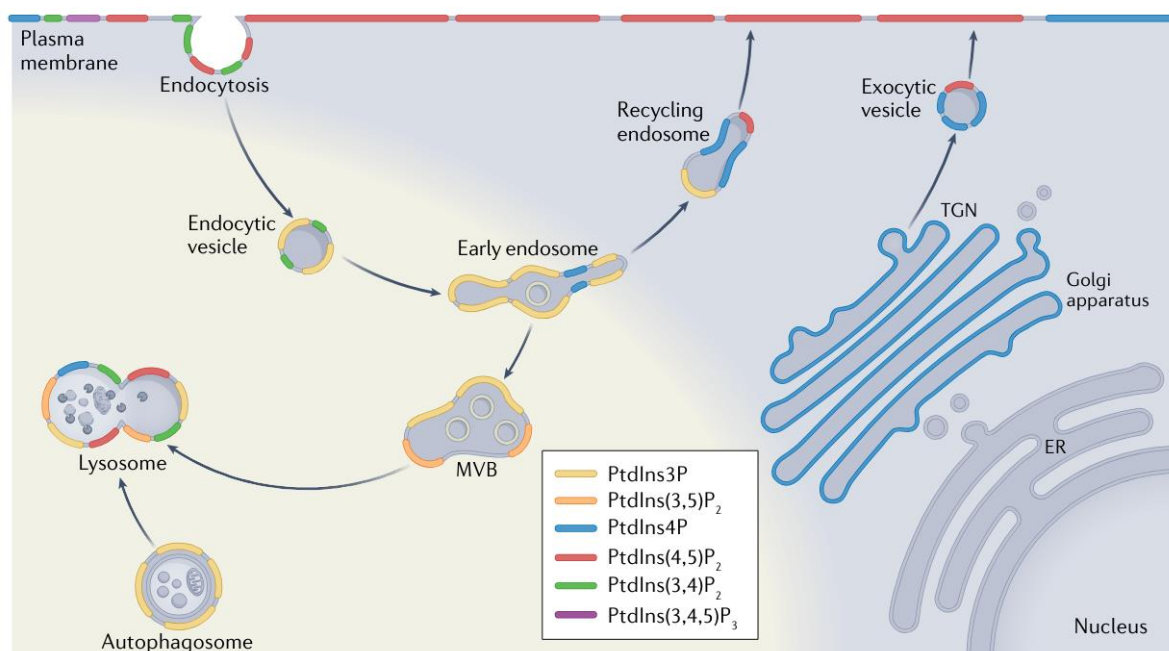
**Figure 19:** Membrane identity provided by Rab GTPases

(A) Representation of a eukaryotic cell and its transport pathways along with the localization of some Rab GTPases regulating these processes. Created on [Biorender.com](https://biorender.com). (B) Table recapitulating those Rab GTPase, some of their effectors and their function during vesicular traffic.

## 2. Signaling lipids

In addition to GTPases, signaling lipids can also be found in specific membrane compartment, modulating various steps of vesicular trafficking and controlling MCS formations. For instance, PIPs, with their complex metabolism regulated by PI-kinases and PI-phosphatases, (Fig. 6B) are defined by their specific locations within the membranes of specific organelles (Fig. 20). For example, PI4P is mostly present in the Golgi apparatus,

and participates in AP recruitment for vesicle biogenesis (Wang et al., 2003). PI4P is also crucial for the lipid exchange at MCS, facilitated by LTPs enabling counter-gradient transport of other lipids, like OSBP for cholesterol (Mesmin et al., 2013) or CERT for ceramides (Kumagai et al., 2007). PI(4,5)P<sub>2</sub>, the main PIP of the PM, is involved in vesicle docking (Chen et al., 2021), serves as an anchor for LTPs such as E-Syt (Giordano et al., 2013), and recruits AP-2 to initiate clathrin-mediated endocytosis (Kelly et al., 2014). PI(3,4,5)P<sub>3</sub>, another PIP predominantly found at the PM, regulates PH domain-containing proteins like the kinase Akt (Watton and Downward, 1999), the Arf6 GEF ARNO (Macia et al., 2000), or the Rac1 GEF Tiam1 for actin reorganization (Singleton et al., 2005). The endo-lysosomal system is characterized by PIPs with phosphate on the position 3 of the inositol ring. PI(3,4)P<sub>2</sub> is found on the surface of the PM and EE, where it participates in the recruitment of PX/BAR domain-containing proteins, such as sorting nexin proteins 9, to sever endosomes from the PM (Schöneberg et al., 2017). EEs are also marked by PI3P, which associates with the FYVE domain of proteins like EEA1 for EE tethering (Zoncu et al., 2009) or Hrs (Gillooly et al., 2000). The transition from EE to LE implies production of PI(3,5)P<sub>2</sub> on the surface of endosomes, which binds to cortactin to regulate actin dynamics (Hong et al., 2015) or the sorting nexin 14 via its PX domain (Akizu et al., 2015). Finally, PI5P, the least studied of all PIPs, may play a role in lipid droplet growth by interacting with septin 9 (Akil et al., 2016) and in autophagosome formation (Vicinanza et al., 2015).



**Figure 20:** Membrane identity provided by phosphoinositides

Distribution of various PIPs along vesicular trafficking pathways in a eukaryotic cell (Posor et al., 2022).

The question of whether other signaling phospholipids such as PA can also confer identity to specific membranes remains open. At a first glance, this might seem counterintuitive since, unlike PIPs, PA is present in multiple membrane compartments with the same headgroup, seemingly unable to differentiate between effector proteins ([Tanguy et al., 2020b](#)). However, like PIPs, the synthesis of signaling PA is tightly regulated within the cell. For instance, PLD1, the main enzyme for PA synthesis in neuroendocrine secretion, is a downstream effector of small GTPases like Arf6 ([Vitale et al., 2002](#)), or RalB ([Vitale et al., 2005](#)) and various kinases such as protein kinase C (PKC) ([Exton, 1999](#)) and RSK2 ([Zeniou-Meyer et al., 2007](#)). Moreover, PA sensors exhibit preferential binding to PA in specific membrane compartments. For example, the PABD of Spo20p interacts mostly with PA at the PM, whereas Opi1p interacts with ER PA, and PDE4A1 with Golgi PA ([Kassas et al., 2017](#)). This suggests that there may be underlying selection mechanisms that enable proteins to distinguish the membrane compartment in which PA is located. Therefore, further investigation is necessary to determine if PA could also serve as an identification Tag for specific membrane compartments.

### 3. Inter-organelle lipid asymmetry

One last mechanism for providing identity to membrane compartments is based on their intrinsic asymmetry of membrane compartments within cells. Indeed, in addition to not being symmetrical laterally and from one leaflet to another, membrane compartments of eukaryotic cells differ also in lipid composition.

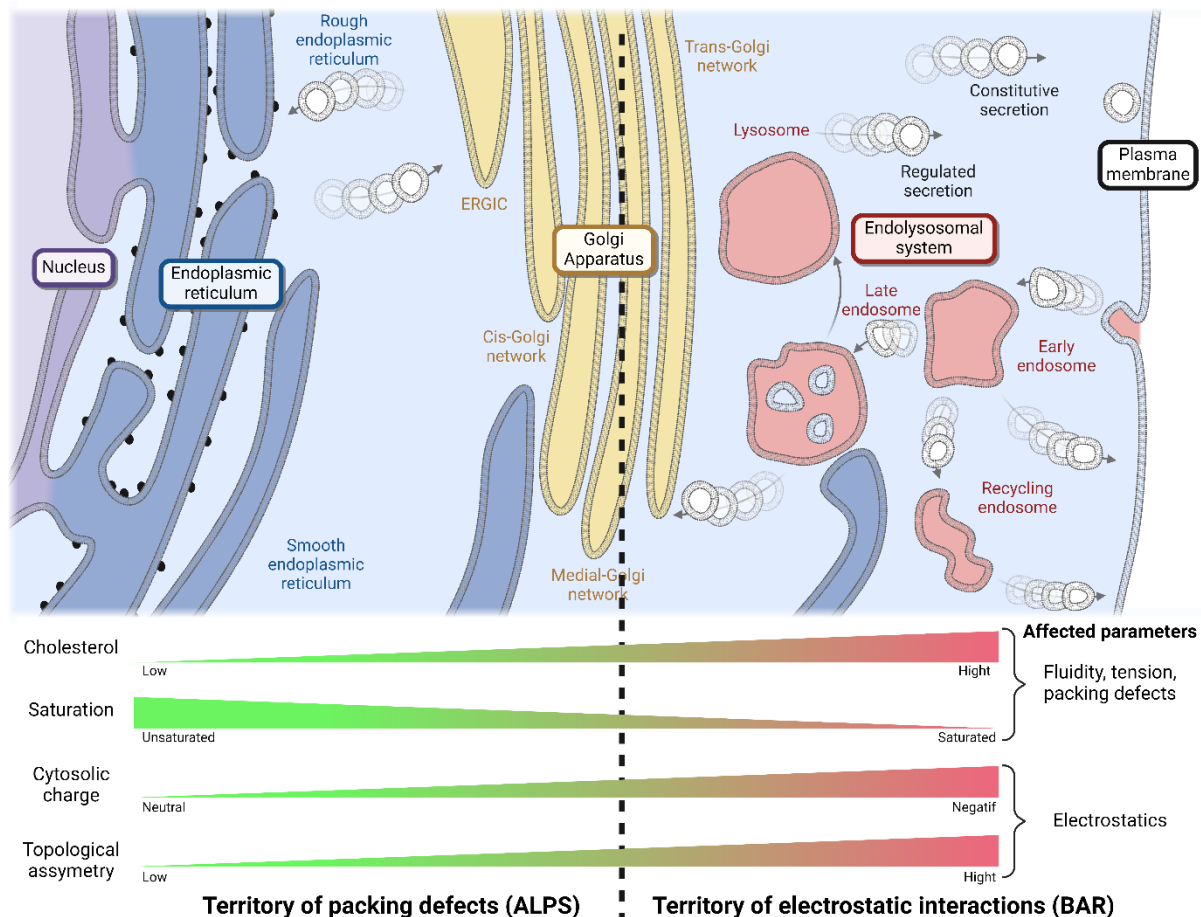
Cholesterol, for instance, is distributed asymmetrically, with 90% of the cell's total cholesterol content found at the PM, and only 10% present in intracellular compartments ([Lange et al., 1989](#)). The cholesterol content of the ER is extremely low (0,5%) in normal conditions ([Lange et al., 1999](#)) and increases gradually along the secretory pathway. In the endo-lysosomal system 80% of cholesterol is found inside the RE, LEs and in the intraluminal vesicles of MVB, with low cholesterol level in EE and lysosomes ([Möbius et al., 2003](#)). Similarly, fatty acid composition of phospholipids varies, with unsaturated acyl chains being more prevalent in the ER (45% of total phospholipid), then the Golgi (36%), and finally PM (29%) ([Keenan and Morre, 1970](#)). Specialized transport vesicles in nervous cells, such as synaptic vesicles or secretory granules, also have a high concentration of polyunsaturated phospholipids, particularly DHA (22:6,  $\omega$ -3) ([Takamori et al., 2006](#);

[Tanguy et al., 2020b](#)). These differences in cholesterol concentration and unsaturation create a gradient in lipid packing defects, fluidity, and tension within cellular membranes. From the ER (low cholesterol and high unsaturation levels) to the PM (high cholesterol and low unsaturation levels), lipid packing defects and fluidity decreases while tension increases (Fig. 21).

Phospholipids are also distributed unevenly between compartments based on their head groups. For example, PS, the most abundant negatively charged phospholipid at physiological pH represents over 12% of total lipids at the PM and less than 5% in the ER ([Kay and Fairn, 2019](#)). This distribution induces an overall increase in charge towards the PM. This effect is further accentuated by a loss of bilayer asymmetry towards the ER, where PS can be detected on the luminal leaflet ([Fairn et al., 2011](#)). This may be due to the increased tendency of polyunsaturated lipids to perform passive flip-flop ([Armstrong et al., 2003](#)), and the presence of integral reticular scramblases like transmembrane protein 41B ([Huang et al., 2021](#)). Additionally, the PM is characterized by an abundance of charged signaling lipids like PI(4,5)P2, PI(3,5)P2, PI(3,4,5)P2 ([Posor et al., 2022](#)), as well as a rapid production of PA under certain circumstances such as exocytosis ([Zeniou-Meyer et al., 2007](#)). These differences in negatively charged lipids, along with the loss of bilayer topological asymmetry, create an electrostatic gradient. The cytosolic leaflet of the ER is not very charged, but the charge increases towards the PM (Fig. 21).

Based on these observations, Bigay and Anthony proposed that eukaryotic cells can be divided into two membrane territories : 1) the territory of packing defects, found before the medial part of the Golgi apparatus (low cholesterol, and high lipid unsaturation), and 2) the territory of electrostatics, after the medial Golgi (high levels of negatively charged headgroups on the cytosolic side) ([Bigay and Antonny, 2012](#)) (Fig. 21). These two membrane territories interact differently with proteins curvature-generating/sensing proteins, providing an additional way to confer identity to cellular membranes. For example, proteins with a rigid scaffolding BAR domain such as Bin1, involved in the formation of clathrin-coated pits, interact with negatively charged lipids of the PM ([Lee et al., 2002](#)). In contrast, proteins with an amphipathic wedging ALPS domain, like Sar1 involved in the formation of COPII vesicles, preferentially bind to ER membranes ([Lee et al., 2005](#)) (Fig. 21).





**Figure 21:** Membrane identity provided by inter-organelle lipid asymmetry

Figure depicting lipid asymmetry between intracellular membrane compartments, the affected membrane properties and the two resulting territories binding different curvature sensing/promoting proteins with an ALPS or a BAR domain. Created on [Biorender.com](https://www.biorender.com).

In summary, eukaryotic cells present multiple membrane compartments, each with specific functions essential for cellular integrity and survival. Transport of molecules across cells involves two distinct processes: vesicular trafficking and transport at MCS, which facilitates the exchange of various molecules between compartments. Vesicular trafficking is a multistep process that includes vesicles biogenesis from a donor compartment, transport, tethering and fusion. MCS involve the close apposition of two membrane compartments to enable signaling or lipid exchange. Cells express numerous proteins to ensure the proper execution of these processes. However, over 3.5 billion years, cells also have evolved to exploit the unique properties of lipid bilayers to their fullest. This is evident in several ways:

- Lateral asymmetries generate signaling platforms for cargo selection, favoring membrane fission and fusion sites, or positioning of MCS.

- Topological asymmetries provide membrane identity and keep signaling lipids or their precursors accessible to cytosolic proteins.
- Membrane curvature and tension are essential for vesicle biogenesis and fusion, as well as for maintaining MCS.

The overall lipidic composition of membrane is therefore tightly regulated by adapting lipid metabolism, vesicular trafficking and MCS. Specific signaling lipids recruit target proteins at the right time and place, allowing for the proper execution of complex molecular sequences. Among all signaling lipids, PA stands out as the only conical phospholipid with a negative charge, generating both packing defects/negative curvature and electrostatic anchor points. Various forms of PA are also found in membranes based on their fatty acid composition with up to fifty different PA species ([Kassas et al., 2017](#)). Each of these different PA species may have different effects on membrane geometry and interact with different proteins. Furthermore, PA is central to lipid metabolism, easily produced from various cellular products with single enzymatic reaction and inactivated by rapid transport at MCS and conversion into other phospholipids (LPA and DAG). However, this versatility also complicates the full understanding of PA's role within cells, highlighting the need for faster tools to gain a comprehensive view of this unique phospholipid's functions.



## IV. Neurosecretion

Membrane trafficking is a versatile process managed by cells for exchanging material between different membrane compartments. However, cells also use this tool to perform exocytosis and secrete molecules in the extracellular medium. This can be done in a constitutive manner which is a common mechanism virtually used by all cell types. However, specialized cells, mainly of neuronal origin, have developed a regulated secretory pathway to adapt to external stimuli. Over the 90's and the early 2000's, researchers have unveiled the major proteins enabling this peculiar use of vesicular trafficking. However, besides proteins, more recently lipids have also been suspected to play crucial roles in these mechanisms. Despite the now well known implication of lipids in various cellular functions, including but not limited to signaling, their key role in the secretion of molecules from cells remains to be better understood. Indeed, although growing evidence suggest that signaling lipids, like PA, are as important as proteins in this process, their biochemical nature, complex and often interconnected synthesis pathway, interaction within cells and implication in various other cellular processes, together with the lack of modern tools has hindered progress on this aspect.

### A. Definition

The concept of neurosecretion was initially proposed in the 1930's by the German scientists Berta and Ernest Scharrer. They hypothesized the existence of a previously undescribed type of cell within the central nervous system of all metazoans, referred to as "*gland-nerve cells*", now commonly known as neurosecretory or neuroendocrine cells. Those cells, which may be neurons or share a common origin with neurons, are characterized by the presence of secretory granules (SG) or large dense core vesicles which can be released into the circulatory system. They also exhibit a large active nucleus to support their high metabolism activity. Neuroendocrine cells can sometimes aggregate in large numbers, forming well-defined glands ([Scharrer and Scharrer, 1937](#)). Examples of such glands in mammals were identified in the 1950's, including the ventral hypothalamus and the neurohypophysis ([Bargmann and Scharrer, 1951](#)). Additionally, neurosecretory glands were found in the peripheral nervous system, such as the pineal glands ([Lerner et al., 1958](#)) or chromaffin cells of the adrenal medulla ([Hillarp et al., 1954](#)).

It is important to distinguish these neurosecretory adrenal chromaffin cells (which will be referred to as chromaffin cells in this manuscript) from enterochromaffin cells.

In the mid-20<sup>th</sup> century, the peptidic nature of secreted molecules from neuroendocrine cells was also discovered, identifying multiple peptides contained in SG, such as oxytocin (Vigneaud et al., 1953), melatonin (Lerner et al., 1958) or thyrotropin-releasing hormone (Schally et al., 1962). The advent of electron microscopy revealed that these peptide-filled SG originate from the TGN (Scharrer and Brown, 1961) and release their contents in a regulated manner, through “reverse pinocytosis”, now known as regulated exocytosis (De Robertis and Ferreira, 1957).

Currently, neurosecretion is defined as “*the production of molecules (usually proteinaceous) in a neuron that are secreted directly into the blood to act at distant target organs*” (Lemos and Dayanithi, 2020). Additionally, it is now known that most neurons produce neuropeptides contained in SGs, which can be released into the cerebrospinal fluid (van den Pol, 2012). These SGs are distinct from small synaptic vesicles, which are released exclusively at active zones of chemical synapses and are heavily dependent on neuronal inputs and complex local recycling mechanisms to generate synaptic plasticity for information integration (Südhof, 2004). In contrast, SGs are exclusively produced at the TGN and can fuse with the PM in all parts of neurons. Importantly these neuropeptides released by SGs, such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3, play crucial roles in modulating neuronal survival, synaptogenesis, and long-term plasticity (Dieni et al., 2012; Wu et al., 2004). Moreover, SG release has also been observed in glial cells such as astrocytes (Hur et al., 2010; Ramamoorthy and Whim, 2008).

## **B. Chromaffin cells**

Neurosecretory chromaffin cells are found in the adrenal gland. Mammals possess two adrenal glands situated above the kidneys, and are histologically, embryogenically, and functionally divided into two parts:

- The adrenal cortex, of mesodermal origin, constitutes the endocrine portion of the gland and is responsible for secreting multiple steroid hormones into the bloodstream. It is composed of three layers from the exterior to the interior: 1)

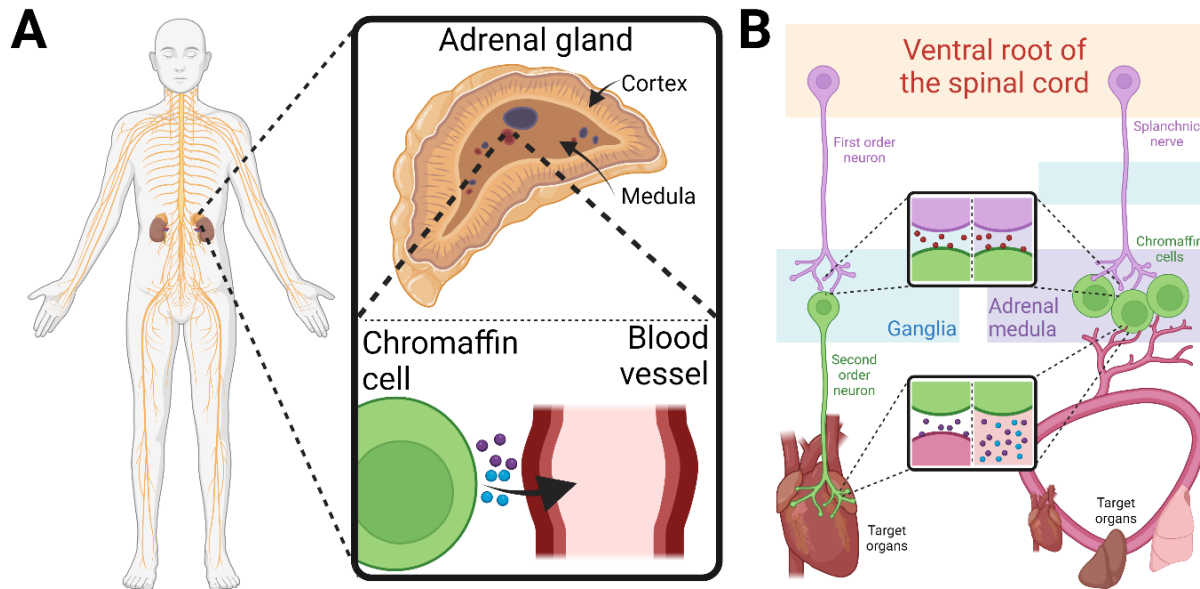
the *zona glomerulosa*, producing mineralocorticoids (aldosterone), 2) the *zona fasciculata*, producing glucocorticoids, and 3) the *zona reticularis*, also producing glucocorticoids and sex steroids in some species (Rosol et al., 2001) (Fig. 22A).

- It has long been thought that the adrenal medulla cells derive from sympathetic neurons originating from neural crests, but it has been recently found that they originate mostly from Schwann cell precursors (Furlan et al., 2017). The adrenal medulla represents the neuroendocrine part of the gland and is mainly populated with chromaffin cells. These cells produce and release small hormones (adrenalin and noradrenalin) alongside neuropeptides like met-enkephalin, substance P, neurotensin, neuropeptide Y and chromogranins (Rosol et al., 2001) (Fig. 22A).

Chromaffin cells are part of the autonomous sympathetic system and are directly controlled by the splanchnic nerve. During stress, the “*fight or flight*” response activates neurons in the lateral horn of the spinal cord, stimulating the splanchnic nerve. This nerve directly projects onto the adrenal medulla, releasing acetylcholine, among other molecules (Feldberg et al., 1934). Acetylcholine binds to nicotinic receptors, enabling sodium entry into the cell, which activates voltage-gated calcium channels to induce SG release (Marley, 1988). This results in a substantial increase in circulating catecholamines, up to 300-fold (Carmichael and Winkler, 1985).

Two different types of chromaffin cells have been reported depending on the nature of catecholamines they are secreting. The occurrence of these two types of cells is dependent on the species. For example, bovine adrenal medulla contains 90% adrenergic chromaffin cells and 10% noradrenergic cells (Moro et al., 1990). During regulated exocytosis, calcium entry can be further amplified by the co-release of pituitary adenylate cyclase-activating polypeptide (PACAP), which activates metabotropic  $G_q$  receptors to increase calcium channel conductance (Kuri et al., 2009). Alternatively, activation of muscarinic receptors by acetylcholine can trigger the release of intracellular calcium stores further increasing intracellular calcium levels (Forsberg et al., 1986). Moreover, *in vivo*, chromaffin cells are coupled with gap junctions, allowing the diffusion of calcium and sodium from one cell to another to synchronize their activity (Martin et al., 2001). This process can be regulated by autoinhibition, where factors co-released during chromaffin

cell exocytosis, such as ATP, bind to metabotropic purinergic receptors to inhibit further secretion ([Harkins and Fox, 2000](#)).



**Figure 22:** The adrenal gland and chromaffin cells

(A) Position of human adrenal glands and its structure. (B) Typical organization of the autonomous sympathetic system and its similarities with chromaffin cell innervation (red spheres: acetylcholine, violet spheres: adrenaline and blue spheres: noradrenaline). Created on [Biorender.com](#).

There are notable similarities between the classical autonomous sympathetic system where a cholinergic neuron stimulating an adrenergic neuron and chromaffin cells releasing adrenaline and noradrenaline in the circulation (Fig. 22B). Consequently, chromaffin cells have long been used as model to study the molecular mechanisms of neurosecretion ([Bader et al., 2002](#); [Neher, 2018](#)). Located in a peripheral organ, chromaffin cells are easily accessible and can be cultured in large numbers; for example, a single bovine adrenal gland can yield tens of millions of chromaffin cells when properly handled ([Fenwick et al., 1978](#)). This accessibility has facilitated the collection of large quantities of protein material, which has been extensively used to identify the molecular machinery of regulated exocytosis ([Hodel et al., 1994](#); [Morgan and Burgoyne, 1997, 1995](#); [Sudlow et al., 1996](#)).

Moreover, chromaffin cells are excitable and can be depolarized with nicotine or a depolarizing potassium solution to study ionic channels ([Nassar-Gentina et al., 1988](#)). They respond to neuropeptides such as PACAP or vasoactive intestinal peptide ([Anderova](#)

et al., 1998; Kuri et al., 2009), making them valuable for understanding metabotropic signaling. This model has also been used in elucidating the basic mechanisms of neuropeptide maturation, neurotransmitter synthesis, and vesicle loading (Menniti and Diliberto Jr., 1989; Waschek et al., 1987). Chromaffin cells can differentiate into actual neurons and form functional synapses when exposed to nerve growth factor, making them a useful model for studying neurodevelopment (Ogawa et al., 1984; Tischler et al., 1993). In their undifferentiated state, due to their simple morphology, ease of adherence to substrates, and the size of their SGs, chromaffin cells are well-suited for powerful techniques to study exocytosis and endocytosis. These techniques include carbon fiber amperometry (Wightman et al., 1991), capacitance recording (Albillos et al., 1997), total internal reflection microscopy (Oheim et al., 1998), or more recently, live stimulated-emission-depletion (STED) microscopy (Shin et al., 2021).

### C. Neurosecretion in chromaffin cells

The formation, transport, fusion and recycling of SGs in chromaffin cells is an integral part of the regulated secretory pathway. This process has many similarities to the mechanisms outlined in the chapter on membrane trafficking and will thus not be totally redescrbed. However, some precise mechanisms are unique to neurosecretion, and specific molecular actors are also detailed below.

#### 1. Secretory granule biogenesis (granulogenesis) and their transport

The early secretory pathway involves the transcription, folding and secondary post translational modifications of secreted proteins. These proteins are transported through the ERGIC, CGN, medial Golgi before reaching the TGN (Dancourt and Barlowe, 2010). Granulogenesis is part of the late secretory pathway and begins with cargo selection at specific TGN sites characterized by lipid rafts containing cholesterol and SM (Duran et al., 2012). At this location PI4P recruits LTPs such as OSBP for cholesterol (Mesmin et al., 2013), or CERT for sphingolipids (Hanada, 2006) which help forming these microdomains. They also attract transmembrane ionic pumps such as V-ATPase (Schapiro and Grinstein, 2000), or the Cab45 calcium pump (von Blume et al., 2012) and aquaporins (Arnaoutova et al., 2008). Consequently, TGN exit sites have a distinct intraluminal pH of 6.0 (Rivinoja et al., 2012) and high calcium concentration (Pizzo et al., 2011). This

facilitates selection of proteins of future immature SGs, such as CgA, which plays a key role in SG biogenesis, alongside acting as a neuropeptide precursor (Kim et al., 2001). Chromogranins can form condensates with other proteins, that must be included in future SG, at low pH and high calcium conditions (Chanat and Huttner, 1991; Montero-Hadjadje et al., 2008). No coat protein has been reported to participate in the initial budding of SG at the TGN levels. However, it was recently shown that CgA interacts with PA via a specific PABD favoring membrane budding (Carmon et al., 2020). PA's ability to induce negative membrane curvature, especially in high calcium and low pH medium (Kooijman et al., 2003), might thus play a role in the initial budding of immature SGs. Accordingly, this process is partially inhibited by PLD inhibitor (Carmon et al., 2020) (Fig. 23). The neck of the preformed vesicle is subsequently severed by the GTPase dynamin-2 to release immature SG (Yang et al., 2001).

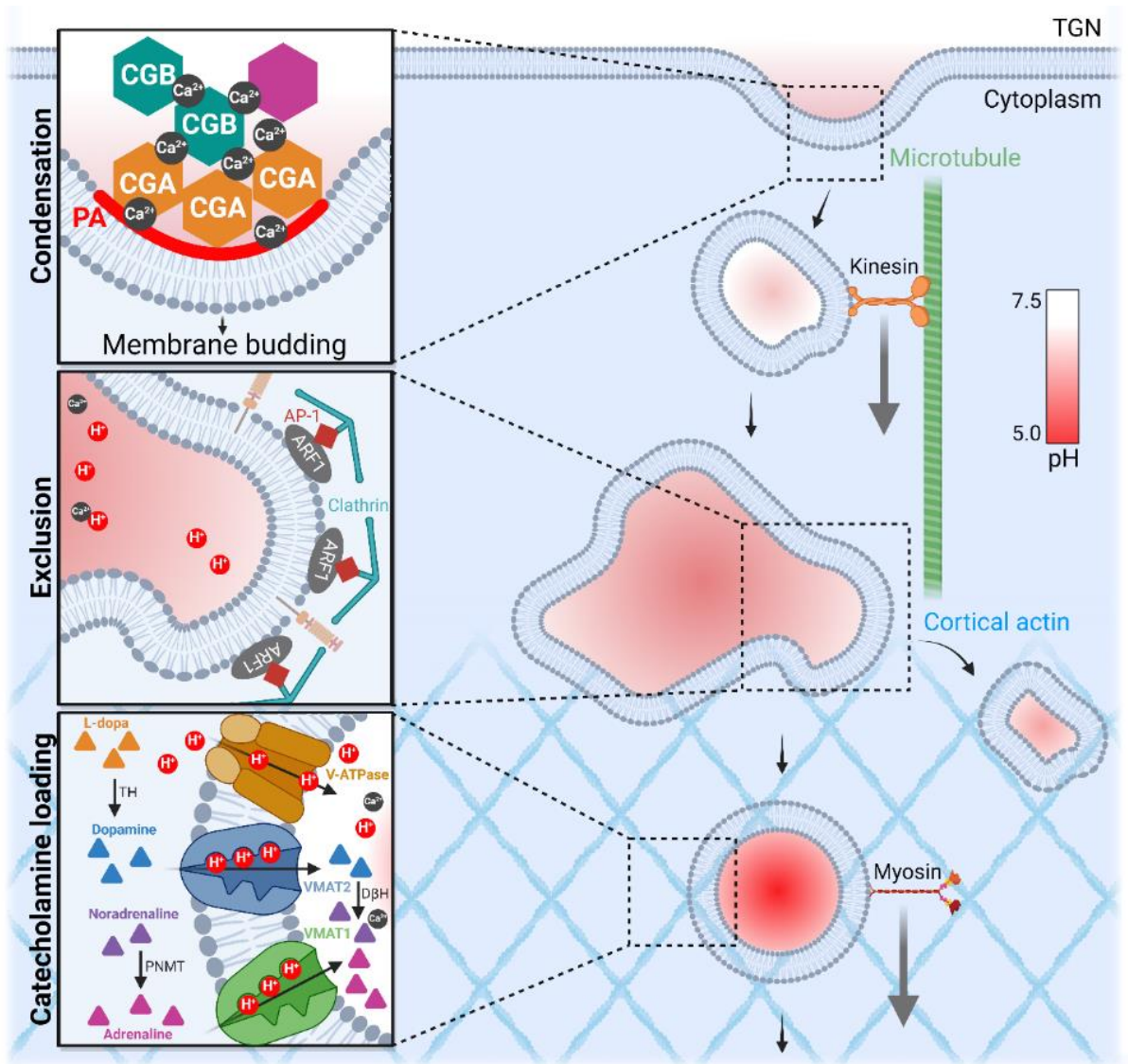
Once released into the cytoplasm, immature SGs undergo a complex maturation process, beginning with homotypic fusion of immature SGs (Urbé et al., 1998), to produce intermediate immature SGs. This fusion involves syntaxin 6 (Wendler et al., 2001) and synaptotagmin 4 (Ahras et al., 2006), which are different from SNARE proteins and regulators implicated in fusion with the PM. The composition of those intermediate SGs is refined by sorting out unwanted proteins and lipids (Eaton et al., 2000). For this, APs are recruited in an Arf1-dependent manner of APs the maturing SG (Austin et al., 2000). AP-1, for example, associates with the cytoplasmic protuberance by the VAMP-4 (Dittié et al., 1996), or GGA (Kakhlon et al., 2006) (Fig. 23). The subsequent formation of clathrin coated vesicles enables exclusion of the homotypic fusion machinery (Ahras et al., 2006; Klumperman et al., 1998), clathrin recruitment proteins (Eaton et al., 2000), misdirected proteins from the TGN, such as the mannose-6-phosphate receptor (Klumperman et al., 1998) and unsegregated lipids. The vesicles generated by this process (not SG) are thought to fuse with the PM in an unregulated manner, sometimes releasing uncondensed neuropeptides but not catecholamines (Glombik and Gerdes, 2000).

During maturation, the pH inside SGs progressively decreases to 5.0 due to the activity of V-ATPases (Apps et al., 1989), while water is expelled by aquaporins (Arnaoutova et al., 2008), further condensing chromogranins and other peptides into the typical dense core of SGs. It is also at this stage that SG are replenished with catecholamines. Dopamine is produced in the cytoplasm from tyrosine, by tyrosine

hydroxylase (Nagatsu et al., 1964) and dopa decarboxylase (Blaschko, 1942), then imported into the SG lumen by the vesicular monoamine transporter 2 (VMAT2) (Henry et al., 1994; Kirshner, 1962) utilizing the proton gradient and further converted into noradrenaline by dopamine- $\beta$ -hydrolase (D $\beta$ H) (Hagen and Welch, 1956). For adrenaline producing cells, noradrenaline is exported by the noradrenaline transporter M2 (Kippenberger et al., 1999), methylated in the cytoplasm by phenylethanolamine N-methyl transferase (PNMT) (Wong et al., 1987), and re-imported back into SGs by VMAT1, using once again, the proton gradient (Henry et al., 1994) (Fig. 23). The refinement of the composition of SG, alongside matrix formation ultimately cause a shrinkage of the vesicle adopting a spherical shape of 100-200nm.

SG maturation coincides with their transport towards the cell periphery, requiring both microtubule and actin-dependent transport (Fig. 23). After budding off from the TGN, initial transport relies on microtubules, while peripheral transport depends on actin (Ñeco et al., 2003; Rudolf et al., 2001). Although no kinesin has been identified for SG transport in chromaffin cells, they express kinesin-1 family members. The latter have been reported to transport SGs in other neurosecretory cells such as neurohypophysis cells (Senda and Yu, 1999) and pancreatic  $\beta$  cells (Varadi et al., 2003). Actin-based transport is mediated mainly by myosin V (Rosé et al., 2003), recruited by Rab27A (Desnos et al., 2003). Whereas no definitive implication of lipids has been reported to date in the transport process of SG, PLD inhibition has been shown to slow down the initial directed motion of SG suggesting a role for PA in this process (Tanguy et al., 2021). Interestingly, PA has been shown to interact with members of the kinesin 1 family such as Kif5b in tumoral cells (Wang et al., 2017).





**Figure 23:** Granulogenesis and transport of secretory granules

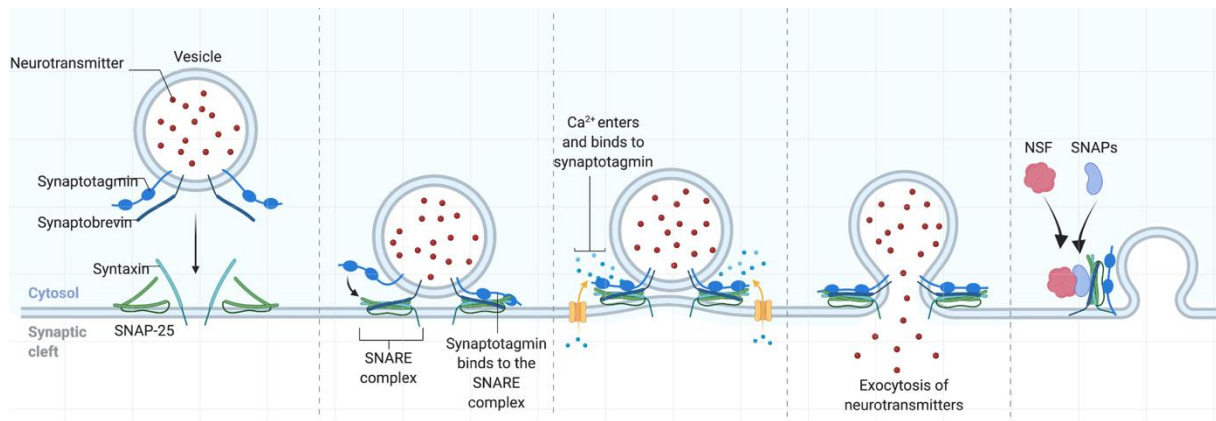
Schematic representation of granulogenesis. 1) neuropeptides condense on the luminal leaflet of the TGN and bud off, 2) After homotypic fusion, undesired proteins and lipids get excluded by generation of clathrin vesicles and 3) Catecholamines get synthesized in the cytoplasm and transported in lumen of SG. Created on [Biorender.com](https://www.biorender.com).

In chromaffin cells, before reaching the PM, SG must first cross a cortical actin mesh that is modulated by small GTPases of the Rho family ([Gasman et al., 1998](#)). In resting condition, actin forms a barrier of parallel fibers to the PM. However, upon calcium entry, these parallel fibers are partially disassembled and newly synthesized actin fibers that are perpendicular to the PM guide SG through 300-400nm channels toward the PM ([Giner et al., 2005](#)). When chromaffin cells are stimulated, the activation of PM neural Wiskott-Aldrich syndrome protein (N-WASP), a downstream effector of Cdc42, induces a reorganization of the cortical actin mesh ([Frantz et al., 2002](#); [Gasman et al., 2004](#)). This results in increased vesicle mobility preceding fusion ([Degtyar et al., 2007](#)). Additionally,

SG are anchored to the PM via actin bundles recruited by annexin A2 ([Gabel et al., 2015](#)). These proteins can be recruited to active exocytosis sites by negatively charged lipids like PI(4,5)P2 ([Chasserot-Golaz et al., 2005](#)), or PA ([Gabel et al., 2019](#)). In line with those observations, PA produced by PLDs is known to modulate cytoskeleton dynamics ([Ammar et al., 2014](#)). However, overexpression of a dominant-negative form of PLD1 did not modulate actin depolymerization in stimulated chromaffin cells, arguing against a major role for PLD1-generated PA at this actin depolymerization step ([Vitale et al., 2001](#)).

## 2. Docking, priming and fusion

Upon reaching their target destination, vesicles associate with specific fusion sites on the PM ([Salaün et al., 2005](#)). These sites position syntaxin1a and the 25kDa synaptosome associated protein (SNAP25) on the PM. These t-SNAREs loosely associate with VAMP-2 present on SGs, inducing docking to form the core SNARE complex, composed of four  $\alpha$ -helices (one from VAMP-2, one from syntaxin1A and two from SNAP-25). This assembly is mediated by the SM protein Munc-18, which binds and opens syntaxin1A for SNAREpin assembly ([Jahn, 2000](#); [Pevsner et al., 1994](#)). Interestingly, Munc-18's MUN domain is structurally like MTCs, suggesting a parallel between initial SNARE complex assembly and vesicle tethering ([Li et al., 2011](#)). The alignment between syntaxin and VAMP occurs primarily in a parallel arrangement and a coiled-coil structure that is bent rather than fully extended, suggesting that the binding of cognate SNARE proteins could serve as the final specificity step before vesicle fusion even while driving the fusion itself. The notion that formation of preassembled SNARE complexes between two membranes is the key for fusion is now widely accepted, and the activity of SNAPs-NSF is believed to be required for disassembly of closed SNARE complexes to recycle the SNAREs (Fig. 24).



**Figure 24:** The SNARE complex cycle

Syntaxin exists in a closed conformation that needs to open to initiate core-complex assembly (nucleation) of the SNARE complex. “Zippering” of the four-helix bundle of syntaxin, SNAP-25 and synaptobrevin/VAMP towards the carboxyl terminus brings the synaptic vesicle and PM towards each other, favoring fusion of the vesicular and PM. After fusion, NSF and SNAPs disassemble the Closed SNARE pin that remain on the same membrane to recycle them for another round of fusion. Created on [Biorender.com](https://www.biorender.com).

Additionally, signaling lipids such as PI(4,5)P2 and PA also participate in positioning fusion sites by directly binding syntaxin1a ([Lam et al., 2008](#); [Umbrecht-Jenck et al., 2010](#)). In resting condition, most of the peripheral SG in chromaffin cells are docked at the PM ([Vitale et al., 1995](#)), with only a minor fraction (1%) able to fuse immediately upon stimulation ([Neher and Zucker, 1993](#); [Sorensen, 2004](#)). Therefore, a calcium- and ATP-dependent priming step is necessary for subsequent fusion. This process involves the small GTPase Rab3a present on the vesicle’s surface; upon activation Rab3 recruits multiple effectors with calcium-sensible C2 domains, such as rabphilin3A ([Shirataki et al., 1993](#)) and Rab interacting molecule (RIM) ([Wang et al., 1997](#)). RIM, in particular, is able to recruit Munc-13, stabilizing this complex in a fusion-ready state ([Stevens et al., 2005](#)). Other proteins like syntaptotagmin-1 and complexin-2, integrate into the SNAREpin. Complexin acts as a clamp to prevent premature closure ([Chen et al., 2002](#); [Dhara et al., 2014](#)), while synaptotagmin functions as a calcium sensor present on SG ([Brose et al., 1992](#)). NSF and  $\alpha$ -SNAP also contribute to the priming process in chromaffin cells by dissociating loosely assembled SNAREpins, enabling a ready state, which explains the ATP dependency of this process ([Xu, 1999](#)). Interestingly, PA can bind NSF, hindering the priming process in liposome fusion assays ([Starr et al., 2019](#)), although *in cellulo* consequences of this interaction remains to be evaluated. PI(4,5)P2 also participates in priming the SNARE complex as increasing its levels favored SG fusion ([Milosevic et al., 2005](#)), while sequestering cellular PI(4,5)P2, using the HIV-1 transcriptional activator

TAT reduced the number of fusion events without affecting their kinetics ([Tryoen-Tóth et al., 2013](#)).

In chromaffin cells, SG fusion is primarily induced by massive calcium influx from the extracellular medium following depolarization and activation of voltage gated calcium channels ([Neher and Marty, 1982](#)). It has been established that locally calcium levels can be in the order of several hundreds of micromolar ([Chow et al., 1994](#)). Synaptotagmin family members are widely accepted as the main calcium sensor in neurosecretion. Mammals express 17 synaptotagmin isoforms, where most studies have focused on isoforms 1, 2, and 7, which are involved in synaptic vesicle exocytosis. Intriguingly these various isoforms also display different affinity for calcium, which might partially explain the difference in calcium sensitivity of different neuronal types and within the same neuron of different types of synaptic vesicles ([Wolfes and Dean, 2020](#)). Generally, in the presence of high calcium concentrations, synaptotagmins associate with negatively charged lipids such as PI(4,5)P<sub>2</sub>, or PS on the PM ([Brose et al., 1992](#); [Schonn et al., 2008](#)). During this process, synaptotagmin displaces complexin from primed SNAREpins, leading to the rapid zippering of the SNARE pin ([Giraud et al., 2006](#)) and bringing the PM and SG closer together.

The formation of a fusion pore has also been proposed to depend on the formation of hemifusion intermediates based on the geometric arrangement of lipids in membranes as described previously. PA production by PLD1 may play a key role in this step and subsequent pore expansion thanks to its cone-shaped structure. Silencing PLD1 does not only reduce the number of fusion events but also slows down the kinetics of the remaining events. These kinetic effects are likely to be influenced either by PA interacting proteins and by the negative membrane curvature induced by this lipid. In line with this model the inhibitory effect on exocytosis of the expression of a dominant-negative catalytically dead PLD1 mutant can be rescued by adding lysophosphatidylcholine (a lipid with an inverted cone-shaped geometry, the opposite geometry of PA) on the external leaflet of the PM ([Zeniou-Meyer et al., 2007](#)).

Four modes of fusion have been identified in chromaffin cells, depending on the type of stimulation and on cytoplasmic free calcium levels ([Marengo and Cárdenas, 2018](#)) (Fig. 25):

- **Kiss-and-Run:** The fusion pore opens transiently, releasing a small amount of catecholamine but not larger molecules such as peptides due to flickering and low diameter of the initial pore during membrane fusion (Fulop et al., 2005). Kiss-and-Run is believed to occur at low frequency stimulation and low cytoplasmic calcium concentrations (Elhamdani et al., 2006a)
- **Cavicapture:** The fusion pore opens enough to release catecholamines and neuropeptides but stops before the vesicle releases all its content. During cavicapture SG retain their  $\Omega$  shape and do not fully flatten with the PM (Perrais et al., 2004; Taraska et al., 2003).
- **Full collapse:** The SG flattens and releases all its content, leading to the classical flat shaped fusion vesicle (Elhamdani et al., 2006a). This is the main mode of exocytosis in chromaffin cells upon intense stimulation.
- **Compound exocytosis:** A second (or multiple) SG fuses with another vesicle that is currently fusing, enabling the release of the content of multiple vesicles from one fusion site on the PM (Ge et al., 2022).

### 3. Compensatory endocytosis

Although endocytosis of SG material after neurosecretion has been less studied than exocytosis, it should not be considered separately, as it is essential altogether for maintaining cellular homeostasis, membrane integrity and the proper functioning of cellular activities. Furthermore, compensatory endocytosis has been proposed to control the amount of catecholamines and neuropeptides released by prematurely closing the fusion pore (Perrais et al., 2004). Recently, four modes of endocytosis have been identified in chromaffin cells: ultrafast ( $>0.6\text{sec}$ ), fast ( $>6\text{sec}$ ), slow ( $<6\text{sec}$ ) and bulk endocytosis (Shin et al., 2021). These recycling mechanisms are heavily dependent on cytosolic calcium concentrations and are closely related to exocytic modes (Fig. 25).

Ultrafast endocytosis, given its rapid kinetic has been proposed to correspond to the retrieval part of the kiss-and-run exocytosis of SG (Fulop et al., 2005; Perrais et al., 2004; Shin et al., 2021).

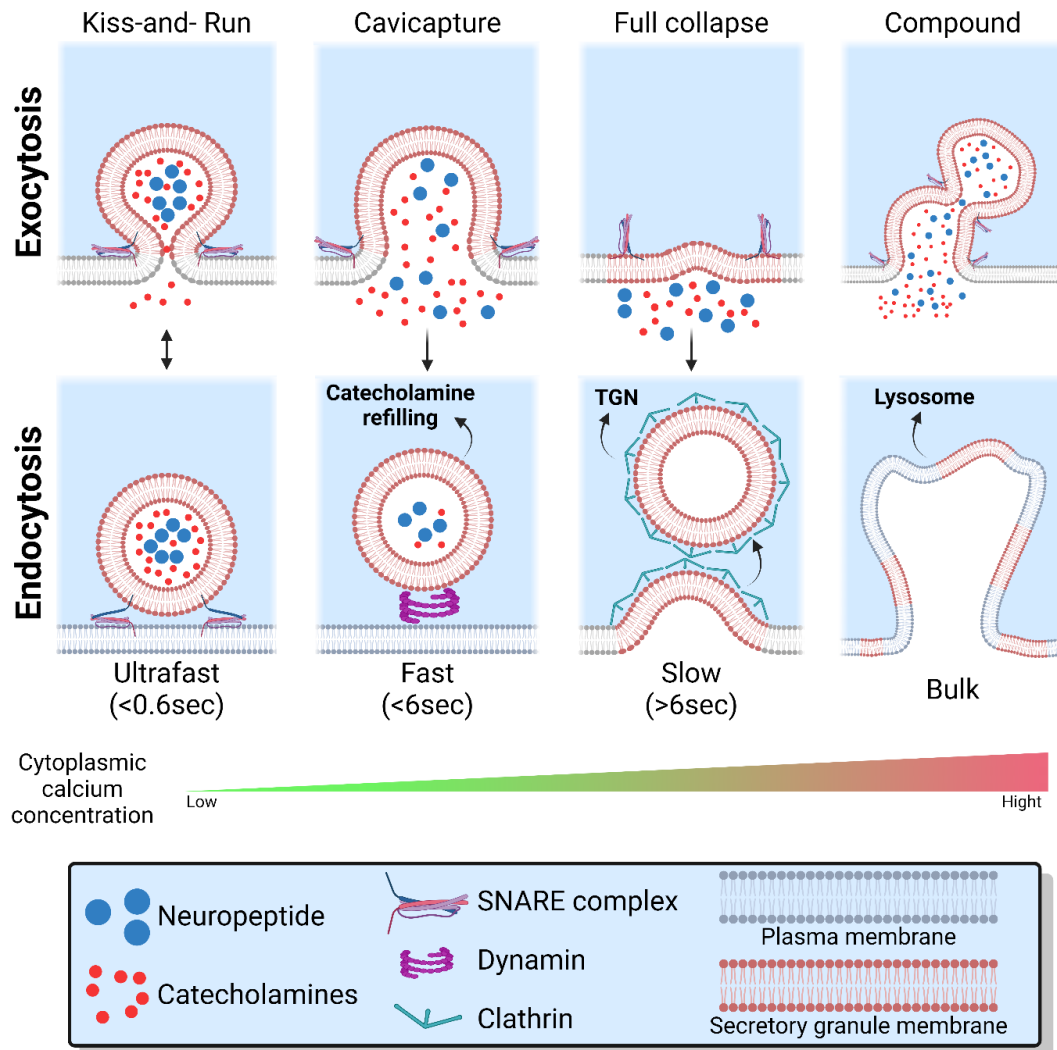
Fast endocytosis has been linked to cavicapture, halting the release of catecholamines and neuropeptides before the vesicle releases all its content (Perrais et al., 2004; Taraska et al., 2003). While the full mechanisms of fusion pore closure remain unclear, fast



endocytosis is known to be clathrin-independent but requires dynamin-1 to sever the vesicle (Artalejo et al., 2002, 1995). Dynamin is proposed to be recruited to fusing SG by synaptophysin (González-Jamett et al., 2010), a protein present on SG of chromaffin cells (Obendorf et al., 1988). The vesicles retrieved by fast endocytosis can undergo another round of fusion after catecholamine refilling in the cytoplasm (Elhamdani et al., 2001).

Slow endocytosis occurs after full collapse of SG and flattening with the PM. It was the first proposed mechanism for compensatory endocytosis (Phillips et al., 1983). This process is initiated by the recruitment of AP-2 by PI(4,5)P<sub>2</sub> present at the PM (Antonescu et al., 2011), thus triggering the budding of a vesicle in a clathrin-coated pit which is severed from the PM by dynamin-2 (Artalejo et al., 2002), recruited by amphiphysin (Graham et al., 2002). Despite not directly occurring at exocytotic sites, this process fully retrieves the original membrane of the previously fused SG in the newly formed endosomes (one SG can be recycled by multiple endosomes thought) (Ceridono et al., 2011). This peculiarity has been used to determine the recycling pathway of SG after full collapse using antibodies targeting SG membrane proteins such as D $\beta$ H. Endosomes containing recycled SG membrane proteins first fuse with EE (Ceridono et al., 2011), before being dispatched towards the TGN or lysosomes. At the TGN, SG can be reformed and are visible in the cytoplasm as early as 30min after the stimulation (Patzak and Winkler, 1986). However, the precise trafficking pathway of this recycling route still needs to be elucidated. In addition to PI(4,5)P<sub>2</sub>, PA might also play a role as a signaling lipid in slow endocytic recycling, as PLD inhibition delays D $\beta$ H internalization following stimulation, but more detailed investigations are needed to fully understand these mechanisms (Tanguy et al., 2021).

Bulk endocytosis has rarely been studied in chromaffin cells, with only one other group mentioning it besides the team of Ling-Gang Wu (Perez Bay et al., 2007). This process is induced when the PM surface extension exceeds 20% of the normal value occurring after a strong calcium influx, generating long tubular endosomes in the cytoplasm that colocalize with lysosomal markers. It is the only compensatory endocytosis mechanism that cannot specifically recycle the original SG membrane components but rather captures large membrane patches from the PM (Perez Bay et al., 2011).



**Figure 25:** Exocytosis-endocytosis coupling in chromaffin cells

Schematic representation of the 4 modes of exocytosis and endocytosis in chromaffin cells and how they are coupled. Only certain proteins specific to each mode are represented. Kiss-and-run exocytosis is followed by ultrafast endocytosis. Cavicapture is rendered possible by fast endocytosis. Full collapse fusion leads to slow endocytosis. All three of those coupled membrane trafficking events maintain the composition of SGs. Other mode of types of fusion and endocytosis like compound exocytosis and bulk endocytosis have also been reported upon intense stimulations. Created on [Biorender.com](https://www.biorender.com).

In summary, the neurosecretory process in chromaffin cells heavily relies on multiple vesicular trafficking steps. Vesicle biogenesis requires membrane budding from the TGN, which is then followed by their maturation along their transport towards cell periphery. Cytoskeleton-dependent transport displaces the vesicles towards the periphery, and membrane fusion is necessary for granule maturation during homotypic fusion of immature SG. Tethering, docking, and priming precede fusion of the mature SG with the PM, and compensatory endocytosis terminates the cycle. Throughout this process, chromaffin cells use membrane properties to their full extent, generating



microdomains to sequester important proteins in the appropriate regions and promoting membrane deformation. The neurosecretory process also exemplifies the versatility of signaling lipids, with PIPs controlling SG biogenesis, exocytosis and endocytosis. PA, though less studied than PIPs, has been also shown to be involved in neurosecretion through its production by PLDs and interaction with important proteins of the late secretory pathway. For instance, PA interacts with CgA to initiate SG budding ([Carmon et al., 2020](#)), promotes annexin A2 recruitment to facilitate actin bundle fasciculation, thereby anchoring SG to the PM ([Gabel et al., 2019](#)) and interacts with the SNARE protein syntaxin1 ([Lam et al., 2008](#)). PA was also reported to interact with PI4P-5-kinases, promoting PI(4,5)P<sub>2</sub> production ([Roach et al., 2012](#)) which in turn is a positive regulator of PLD1 ([Henage et al., 2006](#)) making it challenging to distinguish between PA or PI(4,5)P<sub>2</sub>-dependent effects with our current molecular tools.

#### **D. The distinct functions of mono- and polyunsaturated phosphatidic acid in neurosecretion**

Over the last two decades, to get a better understanding of the role of PA produced by PLD1 in regulated exocytosis, our team has developed molecular sensors for PA and used diverse genetic (silencing, KO, overexpression of mutants) and pharmacological approaches targeting PLDs, complemented by rescue experiments with different species of PA to probe their potential involvement in distinct steps along the secretory pathway. The following review summarizes the finding of the team on this topic, by mostly focusing on the recent work showing that monounsaturated PA control the number of fusion events and thus likely docking/priming steps, whereas polyunsaturated PA regulate the kinetics of individual fusion events and thus probably the dynamics of the fusion pore ([Tanguy et al., 2020b](#)). A summary of the specific implications of different PA species in neurosecretion is also presented:

#### **Phosphatidic acid: Mono- and poly-unsaturated forms regulate distinct stages of neuroendocrine exocytosis**

Tanguy, E., [Wolf, A.](#), Montero-Hadjadje, M., Gasman, S., Bader, M.-F., and Vitale, N.

*Advances in Biological Regulation* 100772 (2020)

[10.1016/j.jbior.2020.100772](https://doi.org/10.1016/j.jbior.2020.100772)



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## Phosphatidic acid: Mono- and poly-unsaturated forms regulate distinct stages of neuroendocrine exocytosis

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## ARTICLE INFO

## Keywords:

Exocytosis  
Fatty acid  
Phosphatidic acid  
Phospholipase D  
Neuroendocrine secretion

## ABSTRACT

Lipids have emerged as important actors in an ever-growing number of key functions in cell biology over the last few years. Among them, glycerophospholipids are major constituents of cellular membranes. Because of their amphiphilic nature, phospholipids form lipid bilayers that are particularly useful to isolate cellular content from the extracellular medium, but also to define intracellular compartments. Interestingly, phospholipids come in different flavors based on their fatty acyl chain composition. Indeed, lipidomic analyses have revealed the presence in cellular membranes of up to 50 different species of an individual class of phospholipid, opening the possibility of multiple functions for a single class of phospholipid. In this review we will focus on phosphatidic acid (PA), the simplest phospholipid, that plays both structural and signaling functions. Among the numerous roles that have been attributed to PA, a key regulatory role in secretion has been proposed in different cell models. We review here the evidences that support the idea that mono- and poly-unsaturated PA control distinct steps in hormone secretion from neuroendocrine cells.

## 1. Introduction

Lipids found in eukaryotic membranes can be divided in three major classes including glycerophospholipids, sphingolipids, and sterols. Sophisticated techniques led to the identification of more than 30,000 different lipids suggesting that these molecules support many biological processes in addition to the well-known energy provider and compartment delimitation roles. Although their synthesis pathway, subcellular distribution as well as mechanisms by which they move from one compartment to another are getting better

**Abbreviations:** ADR, Acyldihydroxyacetone-phosphate reductase; CDP, Cytidyl phosphatidate; CDS, CDP-diacylglycerol synthase; CgA, Chromogranin A; CL, Cardiolipin; DAG, Diacylglycerol; DGK, Diacylglycerol kinase; DHAP, Dihydroxyacetone phosphate; DHAP AT, DHAP acyltransferase; FIPI, 5-Fluoro-2-indolyl des-chlorohalopemide; G3P, Glycerol 3-phosphate; GPAT, G3P acyltransferase; LPA, Lysophosphatidic acid; LPAAT, Lysophosphatidic acid-acyltransferases; PA, Phosphatidic acid; PABD, PA-binding domain; PAP, Phosphatidic acid phosphohydrolase; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PI, Phosphoinositides; PLA, Phospholipase A; PLD, Phospholipase D; PS, Phosphatidylserine; TG, Triglycerides.

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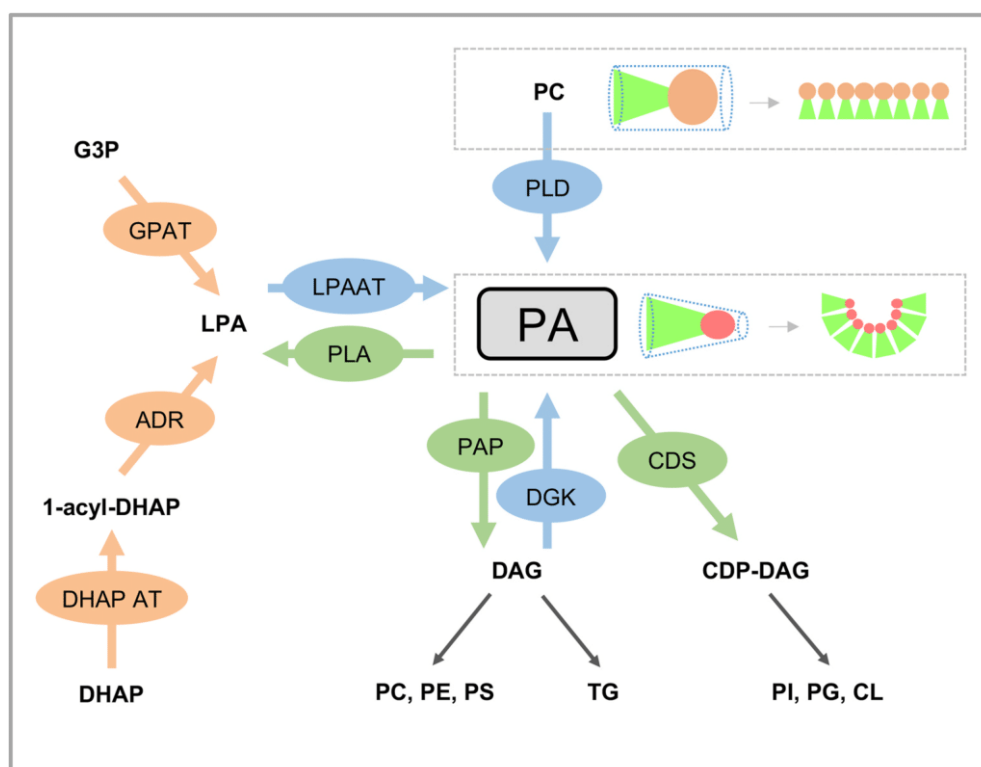
Received 10 November 2020; Received in revised form 16 November 2020; Accepted 25 November 2020

Available online 28 November 2020

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The acyl chain diversity in glycerophospholipids has been studied for decades, but our understanding of its contribution to cell function has remained elusive for many years, largely because of technical and conceptual limitations. Phospholipids are divided in families based on the chemical nature of their headgroup. By order of quantities found in eucaryotic membranes, the main families of phospholipids are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphoinositides (PI) and phosphatidic acid (PA). They can exist both as monoacyl- or diacyl-glycerophospholipid forms. Variety in acyl chain in position *sn*-1 and *sn*-2 further extends the diversity of phospholipids. These chains are divided in three classes: saturated, mono- and polyunsaturated. Based on the location of the polyunsaturation, the latter class has been further divided in subclasses of which the omega-3 ( $\omega$ 3, e.g. docosahexaenoic acid, C22:6) and the omega-6 ( $\omega$ 6, e.g. arachidonic acid C20:4) are the most popular.

Among phospholipids, PA is an important intermediate metabolite in the synthesis of all membrane glycerophospholipids and is therefore playing an important structural role in living cells by contributing to membrane biogenesis. Chemically, PA consists of a glycerol backbone to which are attached, through esterification, two fatty acyl chains and a phosphate at positions *sn*-1, *sn*-2, and *sn*-3, respectively. The unique feature of PA compared to the other diacyl-glycerophospholipids is its phosphomonoester link to a small anionic phosphate headgroup (Jenkins and Frohman, 2005; Tanguy et al., 2018). The small headgroup of PA predicts that it adopts a cone-shaped structure in membranes and X-ray diffraction studies have contributed to postulate that local accumulation of PA in one leaflet of membranes modifies their topology by creating a negative curvature (Kooijman et al., 2005). This property of PA is often linked to its contribution in diverse membrane trafficking events (Ammar et al., 2013b; Tanguy et al., 2016). PA serves also as an essential signaling molecule in diverse cellular processes, through the recruitment of a broad range of cytosolic proteins to appropriate membrane locations (Kim and Wang, 2020; Tanguy et al., 2018, 2019b).



Enzymatic pathways highlighted in orange are involved in structural PA synthesis, whereas enzymes in blue lead to the formation of signaling pools of PA. Among this last category, PLD activity transforms the cylindrical phospholipid PC into PA, a conical one, potentially inducing local negative membrane curvature. Enzymes triggering PA catabolism are shown in green.



and neuroendocrine exocytosis (Bader and Vitale, 2009; Vitale et al., 2001; Waselle et al., 2005), mast cell degranulation (Choi et al., 2002), endothelial cell secretion (Disse et al., 2009), acrosomal exocytosis (Lopez et al., 2012), exosome biogenesis and secretion (Ghossoub et al., 2014) and even the fast synaptic neurotransmission (Humeau et al., 2001; Schwarz et al., 2011; Raben and Barber, 2017). PA is also implicated in autophagy (Holland et al., 2016) and phagocytosis (Corrotte et al., 2006; Tanguy et al., 2019a), intracellular pathways such as tubular endosome biogenesis (Giridharan et al., 2013) and Golgi vesicle formation (Ktistakis et al., 1996), membrane wound repair (Vaughan et al., 2014) and neurite outgrowth (Ammar et al., 2013a). However, despite or perhaps due to these large implications, precise functions of PA along the secretory pathway remain unclear. The diversity of the PA biosynthetic routes together with the possible occurrence of many different PA species based on their fatty acyl chain composition open the possibility for multiple roles in a given cellular function.

## 2. The different sources of phosphatidic acid

In mammals, cellular PA production involves several major routes (Pokotylo et al., 2018; Zegarlińska et al., 2018; Tanguy et al., 2019b) (Fig. 1). Structural PA is synthesized through two acylation reactions: the glycerol 3-phosphate (G3P) pathway and the dihydroxyacetone phosphate (DHAP) pathway, both leading to lysoPA (LPA), a mono-acylated form of PA. LPA is subsequently transformed into PA during a second acylation step (Vance and Vance, 2004). PA generated from these *de novo* pathways is the main intermediate in the synthesis of all triacylglycerols and glycerophospholipids, as it is actively transformed by PA phosphohydrolase (PAP) into diacylglycerol (DAG) in the endoplasmic reticulum (Vance and Goldfine, 2002). PA can also be transformed by cytidyl phosphatidate (CDP)-DAG synthetase (CDS) into CDP-DAG, which fuels the synthesis of PI, phosphatidylglycerol (PG) and cardiolipin (CL). Different phospholipases A (PLA) can also deacylate PA into LPA.

Additionally, three alternative biosynthetic pathways use distinct lipid precursors to produce so-called “signaling” PA (Fig. 1). The first pathway involves phosphorylation of DAG to produce PA using ATP as a phosphate source by one of the 10 different DAG kinase (DGK). The second pathway involves acylation of LPA by specific LPA-acyltransferases (LPAAT) to generate PA (Jenkins and Frohman, 2005). Six human LPAAT isoforms have been cloned and characterized (Leung, 2001), but more proteins displaying LPAAT activities have recently been identified. For instance, the RIBEYE protein in synaptic ribbon enabling ultra-fast synaptic release possesses an LPAAT activity providing PA (Schwarz et al., 2011). The third pathway involves phospholipase D (PLD), which catalyzes the hydrolysis of the distal phosphodiester bond of PC to form PA and choline. Six isoforms of PLDs have been identified based on sequence homology, among them PLD1 and PLD2 being the best characterized isoforms. It is of note that until now no direct PLD activity *per se* has been documented for the PLD3-5 isoforms (Frohman, 2015). The complexity and diversity of PLD, DGK and LPAAT families suggest that they are involved in various specific cellular functions which are, probably, not redundant. In general, signaling PA generated from these enzymes is implicated in multiple processes such as cytoskeleton organization, cell survival, proliferation, membrane and vesicle trafficking (Nelson and Frohman, 2015). Intriguingly PC to PA conversion by PLD transforms a cylindrical phospholipid into a conical one. Since biophysical models have proposed that intermediates of the membrane fusion reaction require such cone- or inverted cone-shaped lipids, a direct role of PA as a fusogenic lipid has been proposed (Vitale, 2010). Interestingly, PLD localization to specific membranes compartments depends on key domains of the enzymes (Du et al., 2003) and probably involves deciphering the phosphoinositide landscape of intracellular membranes (Sztacho et al., 2019).

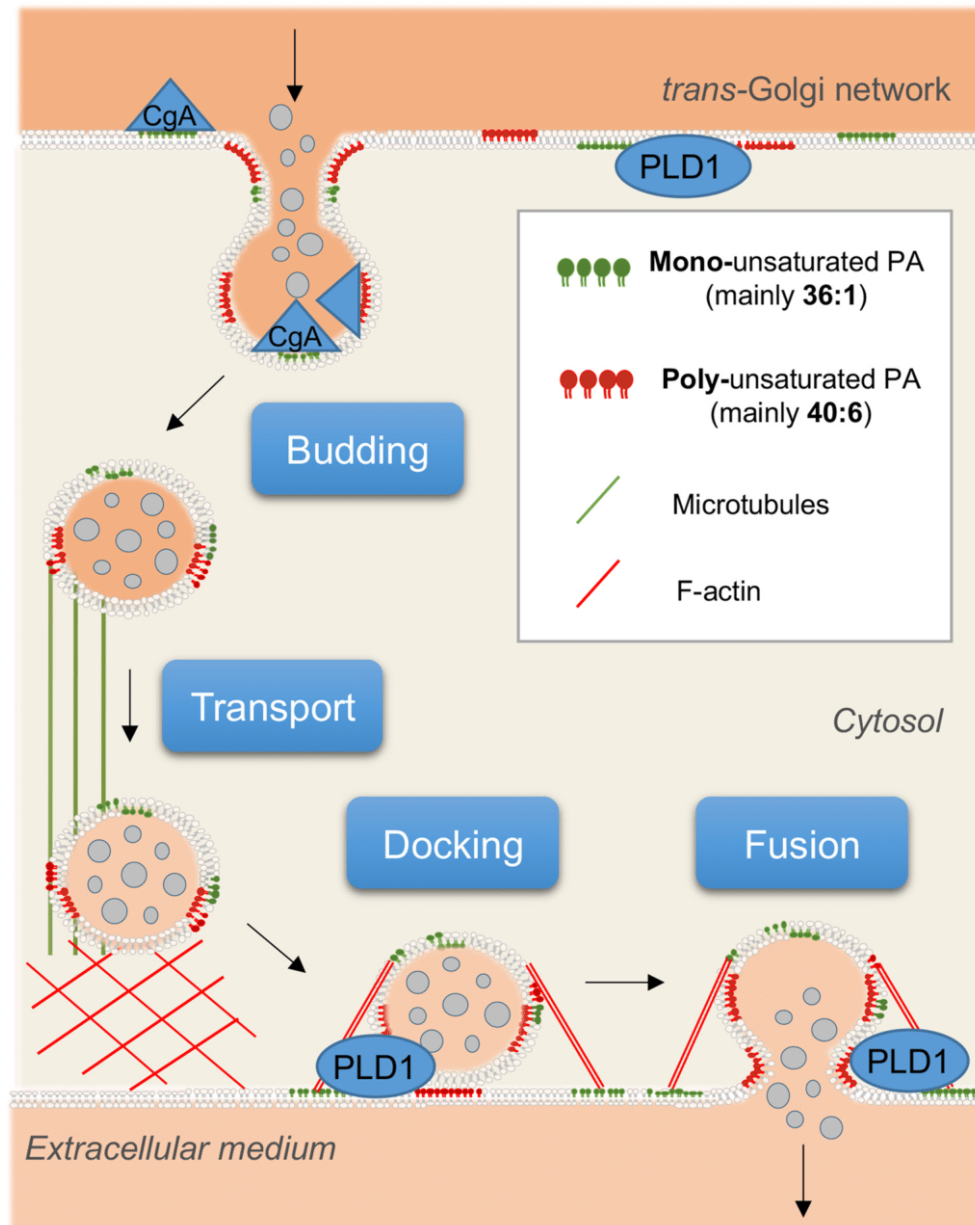
## 3. Role of phosphatidic acid in neuroendocrine exocytosis

The requirement for PA in calcium-regulated exocytosis in neuroendocrine and endocrine cells is probably one of the best documented role for PA in membrane trafficking (Bader and Vitale, 2009). Early studies in bovine chromaffin cells reported the inhibitory action of butanol on exocytosis through the inhibition of PA synthesis by PLD (Caumont et al., 1998; Galas et al., 1997). These observations were supported by experiments in different cell models (Choi et al., 2002; Hughes et al., 2004; O’Luanaigh et al., 2002), but the lack of specificity of butanol has later been questioned (Su et al., 2009). Overexpression of wild type and dominant-negative PLDs provided the first direct molecular evidence supporting the involvement of PA generated by the PLD1 isoform in hormone release in neuroendocrine chromaffin cells (Vitale et al., 2001). PLD1 silencing experiments further supported the notion that PLD1 is the major source for PA synthesis during exocytosis in chromaffin cells (Zeniou-Meyer et al., 2007). Furthermore, the use of a molecular sensor for PA revealed a predominant calcium-stimulated accumulation of PA at the plasma membrane in cells undergoing exocytosis (Zeniou-Meyer et al., 2007). Additional studies highlighted a tight regulation of PLD1 activity by several upstream signaling pathways involving small GTPases and the kinase RSK2 (Béglé et al., 2009; Momboisse et al., 2009; Vitale et al., 2005, 2010; Zeniou-Meyer et al., 2008), revealing a finely tuned production of PA at granule docking sites in stimulated chromaffin cells. Finally, in line with the selective production of PA near exocytotic sites at the plasma membrane, capacitance recordings of chromaffin cells silenced for PLD1 indicated that this enzyme controls the population of fusion competent secretory granules at the plasma membrane (Zeniou-Meyer et al., 2007).

More recently, electron microscopy and morphometric analysis on mouse adrenal gland slices revealed a reduced number of secretory granules in *Pld1* knockout mice adrenal chromaffin cells (Carmon et al., 2020) and a 50% reduction of the number of secretory granules visually docked at the plasma membrane (Tanguy et al., 2020). Supporting the idea that PA might play a role in the biogenesis of dense-core secretory granules, the dual PLD1/PLD2 inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) reduced the number of secretory granules synthesized in a COS-7 cell line expressing chromogranin A (CgA), a member of the granin family playing an important role in granule biogenesis (Carmon et al., 2020). In line with these observations, a PA-binding domain (PABD) was found in the CgA sequence that effectively binds to PA in membrane models, and the overexpression of a CgA PABD-deleted form in

endocrine cells significantly reduced the number of secretory granules indicating that the “granulogenic” activity of CgA might involve its ability to bind PA (Carmon et al., 2020).

Carbon fiber amperometry recordings of individual mouse chromaffin cells revealed that *Pld1* knockout decreased by nearly 60% the frequency of amperometric events corresponding to single granule fusion, in good agreement with the apparent reduction of the number of morphologically docked granules seen in the *Pld1*<sup>-/-</sup> adrenal gland (Tanguy et al., 2020). Shape analysis of individual amperometric spikes and pre-spikes, which provide information on kinetic parameters of single fusion events revealed that the kinetics of the pore dilation and the duration of the exocytotic event were affected by the absence of PLD1 (Tanguy et al., 2020). Supporting the



**Fig. 2.** Model depicting the different PA species involved in neuroendocrine secretion.

Secretory granules are formed by budding from the Golgi apparatus and then actively transported toward the plasma membrane, where they undergo regulated exocytosis to release their cargo into the extracellular medium. Among lipids involved in this journey, specific PA species can modulate different steps of the regulated secretory pathway. PLD1 contributes to the synthesis of mono- and poly-unsaturated PA species found in the *trans*-Golgi network membrane, where they may promote granule biogenesis through their interaction with CgA. Stimulation of exocytosis increases the synthesis by plasma-membrane bound PLD1 of mono-unsaturated PA that favors docking of granules to the plasma membrane and poly-unsaturated PA promoting membrane curvature and fusion pore formation. Note that PA can rapidly flip across membrane leaflets when it is not in tight interaction with specific proteins, suggesting that PA can probably distribute between the two leaflets of granule membranes.



physiological relevance of these *in vitro* observations, catecholamine levels in the blood of new-born mice were reduced by *Pld1* knockout (Tanguy et al., 2020). These observations were further substantiated by the use of FIPI and by the isoform-selective PLD1 inhibitor on bovine chromaffin cells that mimicked the effect observed by amperometry on *Pld1*<sup>-/-</sup> mouse chromaffin cells (Tanguy et al., 2020). Altogether, these findings indicate that the production of PA by PLD1 controls several stages of the neuroendocrine secretory pathway including biogenesis of secretory granules, the number of secretory granules undergoing exocytosis, and also at the single granule level, the duration of the initial fusion pore and its expansion required to release neuropeptides and peptide hormones (Fig. 2). This composite role of PA led to the idea that the sequential stages underlying the secretory pathway could actually require distinct PA subspecies involved in specific functions.

#### 4. Specific PA species contribute to different steps in neuroendocrine cell exocytosis

In neuroendocrine cells, subcellular fractionation experiments revealed that Golgi, secretory granule and plasma membranes contained detectable levels of up to 40 different PA species (Carmon et al., 2020; Tanguy et al., 2020). Secretory granules were particularly enriched in poly-unsaturated forms of PA, especially PA 40:6 containing docosahexaenoic acid (DHA), a major  $\omega$ 3 fatty acid (Tanguy et al., 2020). Stimulation of exocytosis selectively increased the plasma membrane level of PA containing long chain mono- and di-unsaturated fatty acids (PA 36:1, 38:1 and 38:2), by more than 6-fold (Tanguy et al., 2020). These peculiar PA are largely decreased in adrenal glands from *Pld1* knockout mice, suggesting that PLD1 known to be activated in stimulated cells is most likely responsible for their synthesis at the plasma membrane (Tanguy et al., 2020).

Incubation of cultured chromaffin cells with PA micelles proved to be an easy and effective approach to identify the PA species able to compensate for the lack of PLD activity at the plasma membrane of PLD1-depleted cells. Indeed, exogenous PA rapidly reached the intracellular leaflet of the plasma membrane as seen using the PA sensor Spo20p-GFP which was recruited to the cell membrane within minutes when cells were incubated with PA micelles (Tanguy et al., 2020). Incubation of chromaffin cells with a mixture of various forms of PA did not significantly affect catecholamine secretion *per se*. However, it rescued secretion from cells treated with PLD1 inhibitors (Tanguy et al., 2020). Interestingly, mono- and di-unsaturated forms of PA rescued secretion in PLD1-depleted cells, whereas poly-unsaturated PA did not (Tanguy et al., 2020). Using a combination of amperometric recordings and ultrastructural observations on plasma membrane sheets, we observed that mono- and di-unsaturated forms of PA are apparently involved in granule recruitment and/or docking thereby controlling the number of exocytotic events (Tanguy et al., 2020). In contrast, poly-unsaturated  $\omega$ 3 PA seem to be specifically implicated in fusion pore dilation (Tanguy et al., 2020). At present, it is not known if secretory vesicle biogenesis requires a specific type of PA. Both PA (36:1) and PA (40:6) identified as predominant species in Golgi and secretory granule membranes interact with CgA and promote CgA-induced membrane deformation and remodeling (Carmon et al., 2020).

Based on these findings, we propose that various steps preceding dense-core granule exocytosis require distinct PA species, with mono- and di-unsaturated forms of PA being preferentially implicated in granule docking whereas poly-unsaturated  $\omega$ 3 PA favors subsequent fusion pore dilation (Fig. 2).

#### 5. Proteins interacting preferentially with specific species of PA

One possibility to explain the differential effects of mono- and poly-unsaturated PA species on exocytosis is that they recruit different effectors involved specifically in vesicle docking or fusion. Among the nearly 50 different proteins that have been shown to date to bind to PA (Pokotylo et al., 2018; Tanguy et al., 2019b; Zegarlińska et al., 2018), their ability to interact with specific forms of PA has not been extensively evaluated, but some information on these aspects are starting to emerge. Using an *in vitro* fluorescence liposome-based assay, we recently found that the PA-binding domain of Spo20p, Opi1p, and PDE4A1 proteins are partially sensitive to the fatty acyl length and saturation, displaying a clear preference for mono- and poly-unsaturated PA over saturated PA (Kassas et al., 2017). Alternatively, the catalytic domains of *Clostridium sordellii* lethal toxin and related large clostridial glucosylating toxins preferentially recognize saturated PA (Varela Chavez et al., 2015). Muscle creatine kinase specifically binds to PA containing saturated fatty acid- and mono-unsaturated fatty acid, but not to poly-unsaturated PA (Hoshino et al., 2019), whereas L-lactate dehydrogenase selectively interacts with poly-unsaturated PA (Hoshino and Sakane, 2020). Thus, PA-binding proteins seem to be able to discriminate between the diverse forms of PA, although the clues for fatty acid selectivity of the PA-binding domain remain obscure.

Another intriguing mode of interaction has been reported for the dynamin-related protein 1 as it interacts with the headgroup of PA and with the saturated fatty acids of nearby phospholipids (Adachi et al., 2016). Moreover, atomistic molecular dynamics simulations of artificial membranes made of PC and PA revealed that the fatty acyl chain composition of PA affected not only membrane fluidity, but also depth of the PA phosphate groups compared to the PC phosphate groups, which can also influence the presentation of PA for peripheral membrane proteins, affecting their accessibility for binding (Kulig et al., 2019). Finally, even though binding between PA and specific proteins are electrostatic at first, it has been predicted that these links are later solidified through hydrophobic interaction between the acyl chains of PA and apolar groups that can be found in the PA-binding domains (Kooijman and Burger, 2009; Tanguy et al., 2018). Therefore, variation in the hydrophobic amino acids composing these PA-binding domains could partially explain an interaction preference with specific PA species. It is important to note that these kinds of interactions could be exclusive to cone-shaped lipids, such as PA, because they induce lipid packing defects enabling a better and deeper penetration into cellular membranes.

The SNARE protein syntaxin-1 is one of the first target candidate that comes in mind as a target protein in the secretory machinery, as it has been shown to bind to several anionic lipids including PI and PA (Lam et al., 2008). Interestingly, mutations in the polybasic site, abolishing the ability of syntaxin-1 to bind PA, resulted in a reduction of the number of amperometric spikes and increased the pre-spike foot duration in PC12 cells (Lam et al., 2008), very similarly to the amperometric traces obtained in *Pld1*<sup>-/-</sup> mice chromaffin

cells or in bovine chromaffin cells with PLD1 activity pharmacologically inhibited (Tanguy et al., 2020). A similar domain enriched in positive charges that could potentially bind to anionic PA has also been recently described in vesicular SNAREs, such as VAMP-2, and reported to be required for membrane destabilization and fusion (Rathore et al., 2019). It is also worth to recall that the yeast PA binding protein Spo20p is the orthologue of the SNARE protein SNAP25. *In vitro* experiments support the idea that PA directly affects SNARE complex assembly and/or zippering (Mima and Wickner, 2009), although the function of the different PA forms has not been yet tested in this system.

The actin cytoskeleton, originally viewed as a physical barrier preventing granule recruitment, is also emerging as an active network to stabilize granule docking sites, control fusion pore lifetime and/or directly expel granule secretory content (Li et al., 2018). Several actin-binding proteins have been shown to bind to PA, including regulators of the Rho family of small GTPases, which play a key role in providing actin structures required for the late stages of exocytosis (Gasman et al., 2004; Malacombe et al., 2006; Houy et al., 2015). Spatial organization of the exocytotic sites at the plasma membrane also appears under the control of large actin structures bundled by annexin A2 (Gabel et al., 2015), a protein that binds anionic lipids including PA (Gabel et al., 2019). The next challenge will be to determine if the function of these regulators in the exocytotic machinery involves their ability to bind PA and whether they require a partnership with specific PA species.

## 6. Do different PA species differentially affect the fusion pore dynamics?

Fusion of two membranes requires the formation of several non-bilayer intermediates resulting in hemifusion, which is ultimately followed by membrane breakdown and formation of a pore (Chernomordik et al., 1995). For most lipids found in biological membranes, the lamellar bilayer is the most energetically favorable structure. However, cone-shaped lipids, such as PA, promote negative curvature (Kooijman et al., 2005; Monck and Fernandez, 1994) and the formation of hemifusion intermediates (Chernomordik et al., 1993, 1995). Thus, the predicted effect of a local elevation of PA due to the activation of PLD at exocytotic sites would be to promote membrane bending, particularly in the presence of calcium (Cornell and Arnold, 1996), thereby facilitating hemifusion and subsequent formation of the exocytotic fusion pore and its dilation.

The ability of PA (40:6) to specifically restore kinetics of individual amperometric events in PLD1-depleted chromaffin cells suggests that poly-unsaturated PA regulate the speed of catecholamine release and thus the dynamics of the fusion pore. Molecular modeling of different species clearly indicates that the overall geometry of glycerophospholipids is affected by fatty acyl chain composition (Hodgkin et al., 1998). It is thus likely that the ability to affect membrane topology and more specifically to modulate the fusion pore stability may be variable among the different PA species. Clearly additional experimental evidence is now required to prove or refute this possibility, but it is of interest to note that a similar model, based on lipid-induced changes in membrane curvature, has been proposed for endophilin I-mediated recycling of synaptic vesicles at the plasma membrane (Schmidt et al., 1999).

## 7. Conclusion

Lipids are now viewed as essential partners for proteins in membrane trafficking and among them the negatively charged cone-shaped PA is recognized as a key mediator in vesicle exocytosis and membrane fusion events in a variety of cell types including neurons and neuroendocrine cells. The observation that  $\omega$ 3 forms of PA modulate fusion pore dynamics as seen by amperometry opens new routes of investigations. It is possible that the higher flexibility of the acyl chains of poly-unsaturated PA is particularly suited for membrane curvature, thereby reducing the energetic cost of various membrane deformation processes and facilitating fission and fusion events (Manni et al., 2018). It is tempting to propose that poly-unsaturated PA could regulate the fusion mode associated with either a large or a narrow fusion pore (Shin et al., 2018, 2020) or trigger the fusion pore dilation required for secretion of neuropeptides and peptide hormones (Ren et al., 2016). An additional challenge will be to determine if specific PA species contribute to diseases that have been associated to an alteration of PA production. These include intellectual disability diseases such as Fragile-X disease (Tabet et al., 2016) and the Coffin Lowry Syndrome (Zeniou-Meyer et al., 2008), but also neurodegenerative diseases such as Alzheimer (Oliveira et al., 2010) and Parkinson diseases where specific PA subtypes (18:1/18:1 PA) induce structural change and favor aggregation of  $\alpha$ -synuclein, (Mizuno et al., 2017). Interestingly, a recent review also highlighted the important contribution of different DGKs isoforms and their product PA in neuronal pre- and post-synaptic functions and their potential contribution to pathologies (Barber and Raben, 2020). PA generated by PLD has also been proposed to facilitate multiple steps in cancer progression including growth, metabolism, angiogenesis, and mobility (Roth and Frohman, 2018). Although the functions of the individual PA species in these pathologies remain to be precisely established, the ability of exogenous PA to restore neuroendocrine exocytosis by provision of the adequate PA opens a route towards a better understanding of the importance of specific fatty acids in diet to improve human health.

## Additional Information

The authors declare no competing financial interests.

## Fund

This work was supported by grants from the Fondation pour la Recherche Médicale (DEI20151234424) and the Agence Nationale pour la Recherche (ANR-19-CE44-0019) to N.V.



## CRediT authorship contribution statement

**Emeline Tanguy:** Writing - original draft, designed the figures, Writing - review & editing. **Alexander Wolf:** Writing - original draft, designed the figures, Writing - review & editing. **Maité Montero-Hadjadje:** Writing - review & editing. **Stéphane Gasman:** Writing - review & editing. **Marie-France Bader:** Writing - original draft. **Nicolas Vitale:** Writing - original draft, designed the figures, Writing - review & editing, Funding acquisition.

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## Aim of the thesis

At the start of my thesis, the contribution of PA in vesicular trafficking was already a commonly accepted fact and has been demonstrated on multiple secretory models such as in catecholamine release from chromaffin cells (Vitale et al., 2001), aplysia neurotransmitter release (Humeau et al., 2001), mast cells degranulation (Choi et al., 2002), pancreatis  $\beta$  cells insulin release (Hughes et al., 2004) or sperm acrosome release (Lopez et al., 2012). In chromaffin cell exocytosis, the first contribution of this signaling phospholipid that was suspected concerned the formation of the fusion pore (Vitale et al., 2001). Supporting these initial findings was biophysical evidence supporting the notion that PA can directly induce negative membrane curvature (Kooijman et al., 2003) and to interact with SNARE proteins *in vitro* (Mima and Wickner, 2009) and in PC12 cells (Lam et al., 2008). Later, more in-depth studies also revealed the importance of PA in other steps of the neurosecretory process. For example, PLD1 knock-down diminished the number of SG in chromaffin cells (Carmon et al., 2020), whereas pharmacological inhibition of PLDs slowed down directed-motion of secretory granules, reduced the number of docked granules upon stimulation and delayed compensatory endocytosis (Tanguy et al., 2021). These results are also in accordance with the neuronal pathologies that were attributed to a defect of PA production such as in the Coffin-Lowry syndrome (Zenjou-Meyer et al., 2008), in the Fragile X syndrome (Tabet et al., 2016), or other more commonly known neurodegenerative diseases. Indeed, PLD2 knock-out improved Alzheimer's disease linked synaptic dysfunction and cognitive defects in a mouse model (Oliveira et al., 2010) and PA was shown to bind  $\alpha$ -synuclein and to favor development of Parkinson's disease (Mizuno et al., 2017).

The underlying contributions of PA in all those mechanisms remain unknown, and we still don't know how a single signaling lipid can participate in all these diverse steps that are necessary for neurosecretion in a coherent and controlled manner. In other words, how is subcellular membrane recruitment of PA-interacting proteins achieved? Moreover, in the context of regulated exocytosis, how can PA specifically recruit effectors that are involved in membrane fusion during stimulation? These questions have been recently reviewed in *"The phosphatidic acid paradox: Too many actions for one molecule class? Lessons from plants"* written by Pokotylo et al., 2018, where the authors explain the

multiple, sometimes conflicting, roles of PA in *Arabidopsis thaliana*. Supporting the conclusion from a study of our group ([Kassas et al., 2017](#)), they suggested that the ability of PA to bind to specific proteins is, in addition to the availability of partner proteins, also dependent on many additional factors including the physiochemical properties affecting PA binding to (pH, calcium concentrations, phospholipid acyl side chain composition, membrane curvature), which characterize in a unique way each membrane compartment. Furthermore, in the latest studies of the team, lipidomic analyses on fractionated membrane shed light on two important facts:

- i) PA is present in different membrane compartments each with specific levels of the PA species, depending on their acyl chain composition ([Kassas et al., 2017](#)).
- ii) The level of up to 10 different PA species is specifically increased at the PM upon stimulation of exocytosis and mono- versus poly-unsaturated forms of PA regulate two distinct steps of neurosecretion ([Tanguy et al., 2020b](#)).

Unfortunately, up to very recently, there was no tool available to modulate PA levels in specific subcellular compartments. Moreover, it remained difficult to target specifically individual PA subspecies in cells. Therefore, it is now important to tackle both those problems by using novel methods to study the role of this signaling phospholipid in the neurosecretory process. During my thesis I have developed two original methods based, respectively, on optogenetic targeting to specific membrane compartments of enzymes involved in PA metabolism and on innovative PA analogues allowing chick chemistry for *in cellulo* studies on primary neurosecretory cells. Hence the main objective of my thesis was to validate these novel tools and to use them to address some of the unanswered questions regarding the functions of PA during neurosecretion.

# Characterization of novel synthetic PA analogues to study the dynamics and interactome of different PA species in neurosecretion

## I. Objective

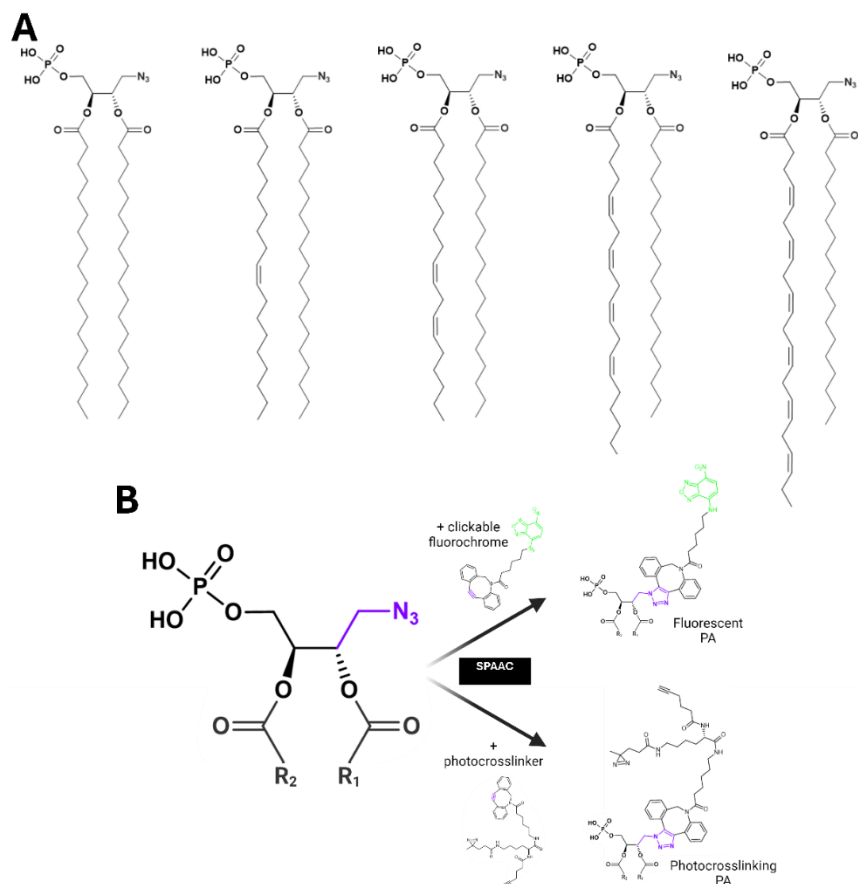
Addition of exogenous PA to cells to perform rescue experiments has proven to be an effective approach to study the functions of various subspecies of this phospholipid ([Tanguy et al., 2020b](#)). We thus reasoned that PA analogues coupled to different molecules (fluorochromes, photocrosslinkers, ...), should be powerful tools to explore the dynamics of various PA species in cells and establish their different interactants. However, while different modified PA analogues already exist and have been successfully used ([Watanabe et al., 2015](#)), those chemical modifications target the acyl chains and in consequence are not suited to distinguish the biological effects of various PA species. Therefore, we worked in collaboration with the chemist team of Dr. Pierre-Yves Renard (COBRA, Rouen, France) to produce new clickable PA analogues with an azide moiety directly linked to the *sn*-1' position of their glycerol backbone (called PA-N<sub>3</sub>). The chemical synthesis of those phospholipids was designed in such a way that, we were able to produce PA harboring the biological diversity of acyl chains in *sn*-2 position such as oleic acid, linoleic acid, stearic acid, arachidonic acid or docosahexaenoic acid (Fig. 26A). Strain-promoted alkyne-azide cycloaddition (SPAAC) was used to modify these lipids by grafting tagged molecules on the azide residue to produce novel fluorescent PA probes (NitroBenzoDiazole (NBD) and Atto647) and photocrosslinkable PAs (PA-DDA1) (Fig. 26B).

Therefore, one of my thesis goals was to characterize these novel synthetic PAs on primary bovine chromaffin cells. This project had three main objectives:

- 1: Determine whether synthetic PAs can enter chromaffin cells and determine whether mono and poly-unsaturated PAs behave differently within cells.
- 2: Functionally validate those new molecules. It was especially crucial to determine whether the distinct effect of natural mono- and polyunsaturated PAs was preserved in the neurosecretory process despite the chemical modifications.



- 3: Establish the protein interactome of mono- and polyunsaturated PAs at different stages of neurosecretion.



**Figure 26:** Novel synthetic clickable PAs

(A) Different synthetic PA-N<sub>3</sub> analogues produced by the group of Dr. P-Y Renard. Each molecule has a stearic acid (18:0) in *sn*-1 position and a different acyl chain in *sn*-2 position, respectively oleic acid (18:1, ω-9), linoleic acid (18:2, ω-9), stearic acid (18:0), arachidonic acid (20:4, ω-6) and docosahexaenoic acid (22:6, ω-3). (B) Example of SPAAC reaction enabling the production of different tools to study each PA isoform (in this case NBD-PA probes and photo crosslinking PAs).

## II. Results

The dynamics of different PA species, functional validation, and their interactome within chromaffin cells is presented in the following paper in preparation for publication:

### **An innovative and versatile synthetic approach in the design of glycerophospholipid tools. Application to phosphatidic acids.**

Antoine Schlichter\*, Alexander Wolf\*, Thomas Ferrand\*, Aurelien Cocq, Lina Riachy, Steven Vertueux, Marine Courvalet, Paul-Joël Henry, Emeline Tanguy, Brice Beauvais, Rémy Ferlet, Fanny Laguerre, Charles Decraene, Alexia Pellissier, Muriel Sebban, Lydie Jeandel, Sarah Cianférani, Jean-Marc Strub, Magalie Bénard, Victor Flon, Valérie Peulon-Agasse, Pascal Cardinael, Stéphane Ory, Stéphane Gasman, Pierre-Yves Renard\*, Maïté Montero-Hadjadje\*, Nicolas Vitale\* & Sébastien Balieu\*

\*: equal contribution to the work

# **An innovative and versatile synthetic approach in the design of glycerophospholipid tools. Application to phosphatidic acids.**

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## Abstract

Glycerophospholipids (GPLs) play an important role in cell compartmentalization, but also contribute to cell signaling. For instance, phosphatidic acid (PA) is a key intermediate in the biosynthesis of glycerophospholipids with more than 40 different members, each potentially displaying specific signaling function. Although many cellular processes in which PA is involved have been identified, the individual role of each PA species remains obscure due to the lack of specific tools. Indeed, the commercially available synthetic PA analogues do not represent the wide structural diversity of natural PA, and their modified versions (fluorophore, (photo)cross linker...) are grafted at the expense of one fatty acyl chain. To circumvent these limitations, we developed a new synthetic approach allowing to preserve the proper fatty acyl chain composition of PA and allowing a versatile grafting of different reporter moieties. This approach revealed the versatility of these novel GPL tools to study their biological activities.

## Introduction

Lipids are essential constituents of all living organisms, but their extreme diversity and the lack of appropriate tools overcomplicated their study<sup>1,2</sup>. Glycerophospholipids (GPLs) are the lipids that constitute the bulk of cellular membranes and play important cellular signaling function. Among GPLs, the phosphatidic acids (PAs) are made of a single phosphomonoester head group, and like all GPLs, naturally occurring PAs vary based on their fatty acid composition<sup>3,4</sup>. PAs have recently attracted considerable attention and have been proposed to play pivotal roles for various cellular functions<sup>3,4</sup>. Accordingly abnormalities in PA producing enzymes are related to numerous diseases including neurodegeneration<sup>5</sup>, intellectual disabilities<sup>6,7</sup>, cancer<sup>8</sup>, fragile X<sup>9</sup> and Coffrin-Lowry syndromes<sup>10</sup>. The coexistence of several hundreds of GPLs in a single cell suggests that they play major yet non overlapping roles. Furthermore, given their rapid and often complex metabolism, it is extremely difficult to know the precise location and role of any given lipid at any given time<sup>11</sup>. GPL analogues aiming at understanding their individual role currently available exhibit modified fatty acid chains (with fluorophore<sup>12</sup> or photocrosslinker<sup>13</sup>) disregarding the role of the fatty acid side chains. Yet, the nature of fatty acyl chains has been proved to play key roles in PA biological activity. To preserve their biological properties, it is anticipated that both the head groups and fatty acyl chains<sup>14</sup> need to be preserved, leaving the glycerol backbone as the relevant place to introduce chemobiological tools. This approach was first developed by Best's team by adding a minimalist clickable azidomethyl moiety on the glycerol core<sup>15</sup>. *In cellulo* studies showed that in this position an azide moiety had little effect on the probe association constants with protein kinase C (PKC) in liposomes, validating this strategy. However, the synthetic route used allows only grafting two identical fatty acyl chains, which does not match the structure of most naturally occurring GPLs. To circumvent these limitations, we developed a novel strategy for the synthesis of azide-based GPL analogues to get appropriate and specific fatty acyl chain positioning for PA species (Figure 1).

## Results and discussion

### Convergent synthesis of GPL-N<sub>3</sub> and GPL probes

To achieve regio-selective grafting of the fatty acyl chains a specific synthetic route was developed together with the grafting of a minimalist clickable moiety on the glycerol core (Figure 1). Furthermore, protection of the phosphate head group to facilitate each purifications steps of the various intermediates was introduced. To obtain the natural stereochemistry of the *sn*-2 position, we chose to begin the synthesis with the dimethyl-L-tartrate as starting material. Several transformations allowed us to differentiate the four carbon atoms of the tartaric backbone giving rise to intermediate **8**, the common building block for all GPL-N<sub>3</sub>. The *sn*-3 position was functionalized with dimethyl phosphate ester, the *sn*-2 was protected with a *p*-methoxybenzyl (PMB) group, the *sn*-1 position was left free and the supplementary carbon compared to the glycerol core, we named *sn*-1', was equipped with an azide moiety. Although the first synthetic steps led to high yields (see Figure 1a and SI), an unexpected deprotection of the phosphate ester occurred during the nucleophilic substitution of the tosylate **7** by sodium azide leading to **9**. Although the reaction between lithium azide, and to a lesser extent sodium azide, is well known for cleaving methyl phosphate monoesters<sup>16</sup>, it was expected that tosylate displacement by an azide would be selective over this ester cleavage. This was not the case whatever the modification in temperature, number of equivalents, azide source, solvents and leaving groups we tested. The best compromise found was to realkylate the deprotected phosphate of **9** using TMSCHN<sub>2</sub><sup>17</sup> giving **8** in an overall 11% yield in 7 steps (Figure 1-a). The second part of the synthetic route afforded four different PA-N<sub>3</sub> **13a-d** from the common building block **8**. The two fatty acid chains were sequentially and regio-selectively added through a sequence of acylation/deprotection/acylation. The first part of the sequence was the acylation of the *sn*-1 position with a saturated fatty acid, in our case stearic acid, leading to ester **10** in good yield thanks to DCC/DMAP as coupling reagent. The second part of the sequence was the deprotection of the PMB moiety in mild conditions. This reaction was carefully monitored by <sup>31</sup>P NMR until completion to convert the resulting *sn*-2 alcohol into esters **12a-d** as quickly as possible to avoid any intramolecular trans-esterification reaction with the stearate introduced in the first part of the sequence. The velocity of this side-reaction was studied by NMR with shorter fatty acids chains and was well characterized (see supplementary materials). Thus, in our reaction conditions (0°C, 5h or RT, 1h) we avoided this side-reaction and consequently maintained regioselectivity higher than 95% for the grafting of fatty acyl chains. To conclude the synthesis, the cleavage of the dimethyl phosphate ester of **12a-e** was performed under mild conditions with BSTFA<sup>i</sup> and TMSBr to obtain the various PA-N<sub>3</sub> **13a-d**, (**13a** PA-N<sub>3</sub> 18:0-18:1; **13b** PA-N<sub>3</sub> 18:0-18:2; **13c** PA-N<sub>3</sub> 18:0-20:4; **13d** PA-N<sub>3</sub> 18:0-22:6) whereas symmetrical PA-N<sub>3</sub> 18:0-18:0 **13e** was also obtained through a shorter synthetic pathway described in the supplementary materials (Figure 1b and 1c).

To obtain PA probes from PA-N<sub>3</sub> **13a-d**, we developed SPAAC ligation reaction to avoid the generation of ROS species with copper (I)<sup>20</sup>, even though this reaction is slower than CuAAC<sup>21</sup>. The chosen cyclooctyne SPAAC partners were obtained from DIBAC derivatives offering a good compromise between stability and reactivity. The DIBAC-C<sub>6</sub>-Amine synthesis<sup>22</sup> was optimized to minimize side products formation and improve the repeatability of the reactions, especially for the dibromation/elimination sequence leading to the cyclooctyne ring (see SI). Thereafter a coupling reaction with activated carboxylic acid derivatives was performed affording PCL-DDA1 **18** and DIBAC-C<sub>6</sub>-ATTO 647N **19** or S<sub>N</sub>Ar reaction to get DIBAC-C<sub>6</sub>-NBD **20** (Figure 2a). These chemobiological tools were first developed with PA-N<sub>3</sub> **13a** and **13d** due to the large difference in fatty acyl chain composition. The SPAAC reactions led to six new PA probes bearing either diazirines<sup>23</sup> as photocrosslinkers with PA-DDA1 **21a** and **21b** or either fluorophore with PA-NBD **22a** and **22d** and PA-ATTO 657N **23a** and **23b** (Figure 2b).

## Biocompatibility and membrane incorporation of synthetic mono- and poly-unsaturated PA analogues in neurosecretory cells

Cell imaging revealed that PA-ATTO 647N (18:0-18:1) **23a** and PA-ATTO 647N (18:0-22:6) **23d** analogues inserted rapidly in the plasma membrane and accessed intracellular membrane compartments without visible cell alteration (Figure 3a, b). PA-ATTO 647N (18:0-22:6) **23d** displayed a more rapid access to the peri membrane zone and accumulated more effectively than PA-ATTO 647N (18:0-18:1) **23a** in internal membrane compartments, in agreement with the well-established cellular gradient of poly-unsaturated GPL decreasing from intracellular organelles towards the PM<sup>24</sup>. Validating the rapid access of the PA analogues to the inner leaflet of the plasma membrane, Spo20p-GFP a plasma membrane PA sensor<sup>25</sup> was recruited from the nucleus to the plasma membrane within minutes of incubation of the PA analogues **13a** and **13d** (Figure 3c, d). Furthermore, these data confirm that once internalized the synthetic PA were not rapidly metabolized as they were recognized by the PA sensor.

## Functional validation of synthetic PA analogues

To validate the functionality of fluorescent PA analogues, we first evaluated their ability to induce membrane remodeling through their interaction with the chromogranin A (CgA) glycoprotein. As previously demonstrated with native PA, this ability depends on the fatty acyl chains, and PA (18:0-18:1) is one of the PA able to induce such a membrane remodelling<sup>26</sup>. We observed that in the presence of CgA, GUV containing PA-NBD (18:0-18:1) **22a** exhibited significant budding events with strong CgA-AF633 co-staining in agreement with what was observed with commercial PA (18:0-18:1). In contrast GUV containing commercial PA-NBD (18:1-6:0) exhibited few buds with a weak CgA staining (Figure 4a). Quantification revealed that the GUV containing PA-NBD (18:0-18:1) **22a** presented more budding events compared to GUV containing the commercial PA-NBD (Figure 4b). These observations revealed that (i) CgA interacts with synthesized PA (18:0-18:1) analogue **22a**, and that (ii) this interaction is functional, by inducing membrane remodeling and vesicle budding, mimicking the effect of native PA (18:0-18:1) found in Golgi and secretory granule membranes, whereas the commercially available PA-NBD (18:1-6:0) failed at mimicking PA (18:0-18:1) activity.

PA synthesized by phospholipase D (PLD) has been shown to contribute to effective secretion from various cell types including neurons, endocrine and exocrine cells<sup>27,28,29</sup>. For instance, we previously reported that phospholipase D1 (PLD1) knock out, knock-down, or pharmacological inhibition severely impaired catecholamine release from chromaffin cells<sup>30,31</sup>. Interestingly, it was also shown that extracellular provision of mono-unsaturated forms of PA rescued the number of exocytotic event, whereas poly-unsaturated PA affected the kinetics of individual events<sup>31</sup>. We probed whether the different analogues of the synthetic PAs produced here fully recapitulated these biological activities using DBH labeling of exocytotic spots as a reporter of the secretory activity. None of the PA analogues tested affected cell size as measured by the cell surface area or by the peripheral area (Extended Figure 1). The potent inhibition of DBH staining after cell stimulation induced by the PLD1 inhibitor CAY93 was as efficiently rescued by synthetic PA (18:0-18:1) harboring either a N<sub>3</sub>-linker **13a**, or a fluorescent NBD group bound to this linker **22a**, or DDA1 bound to this linker **21a** (Figure 5a, b). Importantly, synthetic PA-N<sub>3</sub> (18:0-22:6) **13d** failed to rescue DBH labeling in agreement of data obtained with natural PA (18:0-22:6) (Figure 5a, b). Electrochemical recording of catecholamine secretion from single chromaffin cells confirmed the rescue effectiveness of synthetic PA-N<sub>3</sub> (18:0-18:1) **13a** analogues on the number of exocytotic events from cells treated with CAY93, whereas

synthetic PA-N<sub>3</sub> (18:0-22:6) **21d** analogues specifically rescued the kinetic parameters of individual events (Figure 5c-j and extended Figure 2). Hence, the synthetic PA analogues developed here fully recapitulated the specific functions of natural mono and poly-unsaturated forms of PA, which is not the case for the commercially available PA-NBD (18:0-6:0). These data support the idea that the strategy of modifications hindered on the glycerol backbone of PA did not impeach the functional implication of these PA analogues in the regulation of exocytosis and suggest that the interactome of these analogues might recapitulate that of native PAs.

### Characterization of the PA interactome in neurosecreting cells using synthetic PAs

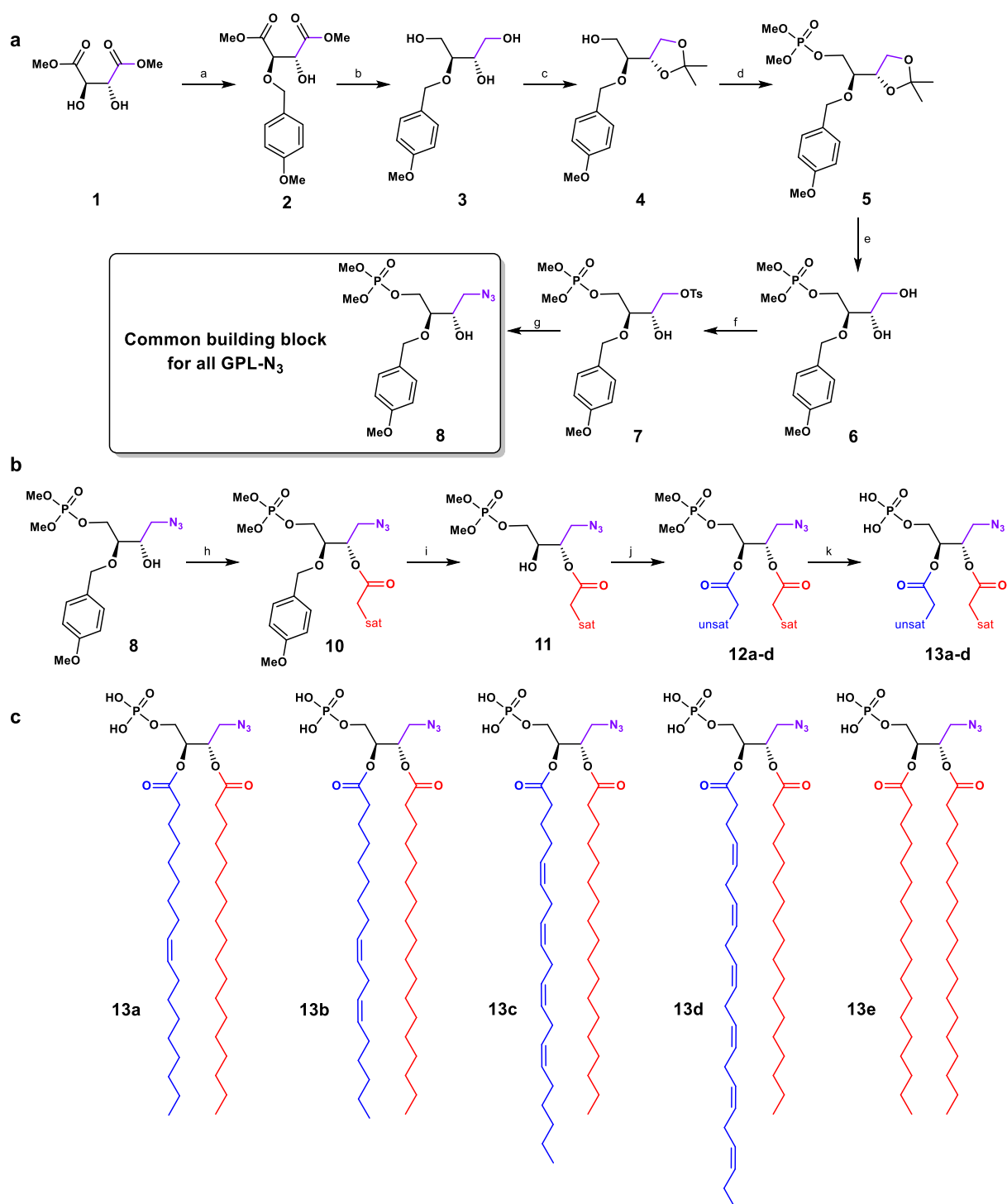
The DDA1 PA (18:0-18:1) **21a** and PA (18:0-22:6) **21d** were used to crosslink PA interactants from chromaffin cells in resting (R), stimulated (S), and post-stimulation (PS) conditions. 1264 polypeptides identified in the absence of photo-crosslinking were considered as background, whereas 778 potential partners of PA specific to photo-illumination were identified. Validating the strategy used, these include proteins that have been identified in previous screens using cell extracts incubated with PA-coupled to agarose beads<sup>32,33</sup> or known PA interactant previously validated biochemically<sup>34,35,36,37</sup> (Extended figure 4) but also many novel unidentified potential PA partners. Among those, 286 were specific to PA (18:0-18:1) **21a** and 341 to PA (18:0-22:6) **21d**, whereas 151 were common to both PA species (Figure 6a). Furthermore, some of these interactant were found specifically in either R, S, or PS condition, whereas others were found in two or three conditions (Figure 6a), highlighting the dynamics of these interactions. Gene ontology analysis for Molecular function, biological process, and cellular component revealed specific enrichments of proteins present in membrane organelles and trafficking with clear differences between the two PA species tested (Figure 6b and extended Figure 3). Furthermore, although less abundant, the profile of the potential partners of PA (18:0-18:1) were clearly more specifically distinct in the three cellular states tested, with a dramatic increase in response to cell stimulation (Figure 6b and extended Figure 3).

The potential PA partners inferred to the GO terms related cellular components were pooled and STRING maps obtained (Figure 6c). Interestingly, the protein chromogranin A (CgA) previously reported to play a crucial role in secretory granule biogenesis in neuroendocrine cells through its interaction with PA<sup>ii</sup>, was found for the two synthetic PA **21a** and **21d** and in all the conditions (Fig. 6c, underlined in red). For PA (18:0-18:1) **21a** an increase in the number of potential partners after cell stimulation was seen, including clusters of proteins related to the vacuolar ATPase and to mitochondria (Figure 4c). Of note, it was recently established that the V-ATPase may regulate the formation of PA near exocytotic site<sup>39</sup>. A cluster of SNARE-related proteins was also detected for PA (18:0-22:6) (Figure 4c) in the S condition, which is consistent with observation that PA interacts with syntaxin1A<sup>40</sup>. Of interest, a cluster of adaptor and membrane coat-related proteins was also detected in the PS conditions, which may be related to the recycling of granular content by compensatory endocytosis. To our knowledge this is the first example of a fishing experiments carried with GPLs embedded in membranes and in living cells under different cellular conditions. Among the many different novel interactant candidates identified here, other potential interesting candidates are involved in lipid transfer, metabolism, and translation (see supplemented data).

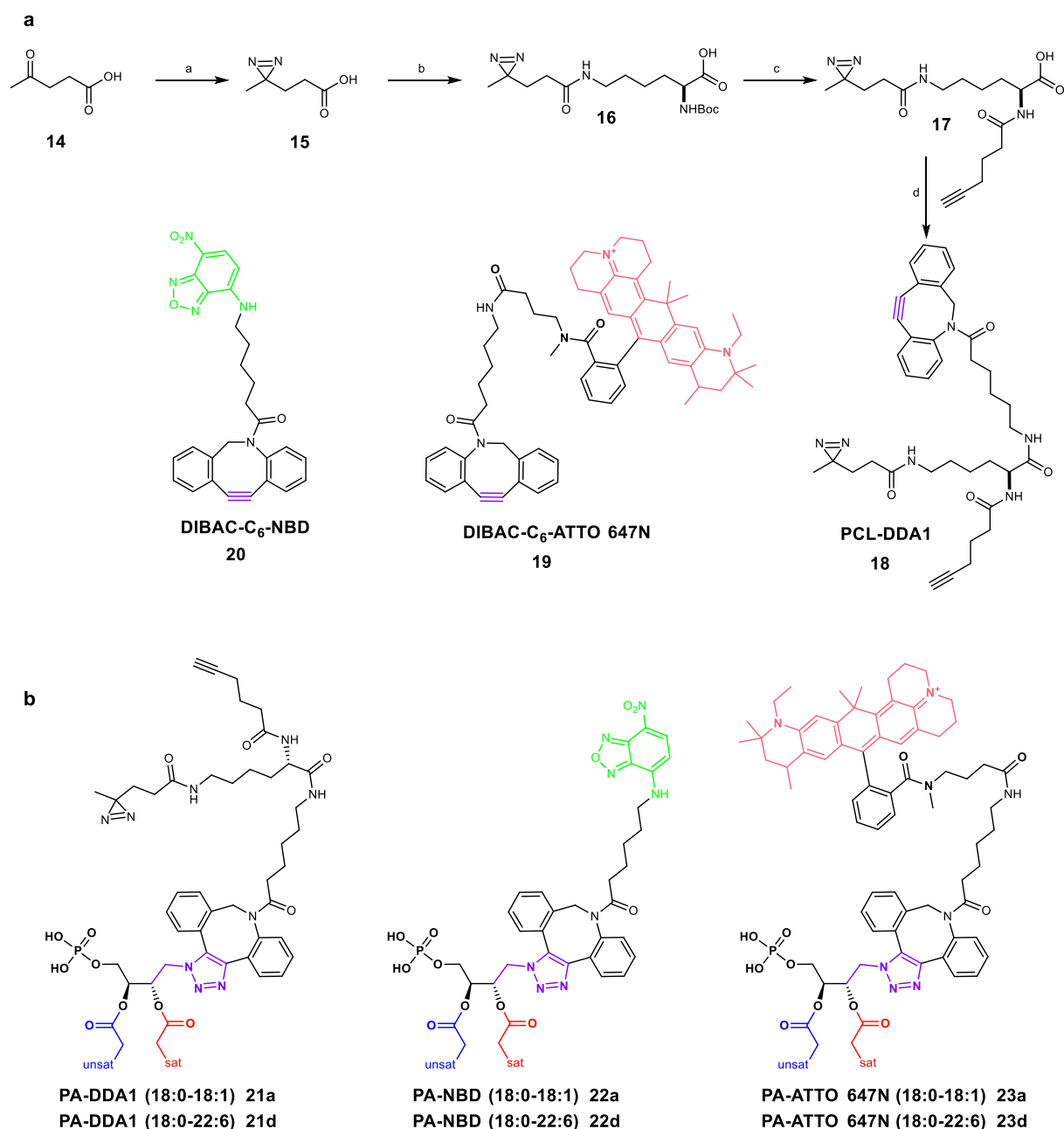


## Conclusion

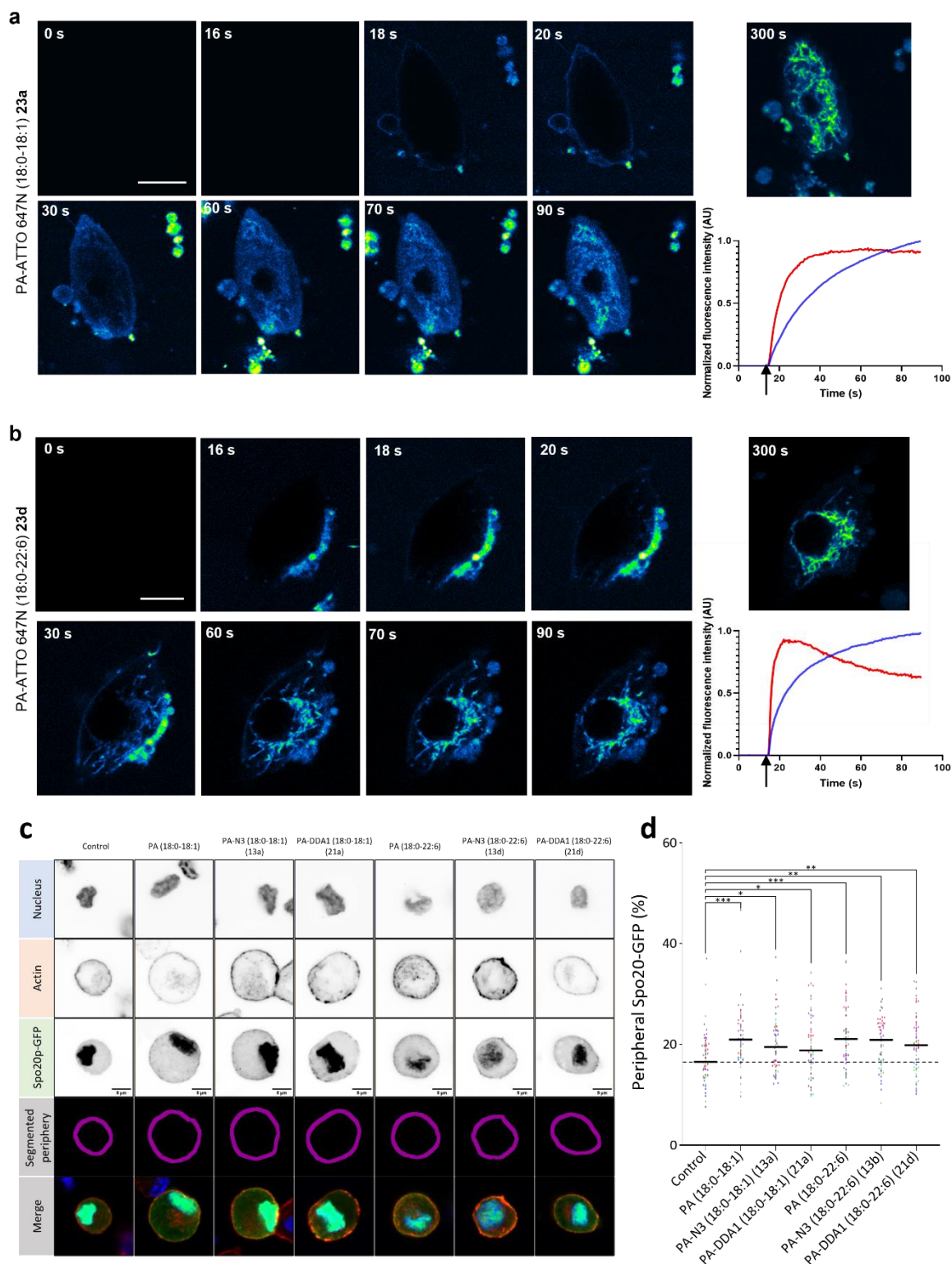
Access to versatile and clickable PA analogues, unmodified neither on the fatty acyl chain, neither on the phosphate head, combined with the biological data confirm the versatility of these novel lipid analogues and their great potential for *in cellulo* use. We were able to demonstrate that, contrary to commercially available PA probes modified on the fatty acyl chains, our probes reproduced the biological behavior of the natural lipids. We have shown that the composition of fatty acid chains is extremely important for all future biological studies involving PA and probably GPL. Finally, the results obtained by photolabeling and fishing not only confirmed the interactions already known with certain proteins such as CgA, but also highlighted interactions with dozens of others unknown proteins, depending on the fatty acyl chain and the cell status (resting, stimulated or post stimulation), thereby broadening our knowledge of PA and their interactome.



**Figure 1: Synthetic route to the common building block 8 leading to PA-N<sub>3</sub> 13a-e.** a, formal synthesis of the common building block 8 having all the *sn* positions differentiated. a) PMBBBr, Bu<sub>2</sub>SnO, Bu<sub>4</sub>NI, CsF, Toluene, 70%. b) NaBH<sub>4</sub>, *i*-PrOH, 90%. c) 2,2-Dimethoxypropane, APTS, acetone, 73%. d) Dimethylchlorophosphate, *t*-BuOK, DCM, 76%. e) HCl, MeOH, 86%. f) TsCl, DIPEA, Bu<sub>2</sub>SnO (0.5% mol.), toluene, 89%. g) 1) NaN<sub>3</sub>, DMF, 2) TMSCHN<sub>2</sub>, MeOH, 53% **b**, formal synthesis of four different PA-N<sub>3</sub> **13a-d** from intermediate **8**. h) DCC, DMAP, stearic acid, DCM, 93%. i) DDQ, DCM, PBS buffer (pH= 7.2). j) DCC, DMAP, oleic acid (**12a**) or linoleic acid (**12b**) or arachidonic acid (**12c**) or docosahexaenoic acid (**12d**), 50% (**12a**, **12b**), 63% (**12c**) 73% (**12d**) over two steps. k) TMSBr, CHCl<sub>3</sub> or TMSBr, BSTFA, CHCl<sub>3</sub>, 31 to 95% **c**, structures of the PA-N<sub>3</sub> (18:0-18:1) **13a**, PA-N<sub>3</sub> (18:0-18:2) **13b**, PA-N<sub>3</sub> (18:0-20:4) **13c**, PA-N<sub>3</sub> (18:0-22:6) **13d** and PA-N<sub>3</sub> (18:0-18:0) **13e**.

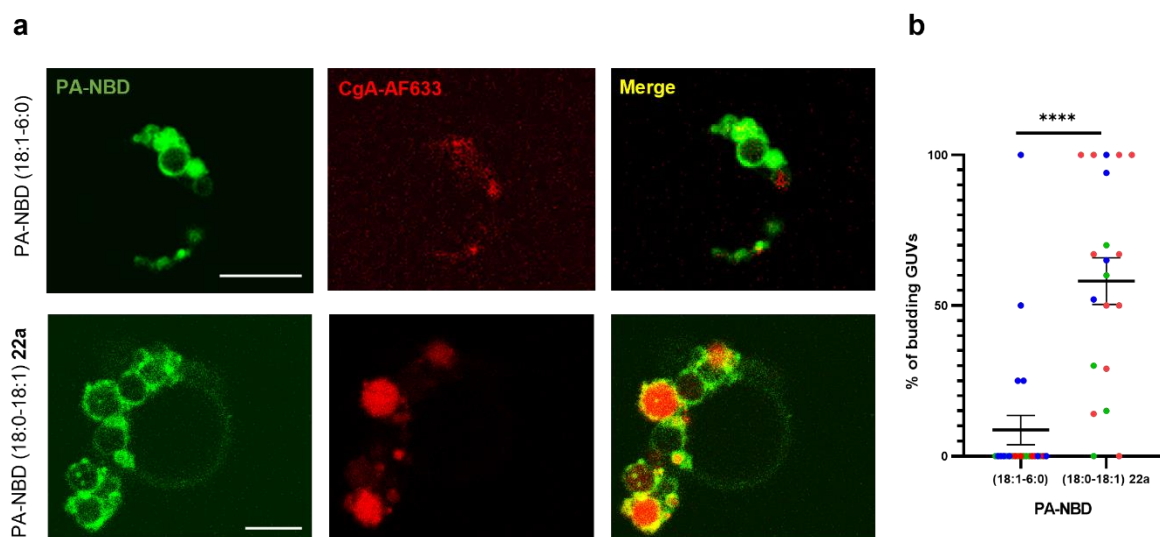


**Figure 2: Synthesis of the PA analogues.** **a**, formal synthesis of the PCL **18** and structure of the synthesized clickable fluorophore **19** and **20**. a) 1)  $\text{NH}_3$ , MeOH,  $t\text{-BuOCl}$ , 2)  $t\text{-BuOCl}$  b) 1) NHS, EDCI, DMF, 2) Boc-Lys-OH, DIPEA, DMF, 90%. c) 1) TFA, DCM, 2) Pent-5-yn- $N$ -hydroxysuccinimide ester, 50% over two steps. d) 1) NHS, EDCI, THF, 2) DIBAC- $\text{NH}_2$ , DIPEA, rt, 30% over two steps. **b**, structures of the PA probes **21-23** obtained by SPAAC click-chemistry between cyclooctynes **18-20** and PA- $\text{N}_3$  **13a** and **13d**.

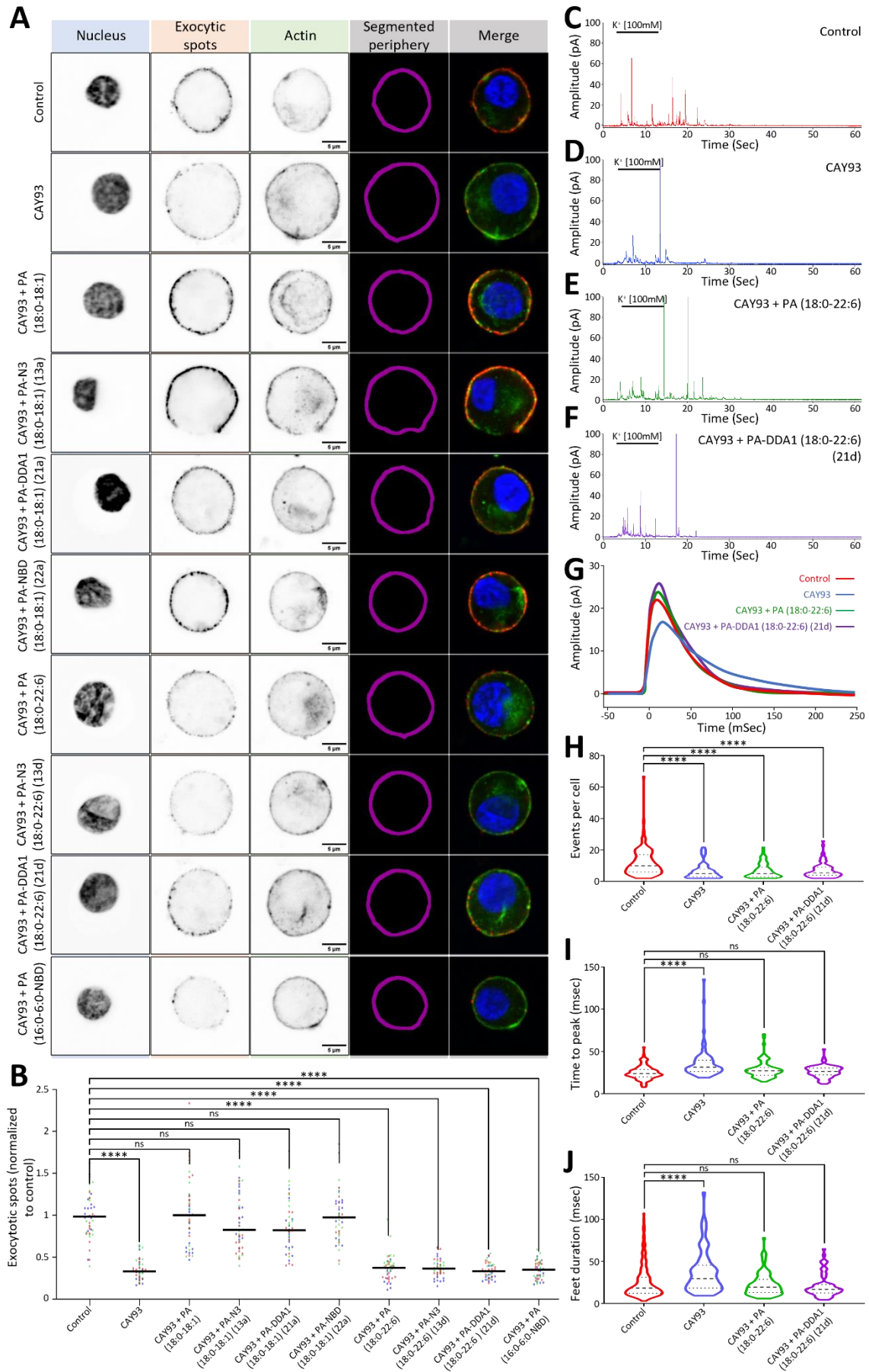


**Figure 3: Live-cell imaging with fluorogenic PA analogues and recruitment of a PA sensor to the plasma membrane. a**, incorporation kinetics of PA-ATTO 647N (18:0-18:1) **23a** on living bovine chromaffin cells. Biomolecules have been added on cultured cells 15 s after the beginning of life-time recording (arrow). Mean fluorescence intensity was analyzed in the whole cell (blue line) and in a perimembrane zone of 500 nm width (red line). The maximum fluorescence intensity was normalized to 1. Analysis of PA-ATTO 647N (18:0-18:1) **23a** incorporation was performed on 6 cells from 4 independent cell cultures. Scale bar, 10  $\mu$ m; a.u., arbitrary units. **b**, incorporation kinetics of PA-ATTO 647N (18:0-22:6) **23d** on living bovine

chromaffin cells. Biomolecules have been added on cultured cells 15 s after the beginning of life-time recording (arrow). Mean fluorescence intensity was analyzed in the whole cell (blue line) and in a perimembrane zone of 500 nm width (red line). The maximum fluorescence intensity was normalized to 1. Analysis of PA-ATTO 647N (18:0-22:6) **23d** incorporation was performed on 7 cells from 4 independent cell cultures. Scale bar, 10  $\mu$ m; a.u., arbitrary units. **c**, Representative confocal images of bovine chromaffin cells overexpressing the PA sensor Spo20p-GFP. Cells were incubated with the mentioned natural PAs and synthetic PA analogues. Each channel is shown individually as an inverted grayscale image, segmented peripheral area in magenta, and merged (Blue: nucleus, red: actin and green: Spo20p-GFP). Scale bar: 5  $\mu$ m **c**, Spo20p recruitment quantification after addition of the indicated natural PA or PA analogues. Data are represented as median, and each point is a measure from an individual cell. Symbols are colored coded (red, blue, green or magenta) according to experimental repeats. Basal recruitment of Spo20p-GFP is indicated by the dotted line. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , Kruskal-Wallis followed by Dunn's multiple comparisons test vs control (N=60 per condition, 15 cells from 2 independent cultures and 4 experimental repeats) performed from 2 independent cultures).

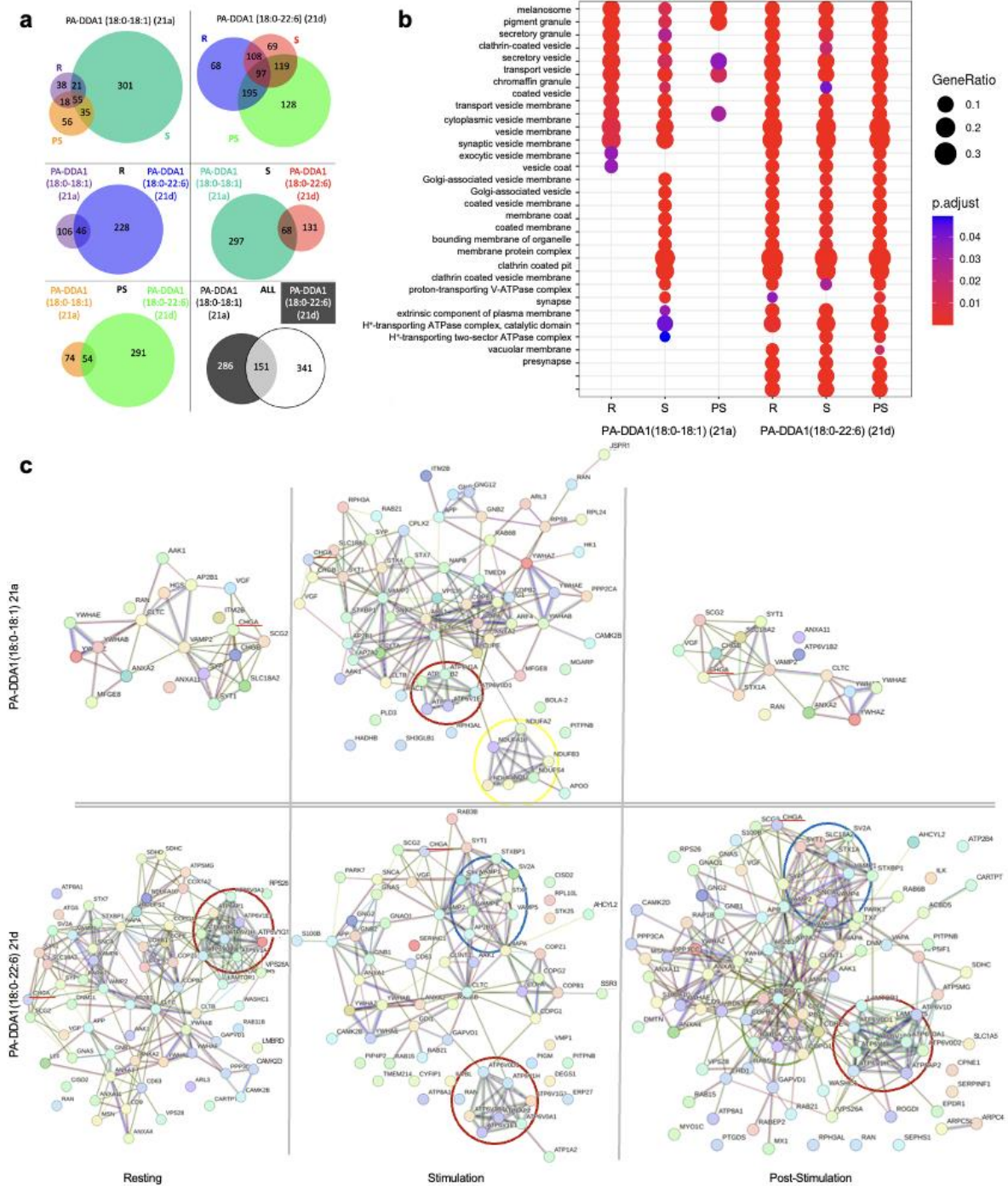


**Figure 4: Liposome-based evaluation of synthetic fluorescent PA analogues.** **a**, formation of GUVs composed of 96% DOPC and 4% PA (18:1-6:0-NBD) or 4% PA-NBD (18:0-18:1) **22a** (green), in the presence CgA-AF633 (red). Scale bar, 10  $\mu$ m. **b**, quantification of budding GUVs composed of 96% DOPC and 4% PA (18:1-6:0-NBD) or 4% PA-NBD (18:0-18:1) **22a** with CgA-AF633. Each point represents the result of GUV analysis from three independent experiments (20 and 22 images for each type of PA-NBD containing-GUVs, respectively). \*\*\*\*  $p < 0.0001$  Mann Whitney test.





**Figure 5: Synthetic PA analogues fully recapitulate the function of natural PA in bovine chromaffin cells.** **a**, Representative confocal images of stimulated BCCs after exocytotic spots staining. PLD1 activity was inhibited with 50nM CAY93 and cells were incubated with the mentioned natural PAs and synthetic PA analogues. Each channel is shown individually as an inverted grayscale image, segmented peripheral area in magenta, and merged (Blue: nucleus, red: exocytotic spots and green: actin). Scale bar: 5 $\mu$ m **b**, Exocytotic spot quantification of BCCs incubated with 50nM CAY93 and the indicated natural PA or PA analogues. Mean peripheral exocytotic spot intensity has been normalized with the mean control condition of the individual experimental repeat. Data are represented as median, and each point is a measure from an individual cell, points are colored red, blue or green according to experimental repeats. ns: non-significant, \*\*\*\*:  $p < 0.0001$ , Kruskal-Wallis followed by Dunn's multiple comparisons test vs control (N=45 per condition, 15 cells from 3 independent cultures), see figure Extended figure 1 for more conditions. **c**, **d**, **e**, and **f**, Example amperometric traces of BCCs incubated with or without PLD1 inhibitor (CAY93) and incubated with the mentioned PA species, or PA analogue. Cells were stimulated with a 100mM K<sup>+</sup> solution (Black line on each trace). **g**, Schematic representation of mean spikes obtained from those recordings. **h**, **i**, and **j**, Violin plots representing respectively the number of exocytotic events, spike time to peak and feet duration of the amperometry experiment (Bars in the plot represent from top to bottom, third quartile, median and first quartile). ns: non-significant, \*\*\*\*:  $p < 0.0001$ , Kruskal-Wallis followed by Dunn's multiple comparisons test vs control (N>82 per condition from 3 independent cultures), see Extended figure 2 for a more detailed analysis of the experiment.



**Figure 6: PA interactome in neurosecretory cells.** *a*, Distribution of the different proteins identified in the fishing experiments performed in bovine chromaffin cells for DDA1 PA (18:0-18:1) **21a** and DDA1 PA (18:0-22:06) **21d** in resting (R), stimulated (S), or after stimulation (PS). *b*, Cellular component of Gene Ontology enrichment analysis of all proteins identified in the different conditions tested. *c*, Protein-Protein Interaction Network Exploration of the pooled proteins identified in the cellular component Gene Ontology enrichment analysis for the potential PA interactors under the different conditions using Cytoscape (STRING data base). Circles highlight clusters of proteins: red ( $H^+$  transport by V-ATPase), blue (SNAREs), yellow (mitochondrial activity), green (membrane coats). CgA found in each condition has been underlined in red.

## Methods

### Reagents and Biomolecules synthesis

Standards reagents were purchased as described in supplemental Table 1. Detailed synthesis and purification of the PA analogues are described in supplemental material. For the cellular incorporation, PA-ATTO 647N 18:0-18:1 **25** and PA-ATTO 647N 18:0-22:6 **26** have been resuspended with 2% EtOH and 98% Phosphate Buffered Saline, pH 7.4 (Sigma). PA solutions have been sonicated 4x30s at 42 kHz 70W with an Ultrasonic cleaner (VTSUC2 Velleman). 3  $\mu$ M lipid solutions were injected in extracellular medium.

### Chromogranin A-Alexa fluorophore 633 (CgA-AF633) coupling

Recombinant CgA (human chromogranin A 19-457aa, His tag, E coli, ATGP0323, Atgen global) was used for CgA-Alexa633 (AF633) coupling. All reactants and solvents were purchased from TCI-Chemicals and Alfa Aesar. CgA labeling was performed according to a modified version of a protocol reported in Chevalier et al (2013). First, 37  $\mu$ L (1.6 eq.) of carboxylic acid AF633 solution (2 mg mL<sup>-1</sup> in anhydrous N, N dimethylformamide) were introduced into a 1 mL round-bottom flask. (O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (70  $\mu$ g, 1.2 eq.) and N,N-diisopropylethylamine (0.04 mL, 1.2 eq.) were added and the reaction medium was placed under argon, with magnetic stirring at RT during 3 hours, to obtain the N-hydroxysuccinimidyl activated ester. Then, 10  $\mu$ L of this solution was added to the solution of 680  $\mu$ g of CgA in sodium carbonate buffer (pH 8.3, 0.1 mg/mL, 1 eq.), for a ratio AF633/CgA 7/1. After 20 min at RT under magnetic stirring, the mixture was cooled down to 0°C and a dialysis was performed at 4°C under magnetic stirring for 20 hours using 10 kDa centrifugation filters (Spin-X UF 500, CORNING) previously conditioned with PBS to remove glycerol and other biocidal compounds. Labeled protein was collected in PBS buffer following three centrifugations of 10 minutes at 7500 rpm. Grafting rate was evaluated by Western blot and UV-Vis spectroscopy ( $\lambda_{abs}$  = 494 nm), with a maximum labeling rate of 10 Alexa633 molecules per protein.

### Primary bovine chromaffin cell culture

Bovine adrenal glands were obtained at the Haguenau municipal slaughterhouse and placed in a 4°C calcium-free Krebs solution (NaCl 154mM, KCl 5.6mM, NaHCO<sub>3</sub> 3.6mM, 5.6mM glucose and 5mM HEPES, pH adjusted to 7.4) supplemented with the broad-spectrum antibiotic, primocine at 100 $\mu$ g/mL. Glands were digested twice for 10min at 37°C with a Collagenase A solution (3.5mg/mL, 5mg/mL, resuspended in Krebs solution). The adrenal medulla was separated from the cortex and minced and digested for 5min with remaining collagenase A solution. BCCs (Bovine Chromaffin Cells) were passed through a 217 $\mu$ m sieve, centrifuged for 10min at 130g resuspended in Krebs solution and passed through a 70 $\mu$ m sieve. BCCs were isolated with a Percoll density gradient generated with a 20min centrifugation at 20,000g. Cells were resuspended in culture medium (low glucose DMEM supplemented with 1mM L-glutamine, 10% FBS, 10 $\mu$ M fluorodesoxyuridine, 10 $\mu$ M cytosine arabinoside and 100 $\mu$ g/mL primocine) and centrifuged at 130g for 10min, resuspended and counted.

For exocytic spots staining, BCCs were seeded at 200,000 cells per well in four-well culture dishes containing 12mm fibronectin coated coverslips. For carbon fiber amperometry

experiments, 200,000 cells were seeded on fibronectin coated 35mm dishes. For photo-crosslinking experiments,  $10 \times 10^6$  cells were seeded on fibronectin coated 60mm dishes. Culture dishes were stored in an incubator at 37°C, 5% CO<sub>2</sub> and humid atmosphere for 48h prior to experiments.

### **PA solution preparation**

10mM PA stock solutions in chloroform were prepared just prior to the experiments in glass microtubes. Chloroform was evaporated using an azote jet to form a lipid film. 37°C MilliQ water was added to the tubes and bath sonication was performed for 10min on a benchtop ultrasonic cleaner (B200 Branson Ultrasonics™).

### **Exocytosis spots staining**

Bovine chromaffin cells were washed twice with 37°C normal Locke's solution before being incubated for 1h at 37°C with normal Locke's solution containing 50nM of the specific PLD1 inhibitor: CAY93 or control (0.01% DMSO). During the last 15min, 1% 10mM PA solutions or MilliQ water was added to the cell bringing the final concentration on the cells to 100μM. Afterwards, for resting conditions, a 37°C normal Locke's solution with either DMSO or CAY93 containing 0.5% of a mouse anti-DβH antibody was added to the cells for 3min ; For stimulated conditions, a 59mM K<sup>+</sup> solution (85.7mM NaCl, 59mM KCl, 2.5mM CaCl<sub>2</sub>, 1.2mM KH<sub>2</sub>PO<sub>4</sub>, 1.2mM MgSO<sub>4</sub>, 11mM glucose, 0.56mM ascorbic acid, 0.01mM EDTA and 15mM HEPES, pH adjusted to 7.2) with either DMSO or CAY93 containing 0.5% of an anti-DβH antibody was added to the cells for 3min. Coverslips were washed once with 37°C normal Locke's solution, and BCC were immediately fixed with a cold 4% PFA solution for 10min. Non-specific sites were saturated with a 3% PBS-BSA solution containing 10% goat serum followed by an incubation with a PBS-BSA 3% solution containing 0.1% secondary goat anti-mouse alexa555 antibodies. Nuclei and cortical actin of BCC were stained for 20min at room temperature with Hoechst33342 and phalloidin-Atto647N. Coverslips were mounted on a microscopic slide using Mowiol 4-88 mounting medium.

### **PA sensor recruitment**

BCCs were transfected directly after the culture. 8,000,000 cells were resuspended in an antimitotic, antibiotic free medium (low glucose DMEM supplemented with 1mM L-glutamine, 10% FBS) and mixed with a plasmid encoding for the Spo20p-GFP PA sensor. Nucleofection was performed by using the bascis primary neuron nucleofector kit (Lonza©) and cells were seeded on fibronectin coated coverslips. 5h after this procedure, antimitotics and antibiotics were added to the medium. The following day, medium of the cells was changed. 18h after transfections, cells were washed twice with normal Locke's solution and 100μM of the indicated PA species or PA analogue was added to the cells for 15min, fixated, nuclei and cortical actin was stained, and coverslips were mounted as described above.

### **Image acquisition**

Images were obtained using a SP5II confocal laser scanning microscope (Leica®) with a 63× oil immersion objective. Acquisition was performed via the LAS AF software in a sequential scanning mode with a pinhole opening of 1 airy unit. The resolution was set at 512×512 pixels

with a scanning speed of 400Hz, a bit rate of 16, no smart offset, a zoom of 6 and a line average of 4. Hoechst33342 (Nuclei) was stimulated with a 405nm emitting laser. GFP (PA sensor) was stimulated with a 490nm emitting argon laser. Alexa555 (D $\beta$ H staining) was stimulated with a 561nm emitting argon laser. Atto647N (Phalloidin) was stimulated with a 633nm emitting helium/neon laser. 15 isolated cells were acquired per condition and experiment, all images were taken with the same settings on each channel and saved as LIF files.

After acquisition images were converted from LIF files into TIF files by using the standard Bioformat plugin on the FIJI software. Segmentation of the peripheral area was performed on the ICY software by using the active contour plug-in and cortical actin staining. Peripheral exocytotic spot intensity was normalized with the corresponding stimulated control condition. Spo20p recruitment was evaluated by dividing the peripheral GFP signal with the total GFP signal in the cell.

### **Carbon fiber amperometry**

BCCs were washed twice with Locke's solution and incubated for 30min with 50nM CAY93 or 0.01% DMSO (Control), during the last 10min, 1% PA solution was added to the cells and recorded for catecholamine release measurements by amperometry for the next 30min. A 5mm Carbon-fiber electrode (ALA Scientific) was held at a potential of 650mV compared with the reference electrode (Ag/AgCl) and approached closely to cells. Cells were stimulated with a 10sec 100mM K<sup>+</sup> puff using a micropipette and recorded during 60sec. Amperometric recordings were performed with an AMU130 (Radiometer Analytical) amplifier, sampled at 5kHz, and digitally low-passed filtered at 1kHz. Analysis of amperometric recordings was done as previously described with a macro (obtained from Dr. R. Borges laboratory; <http://rborges.webs.ull.es/>) written for Igor Pro software (WaveMetrics), allowing automatic spike detection and extraction of spike parameters<sup>31</sup>. Only cell having produced at least 2 analyzable spikes were considered. The number of amperometric spikes was counted as the total number of spikes with an amplitude over 5pA within 60sec. The spike parameters analysis was restricted to spikes with amplitudes over 5pA. All peaks identified by the program were visually inspected and overlapping peaks were manually excluded from the datasets. For foot signal, the analysis was restricted to spikes with foot amplitudes greater than 2 pA.

### **PA fishing experiments**

Bovine chromaffin cells were washed twice with 37°C normal Locke's solution before addition of 1% 10mM PA-DDA1 18:0-18:1 **21** or PA-DDA1 18:0-22:6 **22** solution and incubated for 15min at 37°C under aluminium foil. Cells were incubated for 3min with either normal Locke's solution (Resting), stimulated with 59mM K<sup>+</sup> solution (Stimulated) or stimulated for 3min followed by an incubation in normal Locke's solution (Post stimulation) and placed under a 36W UV lamp (Nailstar™) for 3min during resting, stimulation, and post stimulation. Additional non-illuminated samples also incubated with photo crosslinking PAs were also prepared in resting condition. Following illumination, captured proteins were extracted and enriched using the Click-&-Go™ Plus Protein Enrichment Kit. Cells were incubated at 4°C during 10min with Lysis buffer, extracts were sonicated on ice and centrifuged at 4°C at 10,000rpm for 5min. Protein extracts bound to PA-DDA1 were incubated over night at ambient temperature with picolyl azide agarose resin in copper catalyst solution, ensuring formation of a covalent bound between PA interacting proteins, PA-DDA1 and the resin. The resin was incubated with 10mM DTT at 70°C for 15min and 40mM iodoacetamide in the dark for 30min to reduce disulfure bounds. The resin was washed 5 times with agarose SDS wash buffer, 8M urea/100mM Tris buffer and a 20% acetonitrile solution before trypsin digestion overnight at 37°C. Finally, trypsin

was inactivated by adding 0.2% trifluoroacetic acid to the reaction and samples were stored at -20°C until mass spectrometry analysis. Samples were analyzed by coupling a nanoflow liquid chromatography system (nanoElute, Bruker Daltonics) online to a trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (timsTOF Pro, Bruker Daltonics) equipped with a CaptiveSpray source operating in positive mode (ESI-TRAP (A1=TRAP F=CID A2=TRAP)). The proteomic research was performed on a mouse database created with bovine proteins present in SwisProt database (created 2023-09-23, containing 17289 target sequences plus the same number of reversed decoy sequences).

### Functional and network enrichment analysis.

Functional enrichment analysis was performed using clusterProfiler 4.0 (v4.12)<sup>36,37</sup> and enrichplot (v1.24.0), including Gene Ontology (GO) Molecular function, Biological process, Cellular component, and KEGG pathways of the candidate PA binding proteins (n = 778) enriched from the mass-spectrometry-based analysis. The GO terms were ordered based on the p-value and the top 15 terms were selected. The potential PA partners inferred to the GO terms related cellular components were pooled and Protein-Protein interaction networks obtained using the software Cytoscape at <https://string-db.org/cgi/my?sessionId=b7etya4OTbB3>, using the multiple protein mode and Bos Taurus as selected species.

### PA cellular uptake imaging

150 000 cells were plated per glass bottom dishes (MATTEK). 1 hour before addition of synthesized PA-ATTO 647N 18:0-18:1 **25** and PA-ATTO 647N 18:0-22:6 **26**, the medium was replaced with Dulbecco's Modified Eagle's Medium without phenol red (Gibco, Thermo Fisher Scientific) supplemented with 10% of heat-inactivated and sterile-filtered fetal bovine serum (FBS, Sigma - Aldrich).

PA incorporation in living cells was observed with a STELLARIS 8 inverted confocal microscope equipped with a pulsed white light laser (WLL), a 86X water immersion objective (HC PL APO 86x/1,20 W CORR CS2) and a thermostatic chamber set at 37°C and 5% CO<sub>2</sub> (Leica Microsystems). Synthesized PA-ATTO 647N 18:0-18:1 **25** and PA-ATTO 647N 18:0-22:6 **26** have been injected in the extracellular medium 15 seconds after the beginning of the recording, excited at 647 nm and observed in a 654-750 nm window. Images were acquired at a speed of 2 frames per second for 90 seconds. Images have been false colored with ImageJ's Green Fire Blue LUT. The incorporation kinetic was measured on FIJI software with a home-made macro. Two regions of interest were analyzed: the whole cell and the perimembrane zone, the latter corresponding to 500 nm width at the membrane level. Mean fluorescence intensity has been normalized and graphs were made on GraphPad Prism 8.0.2.

### Giant Unilamellar Vesicles (GUVs) formation

GUVs were prepared as previously described using a polyvinyl acetate (PVA) – assisted swelling protocol (Carmon et al. 2020). Briefly, about 10-20 µL of a lipid mixture (96% DOPC from Avanti Polar Lipids (Alabaster, USA) and 4% PA-NBD 18:0-18:1 **23** synthesized or 4% PA-NBD 18:1-6:0 from Avanti Polar Lipids (Alabaster, USA)) dissolved in chloroform and placed under vacuum overnight at RT to evaporate the solvent. A chamber around the drops of lipids was formed with Vitrex and 10 µL of a CgA-AF633 solution at a final concentration of 4 µM were added for 1 hour at 4°C. Then, the chambers were filled with 150 µL of a 100 mM sucrose



and 150 mM NaCl solution for 1 hour at RT, allowing the formation of GUVs with a suitable size. Next, the buffer containing GUVs is recovered and deposited in LabTek wells (LabTek I non-separable, on glass coverslip, 4 culture chambers, Nunc), which were previously coated with a 1 mg mL<sup>-1</sup> BSA (Bovine Serum Albumin, Sigma—Aldrich) solution. To allow the sedimentation of GUVs, we added the same volume (100 µL) of a solution containing 90 mM of glucose and 15 mM of NaCl in LabTek wells. The sedimentation of the GUVs takes place for about 1 hour at RT, before confocal microscopy acquisitions.

GUVs were observed with an inverse confocal microscope TCS-SP5 AOBS (Acousto-Optical Beam Splitter), equipped with pulsed white light laser (WLL) and with a 63X oil immersion objective (Leica Microsystems). Commercial PA-NBD 18:1-6:0 and synthesized PA-NBD 18:0-18:1 **23** were excited at 470 nm and observed in a 483-587 nm window. CgA-AF633 was excited at 632 nm and observed in a 635-782 nm window.

### Statistical analysis and graphic presentation

Statistical analysis was performed on the Graphpad® Prism 8 software by performing a Kruskal-Wallis test followed by Dunn's multiple comparison test as indicated in the figure captions. Graphs were produced on the same software. Confocal images were cropped to a size of 250×250 pixels and shown without further modifications.

### Fundings

This work was supported by grants from “Fondation pour la Recherche Médicale” (DEI20151234424) and “Agence Nationale pour la Recherche” (ANR-19-CE44-0019) to NV, PYR, SB and MM-H, “Fondation pour la Recherche Médicale” (FDT202304016618) to AW, French Proteomic Infrastructure (ProFI; ANR-10-INBS-08-03) to JMS. INSERM is providing the salary to NV and SG. This work has been partially supported by Université Rouen Normandie, INSA Rouen Normandie, Centre National de la Recherche Scientifique (CNRS), European Regional Development Fund (ERDF), Labex SynOrg (ANR-11-LABX-0029), Carnot Institute I2C, and the graduate school for research XL-Chem (ANR-18-EUR-0020 XL CHEM).

### Acknowledgements

We acknowledge the Plateforme Imagerie In Vitro of ITI Neurostra at CNRS UAR-3256 and the municipal of Haguenau (France) and the slaughterhouse of Bigard Formerie (France) for providing the bovine adrenal glands.

We acknowledge the members of the Plateforme C2I Orga and more particularly Laetitia Bailly, Emily Petit, Albert Marcécual, Mathilde Lauzent and Françoise Ringot.

SB thanks Dr. Antoine Joosten, Dr. Cyrille Sabot and Dr. Alexandre Haefel   for the scientific discussions about this project.

The Universit   de Rouen-Normandie, the CNRS, the INSA de Rouen-Normandie and the INSERM have a patent (FR20220002640 20220324) based on this work. AS, AW, AC, LR, RF, PYR, MM-H, NV and SB are named as inventors on this patent. TF is co-supported by European Union and R  gion Normandie. The other authors declare no competing interests.

Data availability can be requested to the corresponding authors.

## Contributions

PYR, MM-H, NV and SB developed the initial concept. The synthesis of PA-N<sub>3</sub> were performed by AS, AC, RF and BB. The synthesis of DIBAC was performed by LG, AS, SV, PJH and BB. The synthesis of DIBAC-C6-NBD was performed by AS and PJH. The synthesis of DIBAC-C6-ATTO was performed by AS. The synthesis of PCL-DDA1 was performed by SV. The synthesis of PA-NBD was performed by PJH, AC and AS. The synthesis of PA-ATTO was performed by AS. The synthesis of PA-DDA1 were performed by SV. The NMR study about transesterification resulting from the synthesis of the phospholipid model was performed by MS, LG, AP and SV. Purifications of final probes were performed by VF, VAP and PC. GUV experiments were performed by TF, LR and MC. Chromaffin cell cultures were managed by AW, ET, TF, FL and LJ. Live-cell imaging was conducted by TF, MC and MB. AW performed the amperometry analysis, the PA sensor recruitment experiments and the exocytotic site labeling. AW and ET performed the fishing experiments. JMS performed the MS analysis. CD, AW, SO, and NV performed the bioinformatic analysis.

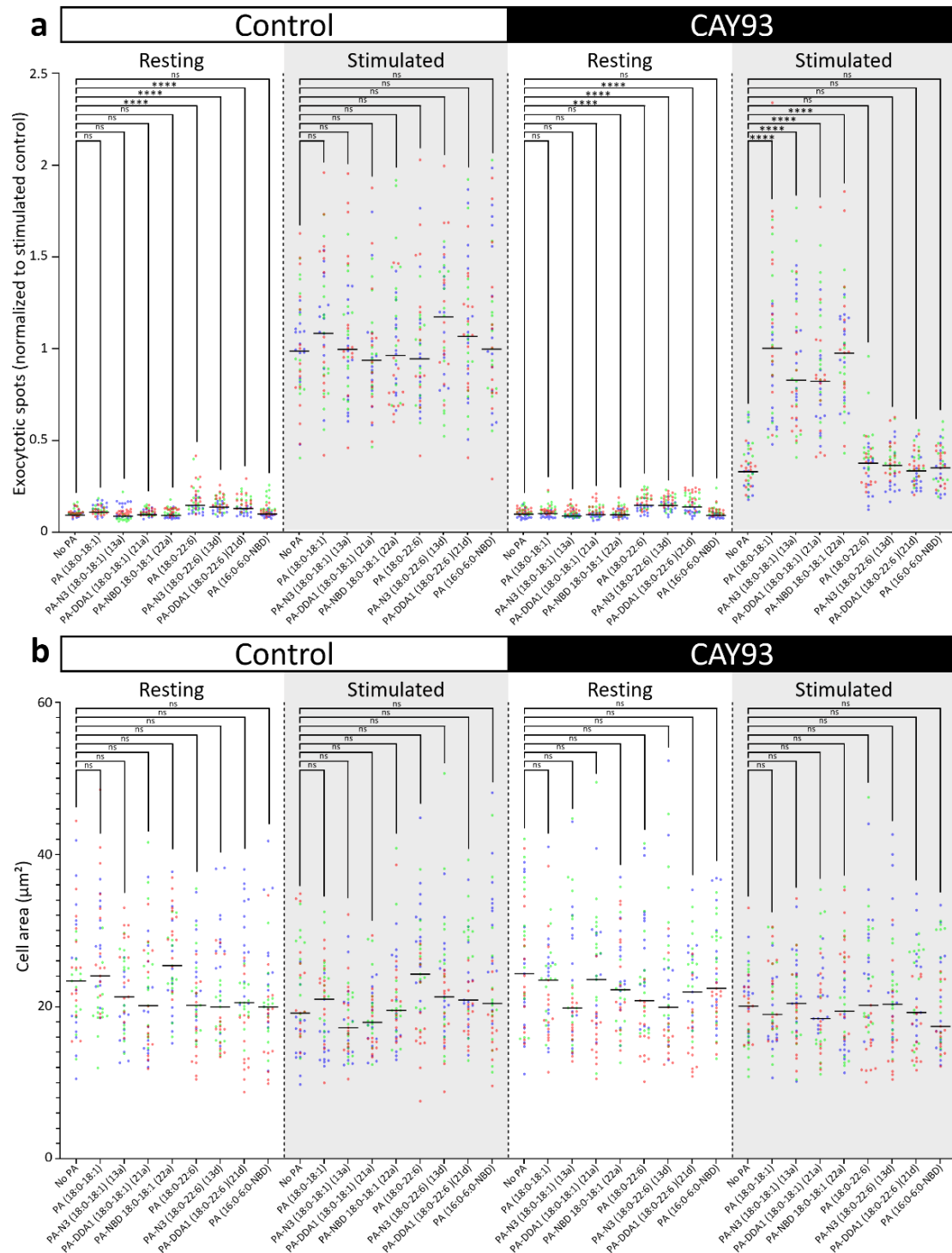
SB, NV, PYR and MM-H discussed the results and wrote the paper. AS, AW, TF, AC, SG, and and edited the paper.

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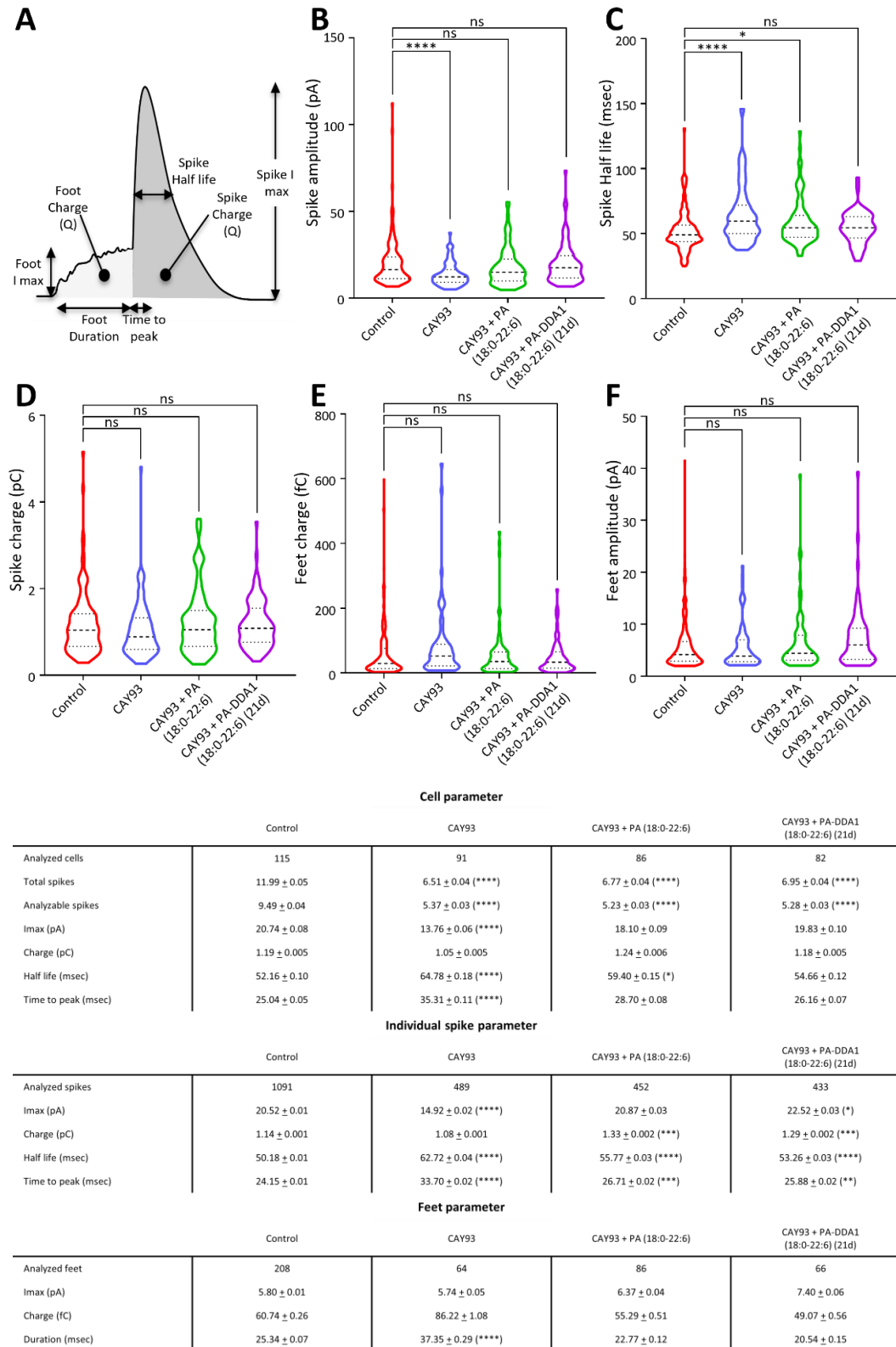
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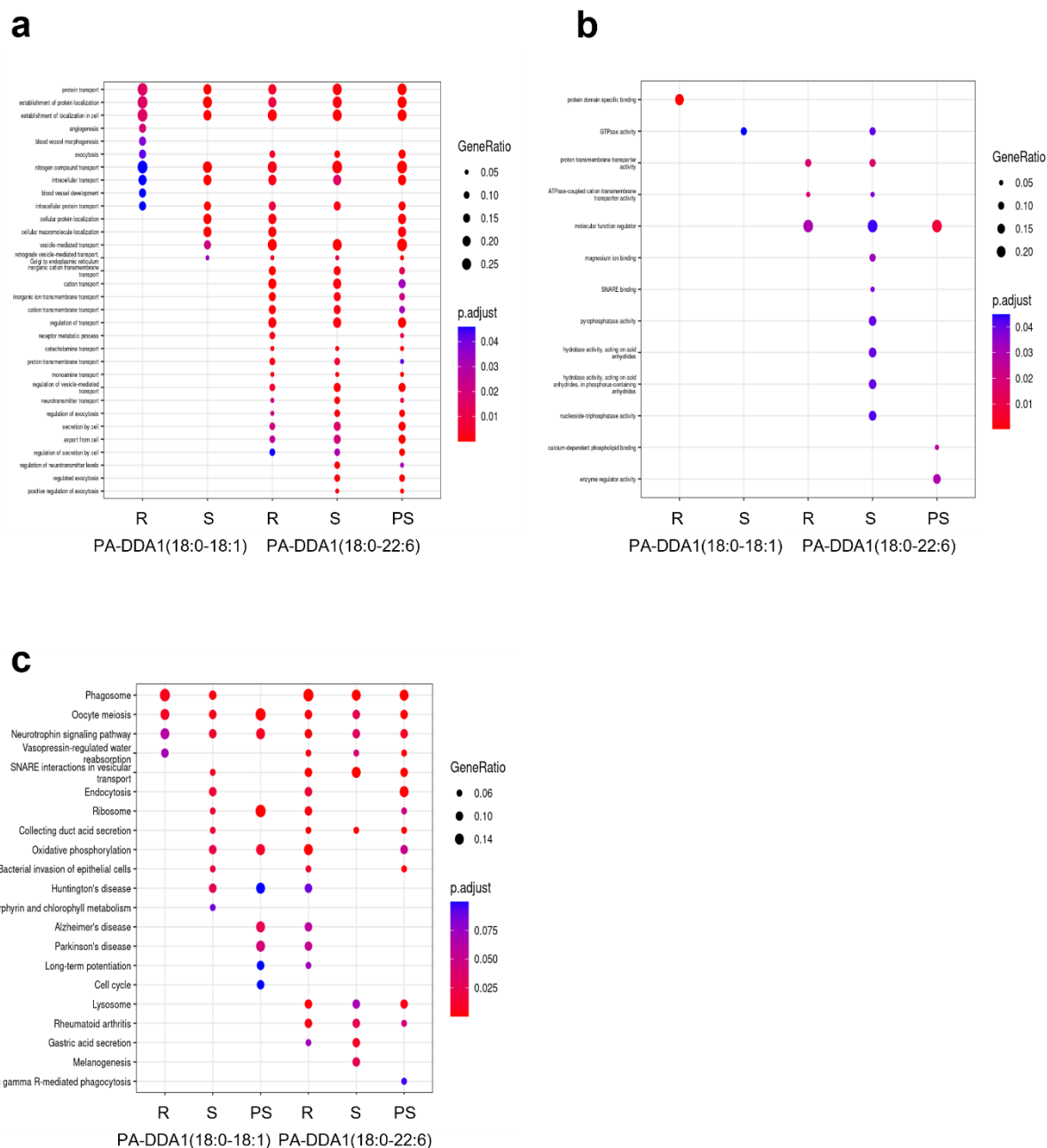


**Extended Figure 1: Effect of the different PA analogues on exocytosis analyzed by measuring cell surface exocytotic sites.** **a**, Quantification of peripheral exocytotic spot of bovine chromaffin cells incubated with DMSO (vehicle), 50nM CAY93, and the indicated PA or PA analogues in resting (3min Locke's solution) or stimulated (3min 59mM K<sup>+</sup>) condition. Mean peripheral exocytotic spot intensity has been normalized with the mean control condition of the individual experimental repeat. **b**, Quantification of the cellular area of each cell. For both graphs, data is represented as a median and each point is a measure from an individual cell. Symbols are color coded (red, blue or green) according to experimental repeats. ns: nonsignificant, \*\*\*\*:  $p < 0.0001$ , Kruskal-Wallis followed by Dunn's multiple comparisons test vs control (N=45 per condition, 15 cells from 3 independent cultures).





**Extended Figure 2: Effect of the different PA analogues on exocytosis analyzed by carbon fiber amperometry.** **a**, Schematic representation of the different individual spike parameters that have been quantified. Violin plots representing respectively the spike amplitude **b**, half-life **c**, charge **d**, feet charge **e**, and amplitude **f**, of the experiment (Bar in the plot represent from top to bottom, 3rd quartile, median and 1st quartile). **g**, Table recapitulating the different parameters that have been analyzed in the amperometry experiment. ns: nonsignificant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ , Kruskal-Wallis followed by Dunn's multiple comparisons test compared to control.



**Extended Figure 3: Functional enrichment analysis of PA fishing experiments. Gene ontology biological processes enrichment analysis. a**, Gene ontology molecular functions enrichment analysis **b**, and KEGG pathways analysis **c** of the proteins identified in the fishing experiments performed in bovine chromaffin cells for DDA1 PA (18:0-18:1) and DDA1 PA (18:0-22:06) in resting (R), stimulated (S), or after stimulation (PS).

| Interactant | Function                                           | PA-DDA1<br>(18:0-18:1)<br>(21a) | PA-DDA1<br>(18:0-22:6)<br>(21d) | Reference |
|-------------|----------------------------------------------------|---------------------------------|---------------------------------|-----------|
| ANXA2       | Annexin, Ca <sup>2+</sup> -dependent lipid binding | +                               | +                               | 1         |
| ANXA3       | Annexin, Ca <sup>2+</sup> -dependent lipid binding |                                 | +                               | 2         |
| ARF1        | GTPase                                             | +                               | +                               | 3         |
| BANF1       | Nuclear assembly                                   | +                               |                                 | 1         |
| CERT        | Lipid transport protein                            | +                               |                                 | 4         |
| CgA         | Secreted peptide and vesicle budding               | +                               | +                               | 5         |
| CLTC        | Coat protein                                       | +                               | +                               | 1         |
| COPB1       | Coat protein                                       | +                               | +                               | 3         |
| DDX3Y       | Protein translation                                |                                 | +                               | 1         |
| DNM1L       | Membrane fission                                   |                                 | +                               | 1         |
| EIF4A2      | Translation initiator                              | +                               |                                 | 1         |
| EIF4A3      | Translation initiator                              |                                 | +                               | 1         |
| FAU         | Ribosomal protein                                  |                                 | +                               | 1         |
| HNRNPR      | Nuclear ribonucleoprotein                          |                                 | +                               | 1         |
| KIF5B       | Kinesin, vesicular transport                       |                                 | +                               | 3         |
| MCCC2       | Methylcrotonyl-CoA carboxylase subunit             | +                               |                                 | 1         |
| MYH10       | Myosin, vesicular transport                        | +                               |                                 | 1         |
| MYH9        | Myosin, vesicular transport                        |                                 | +                               | 1         |
| NAPA        | SNARE associated protein                           |                                 | +                               | 6         |
| NSF         | SNARE associated protein                           | +                               | +                               | 3,6       |
| PARK7       | Parkinsonism associated deglycase                  |                                 | +                               | 1         |
| PCCA        | Propionyl-CoA Carboxylase Subunit                  | +                               |                                 | 1         |
| PITPNA      | Lipid transport protein                            | +                               | +                               | 7         |
| PITPNB      | Lipid transport protein                            | +                               | +                               | 7         |
| PP1         | Protein phosphatase 1 catalytic subunit            | +                               | +                               | 8         |
| PPP1CC      | Protein Phosphatase 1 Catalytic Subunit            | +                               | +                               | 8,9       |
| RAC1        | GTPase                                             | +                               |                                 | 10,11     |
| RBM14       | RNA binding protein                                | +                               |                                 | 1         |
| RHOG        | GTPase                                             |                                 | +                               | 2         |
| RPL10L      | Ribosomal protein                                  |                                 | +                               | 1         |
| RPL15       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPL21       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPL24       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPL3        | Ribosomal protein                                  | +                               | +                               | 1         |
| RPL35       | Ribosomal protein                                  |                                 | +                               | 1         |
| RPL37A      | Ribosomal protein                                  |                                 | +                               | 1         |
| RPL9        | Ribosomal protein                                  | +                               | +                               | 1         |
| RPLP0       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPS11       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPS16       | Ribosomal protein                                  | +                               | +                               | 1         |
| RPS24       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPS26       | Ribosomal protein                                  |                                 | +                               | 1         |
| RPS27       | Ribosomal protein                                  | +                               |                                 | 1         |
| RPS9        | Ribosomal protein                                  | +                               |                                 | 1         |
| SNCA        | α-synuclein                                        |                                 | +                               | 12        |
| SRSF7       | Protein splicing factor                            | +                               |                                 | 1         |
| STAT3       | Transcription factor                               | +                               |                                 | 1         |
| STX1A       | SNARE protein                                      | +                               | +                               | 13        |
| TRA2B       | Protein splicing factor                            | +                               | +                               | 1         |
| TRIM59      | E3 ubiquitin ligase                                |                                 | +                               | 1         |
| TRMT112     | tRNA methylation                                   | +                               |                                 | 1         |
| TUBAC1C     | Tubulin                                            | +                               | +                               | 1         |
| UBA52       | Ribosomal protein                                  | +                               |                                 | 1         |
| VAMP-2      | SNARE protein                                      |                                 | +                               | 14        |
| VAPA        | Lipid transport protein                            | +                               | +                               | 1,15      |
| VAPB        | Lipid transport protein                            | +                               | +                               | 1         |

**Extended Figure 4:** List of the synthetic PA 21a and 21d partners and their corresponding function identified in our screen and found in previous studies using natural PA with the corresponding references.

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### III. Discussion and complementary results

To validate our click chemistry-based tools, these novel synthetic PAs needed first to be validated for their ability to enter cells, to interact with known PA partners, and to behave functionally as natural PAs in membranes.

#### A. Cellular entry and interaction with proteins of synthetic PAs

At first, we validated the ability of various PA analogues to recruit the PA sensor Spo20p-GFP to the PM. Of important note, although this sensor binds *in vitro* to PAs and PI(4,5)P2, this sensor has been reported to specifically target peripheral pools of PAs and does not show any specificity for PA subspecies ([Horchani et al., 2014](#); [Kassas et al., 2017](#); [Zeniou-Meyer et al., 2007](#)). In agreement with our previous observations, incubating chromaffin cells for 15 min with natural commercial PA (18:0-18:1,  $\omega$ -9) and PA (18:0-22:6,  $\omega$ -3), induced a peripheral recruitment of the sensor. Moreover, the addition of PA-N<sub>3</sub> and PA-DDA1, with either mono- or polyunsaturated acyl chains in *sn*-2, induced a similar recruitment (Fig. 3c of the preprint). Hence, these data strongly suggest that the azide group or DDA1 modifications at the *sn*-1' position of PA do not compromise its binding with PA interactants, like Spo20p, and these modifications do not hinder the ability of the synthetic PAs to penetrate cells. Finally, given that Spo20p has a strong *in cellulo* selectivity for PAs, among all other lipids ([Horchani et al., 2014](#); [Kassas et al., 2017](#)), this experiment further suggests that PAs are not rapidly metabolized at the PM, at least within the 15 min incubation time performed here.

We also evaluated the entry dynamics of mono- and polyunsaturated PA-Atto647 into chromaffin cells. Unexpectedly, as commercial NBD-PAs need minutes to access the PM, both fluorescent PA analogues quickly integrated the PM in few seconds and accessed internal compartments in less than one minute following the start of the incubation. This entry does not seem to be primarily dependent on vesicular trafficking as very low signal has been detected in endosomal structures. Interestingly, synthetic polyunsaturated PAs entered cells slightly quicker than monounsaturated PAs and accumulated in a perinuclear compartment (probably the ER), recreating the typical phospholipids saturation gradient within cells ([Keenan and Morre, 1970](#)) (Fig. 3a and b of the preprint). Although, no other similar experiment has been conducted on chromaffin cells, NBD tagged on the *sn*-2 position of phospholipids has been extensively used to evaluate their entry within cell

lines. Originally, the typical entry pathway of PAs has been described as necessitating a conversion into DAG, for transport to the ER, where it can be phosphorylated back into PA. This process is believed to take several minutes ([Pagano and Longmuir, 1985](#)). However, more recent reports suggest an entry kinetic which is more in line with what we have observed. For example, the group of Jeremy Baskin, using click chemistry approaches, to graft a fluorochrome on PA produced by PLD activity at the PM, noticed that the *de novo* produced PA analogues transit in less than 90 s from the PM to the ER and the Golgi in an endosomal-independent manner, and proposed that this transport occurs at MCS ([Tei and Baskin, 2022](#)). It is likely that under these conditions the acyl chains of the produced PA analogues remained unmodified, and PA was not converted into DAG or else, the fluorescent signal on the phosphate headgroup would have been lost. These findings are also in agreement with evidence showing that PIs and PAs are exchanged at the ER-PM MCS to sustain the PLC pathway after intense activation ([Kim et al., 2015](#)).

These experiments highlight the importance of the acyl chain of phospholipids to preserve their entry dynamics within cells. PA species modified on the *sn*-2 position are incorporated way slower into cells and appear to be dependent on DAG conversion to enter cells. Our novel synthetic PAs tagged on the *sn*-1' position entered cell quickly, were not notably converted into DAG, and they illustrated subtleties between various side chains on the *sn*-2 position. Finally, the LTPs present at MCS which may be responsible for the fast transport of PAs within cells are thus likely to have some level of selectivity among the different species of PA.

## **B. Functional validation of mono and polyunsaturated PAs**

To validate the functionality of these novel PAs, we first established their ability to induce membrane curvatures *in vitro*. For this, we reproduced an assay reported previously ([Carmon et al., 2020](#)). CgA addition to PAs containing GUVs induced significant membrane budding. Interestingly, GUVs formed with PA-NBD (18:0-18:1,  $\omega$ -9) were much more prone to budding than GUVs formed with commercial PA (18:1-6:0-NBD) (Fig. 4a and b of the preprint). Additionally, we were able to observe a segregation of PA-NBD signal in membrane buds, which tended to be bigger when the side chains of PAs remained unmodified.



To confirm that the synthetic PAs were able to recapitulate the functional differences between natural monounsaturated PA (18:0-18:1,  $\omega$ -9) and polyunsaturated PA (18:0-22:6,  $\omega$ -3) (Tanguy et al., 2021, 2020b), we performed exocytotic spot staining on chromaffin cells in presence of the specific PLD1 inhibitor CAY93. This induced a 50% decrease of the amount of exocytotic spots after stimulation. Provision of natural monounsaturated PAs totally rescued this effect, while polyunsaturated PAs did not. Monounsaturated PA-N<sub>3</sub>, PA-DDA1, and PA-NBD were able to effectively rescue this phenotype, whereas polyunsaturated PA-N<sub>3</sub>, PA-DDA1 and PA (16:0-6-NBD) did not (Fig. 5a and b of the preprint). This reduction of exocytic events in neurosecretory cells upon PLD1 inhibition is relatively robust, and similar independent studies reported the same reduction using CAY93 or PLD1 knock down (Bills et al., 2024; Vitale et al., 2001; Zeniou-Meyer et al., 2007). Furthermore, in resting condition, independently of PLD1 inhibition, provision of natural polyunsaturated PAs, but also PA-N<sub>3</sub> and PA-DDA1 increased significantly the amount of exocytotic spots (Ext. Fig. 1a of the preprint). The specific ability of monounsaturated PAs addition to rescue this PLD1 inhibition together with an increase of exocytotic spots upon polyunsaturated PAs addition has been established previously by our team (Tanguy et al., 2021, 2020b). To note, PA (16:0-6-NBD) was not able to induce any of those rescue effects, highlighting the fact that PA modifications on the acyl chains likely affect their functionality. We complemented this functional validation, focusing on polyunsaturated PA (18:0-22:6,  $\omega$ -3), by measuring individual exocytotic events on CAY93 treated chromaffin cells using an electrochemical approach: carbon fiber amperometry. The latter allows a fast detection of catecholamine release and provides information on individual fusion events (Chow et al., 1996). As reported previously, PLD1 inhibition reduced the number of exocytotic events, but also decreased their kinetics (increased time to peak, half-life, feet duration, and decreased maximum intensity of spikes). Incubating cells with natural polyunsaturated PAs or PA-DDA1 did not rescue the number of events recorded per cell, however it effectively rescued all kinetic parameters of fusion events (Fig. 5 c-j and Ext. Fig. 2 of the preprint), which is similar to what has been previously obtained on chromaffin cells with the panPLD inhibitor fluoro-indolyl deschlorohalopemide (FIPI) and natural polyunsaturated PAs (Tanguy et al., 2020b). To note, we detected a significant increase in spike charge when incubating cells with natural polyunsaturated PAs or PA-DDA1 (Ext. Fig. 2g of the preprint), which has never been reported previously. This could reflect an effect on catecholamine loading into

SGs or an increased transition from fusion pore/cavicapture to full collapse during fusion events.

Altogether, these experiments strongly suggest that modifications of the acyl chains impaired endogenous functions of natural PAs, as this type of PAs seemed slower to enter cells and seemed to use an alternative pathway independent of MCSs. Furthermore, NBD grafted on the acyl chain of PAs impaired PA-induced membrane budding *in vitro* and failed to rescue the effects of natural monounsaturated PAs or polyunsaturated PAs in chromaffin cells. Additionally, these experiments allowed us to confirm that our novel synthetic PAs, like natural PAs, can rescue exocytosis following PLD1 inhibition in chromaffin cells. Therefore, they are likely to induce similar membrane topological reorganizations, are present at the same locations as unmodified PAs, and interact with same proteins during neurosecretion.

### **C. Establishment of the interactome of different PA species at different stages of neurosecretion**

The crosslinker DDA1 on the *sn*-1' position of mono- and polyunsaturated PAs has two main chemical functions, which allowed us to establish the interactome of those two PA species, at different steps of the neurosecretory process. A diazirine function can form a covalent bond with neighboring proteins upon UV illuminations (350 nm). A secondary alkyne function on the DDA1 arm was used to purify the interactant/PA complexes by performing a copper(I) catalyzed alkyne-azide cycloaddition (CuAAC) reaction with azide tagged beads before mass spectrometry and analysis. Despite the size of the DDA1 arm, PA-DDA1 remained fully functional as described above.

Photo crosslinking procedure was performed in resting, stimulated or after 5 min of compensatory endocytosis conditions of chromaffin cells previously incubated for 15 min with mono- or polyunsaturated PA-DDA1. Control experiments without illumination were performed to identify non-specific interactants. Accordingly, nearly 1000 proteins were identified in the non-illuminated samples and were discarded from the other conditions. Despite this stringent filtration method, we identified 778 potential PA interactants. Among them, only 20% interacted with both PA species, and 40% interacted with mono- or polyunsaturated PAs. Interestingly, among those hits, we found many proteins that have been identified previously as PA interactants on PA-beads fishing

experiments (Ext. Fig. 3 or the preprint) and more specifically in chromaffin cells such as syntaxin1A ([Lam et al., 2008](#)), annexin A2 ([Gabel et al., 2019](#)) or CgA ([Carmon et al., 2020](#)). Of interest, in other signaling lipid photo crosslinking studies, the interactome of various PIPs has also been established directly in HEK cells, and a similar number of proteins has been identified before data filtration ([Müller et al., 2021, 2020](#)). Additionally, we were also able to establish that the binding of some of those interactants are dependent on the state of the cells. For example, CgA was found in all conditions, whereas syntaxin1a only interacted massively with polyunsaturated PA during stimulation. Some of the most interesting candidates we have obtained will be addressed further in the general discussion.

Importantly among the 778 proteins that we fished; we noted many proteins appear to be part of large molecular complexes. It is thus likely that only a portion of those are direct PA interactants. Indeed, larger protein complexes might have been preserved and thus fished out. For example, we have identified numerous subunits of the V-ATPase complex, which might be regulated by PAs in some way, as it is itself controlling PLD1 activation ([Wang et al., 2023](#)), but it is unlikely that all these V-ATPase subunits directly interact with PAs. In line with this hypothesis, we found members of the V1 segment of this complex that transiently interact with the membranous V0 segment; thus, they probably might not directly bind PAs. To reduce this bias, we could play with the experimental conditions, for example by increasing the ionic strength during washes to reduce protein-protein interactions.

Since PA binding proteins are all characterized by an amphipathic alpha helixes PABD ([Tanguy et al., 2018a](#)), we can now perform a bioinformatic analysis on our hits and determine the presence of potential PABD in the aforementioned 778 potential PA partners using the heliquet tool ([Gautier et al., 2008](#)). This software has been useful to establish the PABD of several proteins such as Opi1p ([Hofbauer et al., 2018](#)), Spo20p ([Horchani et al., 2014](#)), PDE4A1 ([Kassas et al., 2017](#)) or CgA ([Carmon et al., 2020](#)). By increasing the number of potential PABD, we may also precise the characteristic of a consensus PABD motif. Additionally, since we obtained specific PA interactant for different PA species and upon different stimulation states of the cell, we may also be able to determine what PABD characteristics could induce such specificity (hydrophobicity, hydrophobic moment, net charge...).

Additionally, several known PA interactants such as PIK5 $\epsilon$ , mTor, or Nir2 have not been identified in our screen. Several hypotheses could explain these discrepancies. Firstly, bovine proteomic databases are less complete than those from more commonly used species in biological research. In fact, the different known interactants that we did not identify in our experiment do not have their bovine gene verified on [uniprot.org](https://www.uniprot.org). Secondly, the main objective of this photo-crosslinking strategy was to establish the interactome of PAs in living primary chromaffin cells, with PAs directly embedded in cellular membranes rather than using classical *in vitro* strategies (PA beads, PA liposomes...). It is now known that the binding of interactants to PAs is dependent on intrinsic cellular membrane properties ([Tanguy et al., 2018a](#)), including membrane curvature, ionic concentrations, membrane tension, packing defects, temperature and probably unknown parameters not yet identified, which could have hindered proper binding to PAs *in vitro*, or induce artifactual binding which never would have happened in cells. Finally, a last explanation for these differences could be that most lipid interactome studies are performed on cell lines. Our experiment has been performed on primary neurosecretory cells, therefore, differences in models could also partially explain those differences. Exemplifying this, recently a study using HEK cells lysates incubated on PA beads found 288 potential interactants of which only 38 were also present in our experiment ([Kattan et al., 2022](#)).

#### **D. Towards caged PA analogues**

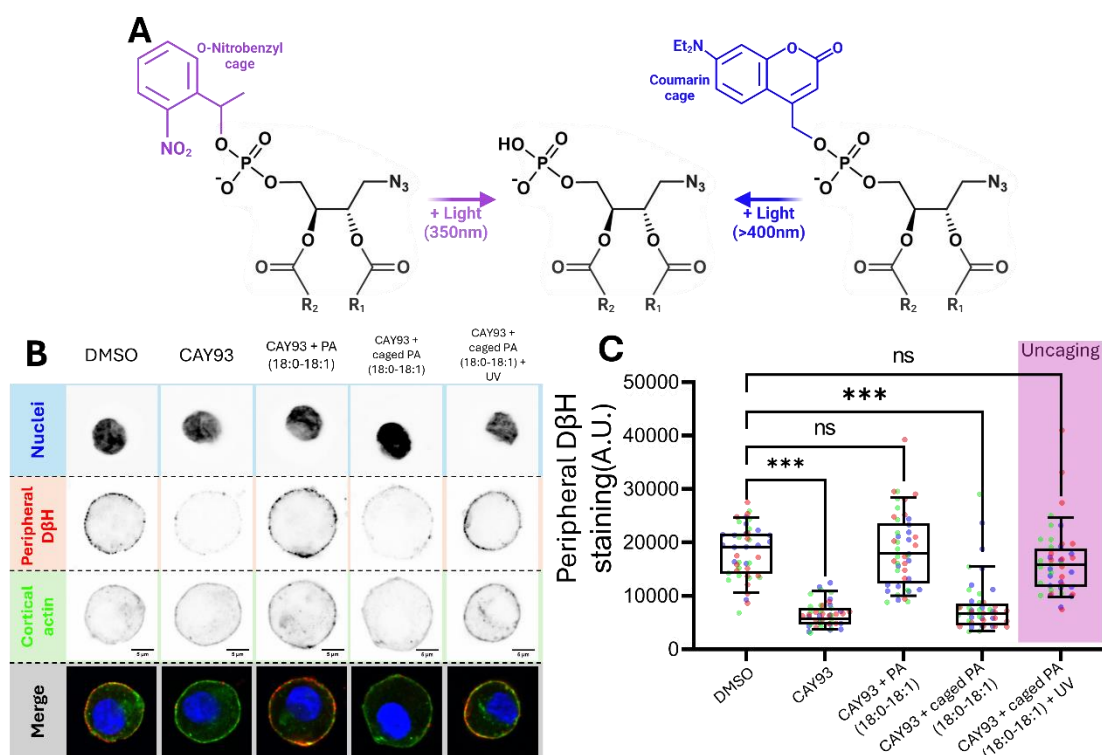
One of the major limitations of these novel PA analogues is their potential conversion into PA down-products by the enzymes involved in PA metabolism. This is especially relevant for PA as it is an important metabolic intermediate necessary for the synthesis of all other phospholipids (see phospholipid metabolism in the introduction). Hence, the dynamics of PA-Atto647 could also reflect the dynamics of DAG and LPA, whereas the interactomes of PA-DDA1 established in live chromaffin cells could also contain interactants of potential down-products of PA's metabolism.

To probe these possibilities, we investigated whether any PA degrading enzymes (PLAs, PAPs and CDSs) were captured during the photo crosslinking experiment. Except for a low signal for CDS2 found in all the conditions including the non-illuminated control condition, we found no enzyme capable of performing the subsequent conversion of CDP-

DAG into PI (PI synthase), PG (PG synthase) or CL (CL synthase). Of note, we identified a PLA2: patatin-like phospholipase 6 which interacted weakly with polyunsaturated PAs upon stimulation, but the relevance of this interaction remains to be established. All together these data suggest that PA is probably not actively metabolized during the short incubation time (15 min) used. An alternate explanation could be that interactions between enzymes and PAs is very transient and not effectively captured by our crosslinking strategy.

A complementary approach that we could have used would be to directly assess the metabolism of these PA analogues by thin layer chromatography. We could have incubated synthetic PAs and recovered membranes from cells after different times. The sample detection would also have been easy, by using a fluorochrome grafted in *sn*-1' position, on acyl chains or even CuAAC/SPAAC with PA-N<sub>3</sub>. Detection of PA's down-products would have enabled us to confirm the metabolic fate of various synthetic PA species within cells. Additionally, subcellular fractionations of these samples could have helped identifying the various membrane compartments in which the different fluorescent PA species accumulate ([Kassas et al., 2021](#)). However, a limitation of this approaches is that it requires a lot of initial cellular material, which is an issue with the usage of primary chromaffin cells and the low abundance of synthetic PA material currently available.

Finally, an alternative strategy we only started to explore is the usage of caged PAs to avoid any "unfortunate metabolic accidents" during incubations. Our chemist collaborators were able to synthesize two different caged PA-N<sub>3</sub> analogues (Fig. 27A). I was able to characterize the UV sensible monounsaturated PA species by using the same rescue approach after PLD1 inhibition during stimulation of chromaffin cells described previously. Without any uncaging, no significant rescue was observed, whereas illumination of the cells treated with the nitrobenzyl-caged PA (18:0-18:1) fully restored secretion to normal levels (Fig. 27B and C). Of note usage of nitrobenzyl caged PA species might not be compatible with DDA1 induced photo crosslinking as there is a high chance of spectral interference between the UV induced diazirine photo crosslinking and uncaging. Hence, we have also developed blue light sensible coumarin caged PAs, but I was not able to characterize them due to a lack of time (Fig. 27A).



**Figure 27:** Characterization of caged PA analogues

(A) Schematic representation of the two caged PA analogues that were synthesized. The first is UV sensible and the second blue light sensible. They have, respectively, an O-Nitrobenzyl or a coumarin group grafted on the phosphate. (B) Representative confocal images of stimulated chromaffin cells after exocytotic spots staining. PLD1 activity was inhibited with 50nM CAY93, and cells were incubated with PA (18:0-18:1) or O-nitrobenzyl caged PA-N<sub>3</sub> (18:0-18:1) analogues. When indicated, cells were exposed to 350 nm UV light. Each channel is shown individually as an inverted grayscale image, and merged (Blue: nucleus, red: exocytotic spots, and green: actin). Scale bar: 5 μm. (B) Mean peripheral exocytotic spot quantifications. Each point is a measure from an individual cell, points are colored red, blue or green according to experimental repeats. ns: nonsignificant, \*\*\*: p<0.001, Kruskal-Wallis followed by Dunn's multiple comparisons; test vs control (N=45 per conditions, 15 cells from 3 independent cultures). The protocol was the same as described in the preprint, uncaging has been realized with a 36 W UV lamp (Nailstar™).

Performing these experiments with the other types of synthetic PAs (fluorescent and photo crosslinking) and exploring whether uncaging impacts the entry dynamics of synthetic PAs into cells or if this induces differences during fishing experiments remain to be done. Additionally, we will also perform similar experiments with different synthetic PAs, for instance containing arachidonic acid, EPA, palmitic acid, linoleic acid in *sn*-2 position) (Fig. 26) to investigate more precisely their potential specific functions in the neurosecretory process.

In conclusion, we took full advantage of the fact that the role of PA in chromaffin cells has already been studied extensively to start the characterization of our novel synthetic PA analogues. Supported by our previous work, we validated the functionality of different PA probes, first depending on their acyl chains composition and secondly with



additional groups grafted in *sn*-1' position. As expected, we verified that these different analogues fully recapitulate the properties tested of their natural counterparts. This enabled us to confirm that the acyl chain composition of phospholipids matters in a cellular context as PA tagged with NBD on their side chain 1) enter cells way slower, 2) cannot induce budding of GUVs, and 3) hardly rescue PLD1 inhibition. We also observed differences between the localization, entry dynamics and protein partners for mono- and polyunsaturated PAs. Chemical biology has been extensively used in the last two decades to expand our understanding of lipids within cells. However, most of the work that has been performed relies on lipids modified on acyl chains ([Müller et al., 2020](#); [Watanabe et al., 2015](#)), which as illustrated for PA could have influenced the outcome of experiments. To further exemplify this, usage of photo switchable acyl chains on lipids, which change their conformation upon illuminations, has gained much attention in the last years. These minor structural changes were shown to massively impact the signaling induced by certain lipids in membranes ([Doroudgar et al., 2021](#); [Frank et al., 2016](#); [Tei et al., 2021](#)).

Finally, we were able to establish the first interactome of two different PA species in living cells. As described previously, being able to perform these experiments directly within cells represents an advantage compared to *in vitro* strategies. Furthermore, the usage of primary cells for these types of experiments has never been attempted before to our knowledge. This is also an important point; especially for the specific case of PA, which is actively involved in many cellular functions, particularly cell survival and proliferation ([Wolf et al., 2022](#)). Post-mitotic primary chromaffin cells do not proliferate whereas cell lines do, therefore, some interactants we identified might be specifically relevant to neuronal cells, while those not identified in our screen might still be relevant in other contexts. Hence, one interesting experiment that we are currently performing is to establish PA interactomes in other cell types. Using more commonly used animal models (such as mice) would also solve the issue of the incomplete bovine database problems we have encountered.

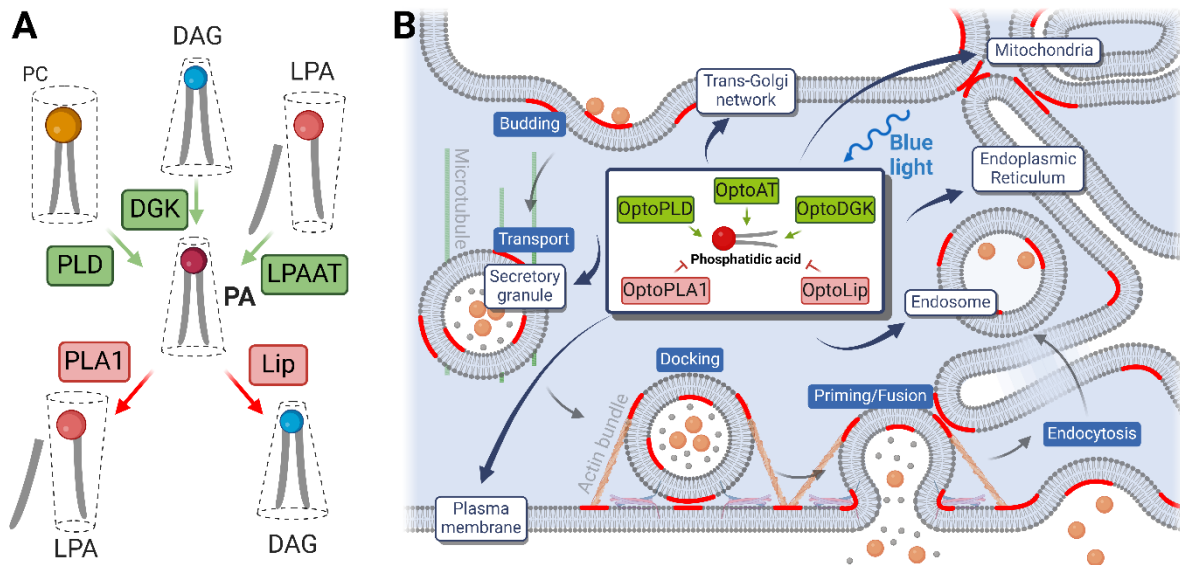
# Development of optogenetic recruitment tools of lipid metabolism enzymes to determine the organelle-dependent functions of PA in neurosecretion

## I. Objective

Although the function of PA is becoming more understood in different cellular contexts, these observations rely exclusively on manipulation of phospholipid levels in cells based on genetic or pharmacological alteration of their metabolic pathways. However, these approaches are not quick enough (at least 30min incubation is necessary to inhibit PLD1 with FIPI ([Tanguy et al., 2020b](#))) and they affect cells, as a whole, and thus cannot offer subcellular specificity, which make them not well-suited to study fast cellular processes bringing into play multiple membrane compartments like neurosecretion. Recently, the group of Dr. Jeremy Baskin has developed an innovative optogenetic tool, called optoPLD, that allowed a fast recruitment of a bacterial PLD to specific membrane compartments to increase subcellular PA levels on demand. This has been realized by linking bacterial PLDs to the cryptochrome 2 (CRY2) and specific membrane markers to the cryptochrome interacting basic helix loop 1 N-terminal domain (CIBN). optoPLD enabled the demonstration that PM PA is required in the activation of the Hippo pathway compared to other PA pools ([Tei and Baskin, 2020](#)). Following this idea, we applied this strategy on chromaffin cells as PA might exercise its function from different membrane compartments, but also at specific moments of the highly choreographed neurosecretory process. Hence, to target different PA metabolism pathways we developed, opto-tools with two additional PA producing enzymes (the LPAAT: RibeyeB, and DGK $\kappa$ ) and two PA degrading enzymes (the PAP: Lipin1b and the PLA1: DDHD1) to produce, respectively, optoAT, optoDGK, optoLip and optoPLA1. Additionally, we expanded the number of targetable organelles by enabling recruitment to two additional compartments of particular interest in the secretory pathway: the TGN (TGN32) and SG (Rab3a) (Fig. 28). Hence for this part of my thesis, I had three main objectives:

- Perform the molecular cloning to generate these additional tools

- Validate the speed and specificity of light induced recruitment of PA metabolism enzymes on primary chromaffin cells
- Establish the consequences of PA modulation on specific organelles during the neurosecretory process.



**Figure 28:** An optogenetic toolkit to quickly modulate subcellular PA levels

(A) Schematic representation of the five metabolic pathways that we targeted to modulate PA levels (B) Representation of the different targeted membrane compartment and their addressing signal: CaaX for the PM, Rab3a for SG, TGN32 for the TGN, Bcl for the mitochondria, TcRb for the ER, and 2xFYVE for endosomes.

## II. Results and discussion

While I successfully fulfilled the first objective for all enzymes and compartments, due to time limitation, for the second and third objective, I focused on optoPLD, optoLip and optoPLA1 and their recruitment to the two membrane compartments most clearly involved in exocytosis: the PM and SG. The characterization of those tools and their functional impact on different steps of neurosecretion are presented in the following manuscript:

### **Optogenetic control of signaling lipid levels revealed essential and specific roles for phosphatidic acid pools in secretory granule and plasma membranes during regulated exocytosis**

Alexander Wolf, Sebahat Ozkan, Micol Vicari, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, Emeline Tanguy and Nicolas Vitale

# **Optogenetic control of signaling lipid levels revealed essential and specific roles for phosphatidic acid pools in secretory granule and plasma membranes during regulated exocytosis**

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Key words: Exocytosis, endocytosis, Lipin, phosphatidic acid, phospholipase A, phospholipase D

## **Abstract**

Calcium-regulated exocytosis is a multi-step process that allows specialized secretory cells to release informative molecules such as neurotransmitters, neuropeptides, and hormones for intercellular communication. The biogenesis of secretory vesicles from the Golgi cisterna is followed by their transport towards the cell periphery and their docking and fusion to the exocytic sites of the plasma membrane allowing release of vesicular content. Subsequent compensatory endocytosis of the protein and lipidic constituents of the vesicles maintains cell homeostasis. Even though lipids represent most membrane constituents, their contribution to these processes remains much less understood than proteins. Using a combination of genetic, silencing, and pharmacological approaches targeting the enzyme phospholipase D1 (PLD1) producing phosphatidic acid (PA) from phosphatidylcholine, we recently reported that PA regulates catecholamine release efficiency by controlling secretory granule biogenesis, transport, docking, fusion, and recycling. Here, we extended this work using an optogenetic-based targeting system to rapidly and selectively modulate PA levels in specific membrane compartments coupled to dopamine  $\beta$ -hydroxylase (D $\beta$ H) labeling and internalization assays. Hence targeting PA metabolizing enzymes revealed specific function of plasma membrane and secretory granule PA pools along the secretory pathway. Thus, among numerous roles that have been attributed to PA our works gives core to the key regulatory role in secretion that has been proposed in different cell models and allows to propose specific contributions of different intracellular PA pools.

## Introduction

Regulated exocytosis in neuroendocrine cells is crucial for hormone and neurotransmitter secretion. During this process, the membrane of a docked vesicle fuses with the plasma membrane, overcoming a high energy barrier. SNARE proteins provide the minimal machinery necessary for fusion and, along with accessory proteins, supply the required energy (Mohrmann et al., 2010; Stepien and Rizo, 2021; Weber et al., 1998). While SNARE proteins are essential for exocytosis, lipid rearrangement also plays a significant role by recruiting protein clusters (Lang et al., 2001), vesicles (Honigsmann et al., 2013) or modulating the stability of the highly curved fusion pore (Chernomordik and Kozlov, 2005; McMahon et al., 2010; Rohrbough and Broadie, 2005). For example, phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P<sub>2</sub>) inhibits fusion pore dilation (Omar-Hmeadi et al., 2023), and cholesterol clusters Syntaxin1a at exocytotic sites, a critical SNARE protein for membrane fusion (Lang et al., 2001).

Eukaryotic membranes contain three major lipid classes: glycerophospholipids, sphingolipids, and sterols. Over 30,000 different lipids have been identified, suggesting diverse biological roles (Yang et al., 2018). Among the glycerophospholipids, phosphatidic acid (PA), plays significant roles in cell function, membrane structure, and signaling. PA production involves several routes, including the glycerol 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) pathways (Vance and Vance, 2004). Signaling PA can be generated by DAG kinases, LPA acyltransferases, and phospholipase D (PLD) (Pokotylo et al., 2018; Tanguy et al., 2019; Zegarlińska et al., 2018). These enzymes are involved in various cellular functions, including vesicle transport and fusion.

Six PLD isoforms exist in mammals, with PLD1 and PLD2 being the most studied (Frohman, 2015). The conversion of phosphatidylcholine into PA by PLD1 requires activation of the enzyme by GTPases and kinases, whereas PLD2 has been shown to be constitutively active (Peng and Frohman, 2012). PLD1 is a crucial lipid-modifying enzyme in exocytosis (Bills et al., 2024; Coorssen and Haslam, 1993; Hughes et al., 2004; Humeau et al., 2001; Jenkins and Frohman, 2005; Stutchfield and Cockcroft, 1993; Tanguy et al., 2020; Vitale et al., 2001; Zeniou-Meyer et al., 2007) and its product PA has been proposed to stabilize negatively curved membranes (Bills and Knowles, 2022; Callan-Jones et al., 2011; Chernomordik and Kozlov, 2005; Kooijman et al., 2003), thereby potentially stabilizing the fusion pore and facilitating exocytosis, but also by potentially recruiting key proteins to specific locations dedicated to exocytosis (Tanguy et al., 2019, 2018).

The involvement of PA in calcium-regulated exocytosis is well-documented (Bader and Vitale, 2009; Bowling et al., 2021). Early studies showed that inhibiting PA synthesis by PLD impairs secretion (Caumont et al., 1998; Galas et al., 1997). PLD1 is the major source of PA during this

process, with its activity tightly regulated by signaling pathways involving small GTPases and the kinase RSK2 (Béglé et al., 2009; Momboisse et al., 2009; Vitale, 2010; Vitale et al., 2005; Zeniou-Meyer et al., 2008). PA is crucial for SNARE complex assembly and fusion pore dynamics (Mima and Wickner, 2009) and a PA-binding domain has been identified in the SNARE proteins syntaxin1 (Lam et al., 2008) and is able to regulate VAMP-2 (Vicogne et al., 2006). Pharmacological inhibition, knockdown, or knockout of PLD1 diminished secretion in chromaffin cells, demonstrating its necessity (Tanguy et al., 2021b). In these cells, PLD1 localizes to the plasma membrane, and its activity increases during stimulation, leading to the formation of multiple PA species (Tanguy et al., 2020; Zeniou-Meyer et al., 2007).

Interestingly different PA species contribute to various steps in exocytosis. Mono- and di-unsaturated PAs are involved in granule docking, whereas poly-unsaturated PAs favor fusion pore dilation (Tanguy et al., 2020). The specific functions of these PA species are most likely linked to their interactions with specific effector proteins. Indeed, some proteins interacting with PA have been reported to discriminate between different PA species. For example, the muscle creatine kinase specifically interacts with saturated PA (Hoshino et al., 2019) whereas the sphingosine kinase interacted preferentially with mono-unsaturated PA and poorly with saturated PA (Guo et al., 2011).

PLD1-generated PA is essential for various steps in exocytosis, from granule biogenesis (Carmon et al., 2020), to transport (Tanguy et al., 2021b), docking, and fusion pore dilation (Tanguy et al., 2021a) with distinct roles for specific PA species, highlighting the importance of lipid diversity in membrane trafficking and signaling. This also implies that PA in different subcellular membrane compartments could play a different role along the secretory pathway. However, until recently, due the lack of tools it was impossible to selectively modulate PA levels rapidly and in specific organelles. To circumvent these limitations, we adapted an elegant optogenetic system that was recently set-up to target bacterial PLD to various organelles allowing to demonstrate the importance of plasma membrane PA in the activation of the Hippo pathway (Tei and Baskin, 2020). By targeting specifically, the PA-phosphatase lipin1b and the phospholipase A1 (PLA1) enzymes involved in PA degradation to secretory granules or to plasma membranes for short or longer time, we were able to test the contribution of these PA pools on different steps along the late secretory pathway. Understanding the contribution of the different subcellular pools of PA will provide novel insights into the molecular mechanisms underlying hormone and neurotransmitter secretion.



## Results and discussion

### *Design of an optogenetic toolbox for quickly recruiting PA metabolism enzymes to various specific membrane compartments*

Existing tools are not suitable for determining whether acute local pools of PA found on specific organelle membranes are directly involved in individual physiological processes. Here, we developed a toolset for modulating physiologically relevant pools of PA with spatial and temporal precision and applied it to define roles for organelle-specific PA pools along the secretory pathway. Thanks to the ribosome skipping P2A, an optogenetic targeting system for enzymes involved in PA metabolism was designed based on the simultaneous and equimolar expression of 1) PA metabolic enzymes fused to mCherry and CRY2 and 2) of targeting peptides fused to CIBN (Fig. 1A). Among several light-mediated heterodimerization systems, CRY2–CIBN was chosen because its dimerization is rapidly induced by nontoxic, blue light (450-495 nm) (Fig. 1A), but its dissociation rate in the dark is relatively slow, making it ideal for sustained heterodimerization (and concomitant PA production/degradation) using relatively minimal sample illumination (Benedetti et al., 2018; Idevall-Hagren et al., 2012; Kennedy et al., 2010; Niu et al., 2016).

To validate this optogenetic approach in a neurosecretory cell model, we first attempted to recruit ectopically expressed PLD1 at the plasma membrane of chromaffin cells, as PLD1 was shown to be the master player in PA synthesis during exocytosis (Tanguy et al., 2021b, 2020). However, mammalian PLD1 proved very difficult to express in conjunction of the different targeting system in these primary cells. We thus decided to express a bacterial PLD from *Streptomyces sp.*, which is not subjected to complex regulation by protein-protein interaction as reported previously (Tei and Baskin, 2020). Interestingly, this well-characterized enzyme exhibits unregulated activity under physiological conditions (37°C, pH 7.4), and the availability of its crystal structure enabled protein-engineering generation of mutants with increased activity (Tei et al., 2023). We tested several mutants with increased PLD activity (10, 30, 50, and 100-fold above basal activity of the wild-type bacterial PLD) and found that the PLD<sub>10X</sub> was the most effective with no noticeable toxic effects on chromaffin cells. Additionally, as PA lies at the center of lipid metabolism, its conversion in additional signaling lipid could also contribute to its effects. Accordingly, our toolbox included Lipin1b that converts PA into diacylglycerol and phospholipase A1 (PLA1) to metabolizes PA into lysophosphatidic acid (LPA), named optoLip and optoPLA1. In the absence of blue light, all three CRY2-mCherry-enzymes were localized in the cytosol with a slight accumulation of PLD<sub>10X</sub> in the nucleus. Upon illumination, these enzymes were effectively recruited to the expected, CIBN-tagged membrane compartments such as the plasma membrane

(CaaX) or secretory granules (Rab3a). Both those compartments are at the center of neurosecretion. Strikingly, recruitment of both PA catabolism enzymes affected the overall shape and size of secretory granules, whereas recruiting PLD had no noticeable effect (Fig. 1C). Hence, recruiting PLA1 or Lipin1b to secretory granules for 30 min induced the appearance of enlarged granules with often non round forms (Fig. 1C).

To further validate the speed efficiency of the CRY2-CIBN system to perform a fast recruitment which would be in line with the kinetics of our assay to monitor exocytosis from bovine chromaffin cells, we performed recruitment of those enzymes to the plasma membrane after only a shorter 3 min and 30 s illumination time. Cellular segmentation revealed a significant increase of the peripheral localization of all three enzymes (Fig. 2). However, optoPLD<sub>10X</sub> recruitment (Fig. 2A) was more modestly recruited than optoPLA1 or optoLip (Fig. 2B and C), probably because part of the optoPLD1 is expressed in the nucleus, preventing rapid accessibility to the plasma membrane of this pool of nuclear optoPLD<sub>10X</sub>. Of note, the expression of these tools did not modify the overall cell surface area (Fig. 2), as this is an important parameter that can bias the recruitment analysis and subsequent functional assay quantification. Interestingly, further experiments revealed that this short 3 min illumination was as effective as 30 min to recruit optoLip and optoPLA1 to the plasma membrane, meaning that a short illumination time is sufficient to recruit most of the enzymes to a given compartment (Fig. 2B and C). Accordingly using an infrared fluorescent protein (iRFP)-tagged CIBN-CAXX, the light-mediated PLD recruitment to the plasma membrane was previously shown to have half time of ~5 s in HEK 293T cells, whereas its dissociation in the dark was much slower ( $t_{1/2}$  ~10–15 min) (Tei and Baskin, 2020).

#### *PA production at the plasma membrane is required for exocytosis*

To perform functional evaluation of the recruitment of optoPLD<sub>10X</sub> to the plasma membrane we have opted to perform immunolabelling of exocytic spots. This approach, based on the addition into the medium of specific antibodies targeting the dopamine  $\beta$  hydroxylase enzyme (D $\beta$ H), an antigen associated to the luminal leaflet of secretory granules. The latter is only available to the extracellular medium after fusion of the granule with the plasma membrane. Hence, this approach enabled us to perform experiments in the darkness to prevent an early unwanted activation of the CRY2 protein. The peripheral D $\beta$ H signal is very weak in resting cells without photoactivation in agreement with a very low basal secretory activity of chromaffin cells (Fig. 3A and B). A five-fold increase in peripheral anti-D $\beta$ H signal was observed after stimulation of exocytosis with a [59mM] K<sup>+</sup> solution independently of photoactivation (Fig. 3A and B). Hence,

overexpression or recruitment of optoPLD<sub>10X</sub> did not seem to interfere with the ability of chromaffin cells to perform exocytosis in a regulated manner. However, when comparing D $\beta$ H signal intensity in resting cells combined with photoactivation, we noticed a significant increase in peripheral D $\beta$ H signal (Fig. 3A and B). Those results are in line with previous data showing that overexpression of PLD1 slightly increased exocytosis in PC12 cells (Vitale et al., 2001), and that provision of exogenous polyunsaturated PA in the cell medium also increased exocytosis in resting chromaffin cells with similar experimental designs (Tanguy et al., 2021a). The effect of PA synthesis at the plasma membrane induced by unstimulated secretory activity might occur because of spontaneous fusion of peripheral pre-docked/primed granules upon provision of fusiogenic lipids independently of stimulation. Interestingly, while not being a definitive proof, this increase of exocytotic spots after peripheral recruitment of optoPLD<sub>10X</sub> in resting condition, shows that this effect is dependent of the presence of PA at the plasma membrane.

To further evaluate the contribution of PA produced by optoPLD<sub>10X</sub> on exocytosis, we used CAY93 a potent and specific inhibitor of PLD1-generated PA synthesis (Scott et al., 2009). This enabled us to explore the resulting consequences of targeted PA production by bacterial PLD at the plasma membrane on exocytosis without the bias of endogenous PA production. Of importance, this pharmacological inhibitor is specific to mammalian PLD1 and should thus inhibit endogenous PLD1, but not interfere with optoPLD<sub>10X</sub> activity. Importantly, the translocation of the overexpressed enzyme from the cytosol to the plasma membrane after photoactivation was not affected by the CAY93 treatment (Fig. 3A). As described previously (Tanguy et al., 2020), the PLD1 inhibitor strongly impaired exocytosis (Fig. 3A and B). However, a short 30 s blue-light exposure followed by a 3 min illumination during the stimulation, inducing the recruitment of optoPLD<sub>10X</sub> to the plasma membrane, increased the number of exocytic sites at the cell periphery upon stimulation at levels close to that of cells not treated with CAY93 (Fig. 3A and B). This rescue effect by optoPLD<sub>10X</sub> is only seen after illumination, suggesting that PLD dependent PA production is required at the plasma membrane during stimulation for efficient exocytosis. Hence, by combining specific pharmacological PLD1 inhibition and optogenetic recruitment of bacterial PLDs, we were able to functionally validate the requirement of PA synthesis at the plasma membrane for efficient exocytosis with an unprecedented spatial and temporal precision.

#### *Fast PA degradation at the plasma membrane impaired exocytosis*

We next probed the impact of the recruitment of PA catabolizing enzymes on the plasma membrane to further assess the contribution of this PA pool on exocytosis. We tested the effect

of short (30 s prior to stimulation) or long-term (30 min prior to stimulation) illumination in cells expressing optoLip associated with the plasma membrane targeting system and found that both profoundly reduced exocytic site labeling compared to non-illuminated cells (Fig. 4). Importantly, a catalytically inactive optoLip with the R799G mutation (optoLip<sub>PD</sub>), which has been reported to abolish the phosphatase activity of lipin1b (Khayyo et al., 2020), did not affect exocytosis, validating the specificity of these observations (Fig. 4). Of note, short or long-term illumination were equally effective in recruiting optoLip or its mutated form (Supplemental Fig. 1). As the effect of Lipin could be attributed either to decrease in PA levels or to an increase diacylglycerol, we used optoPLA1 that converts PA into lysophosphatidic acid and a free fatty acid (Fig. 1B). Like optoLip, optoPLA1 recruitment to the plasma membrane reduced exocytic site labeling after short or long illumination times (Fig. 5). Moreover, a catalytic dead optoPLA1 (optoPLA1<sub>LD</sub>) with the S537A mutation, reported to abolish its lipase activity (Maemoto et al., 2020), did not perturb exocytosis (Fig. 5). As seen for optoLip, no significant differences in the recruitment of optoPLA1 or its mutated form to the plasma membrane was seen with short or long-term illumination (Supplemental Fig. 2). Importantly by targeting two PA degradation pathways, DAG and LPA produced, respectively, by optoLip and optoPLA1 have profound different impact on membrane geometry. Indeed, DAG has a cone-shape structure, whereas LPA has an inverted cone-shape structure, thereby promoting negative and positive membrane curvature, respectively (Tanguy et al., 2018). It is thus unlikely that the inhibitory effect on exocytosis seen here results from the synthesis of DAG or LPA, which have such difference biophysical properties, but rather simply reflects the loss of the plasma membrane PA pool.

Hence, all together these data suggest that hampering with PA levels at the plasma very rapidly impacted exocytosis. As established with optoPLD<sub>10X</sub>, PA production in this membrane compartment is required for efficient vesicle fusion during stimulation. Additionally, by targeting two PA degradation pathways, our results support the notion that the effects seen on exocytosis result from the metabolism of PA rather than from the synthesis of two distinct down products of PA. Therefore, the peculiar combination of a conical shaped lipid harboring an anionic phosphate headgroup seems to confer fusiogenic properties to PA. These results are in line with our previous lipidomic analysis that indicated a dramatic increase in PA levels at the plasma membrane during exocytosis and with rescue experiments in PLD1-inhibited cells in which polyunsaturated forms of PA specifically regulated the late stages of exocytosis by controlling the dynamics of the fusion pore (Tanguy et al., 2020).

### *Long-term PA degradation on secretory granules is needed to alter neurosecretion*

We next focused on the secretory granule pool of PA as the membrane of this organelle is obviously involved in exocytosis. We observed that PA degradation on this compartment produced these granules with anormal shape (Fig. 1C). Unlike the plasma membrane, short recruitment of optoLip or optoPLA1 prior to stimulation did not affect the level of peripheral D $\beta$ H staining upon stimulation (Fig. 6 and 7). On the contrary, 30 min illumination prior to the stimulation strongly inhibited exocytosis when targeting optoLip or optoPLA1 to secretory granules (Fig. 6 and 7). This inhibitory effect is dependent on the ability of both those enzymes to degrade PA as optoLip<sub>PD</sub> and optoPLA1<sub>LD</sub> did not recapitulate these findings (Fig. 6 and 7). Additionally, the overall shape of the secretory granules seemed only affected by a long 30 min illumination and this effect was also dependent on the catalytic activity of the enzymes (Fig. 6 and 7).

As for the plasma membrane, the usage of two distinct metabolic pathways to degrade PA is a strong argument in favor of a direct involvement of the secretory granule pool level during exocytosis and against a role of the PA down products DAG and LPA. The long illumination time needed to see an effect on exocytosis suggests that this specific PA pool might regulate maturation and/or transport of secretory granules, rather than the late steps of exocytosis. An interesting consideration given the increase in granule size is a potential role of PA in granule maturation. Indeed, during this process, exclusion sorting requires the GTPase Arf1 recruitment (Austin et al., 2000), which has already been established as a potential PA binding partner (Manifava et al., 2001). Hence, recruitment of Arf1 on immature secretory granules may be mediated by PA in the granule membrane. A second potential yet non-exclusive explanation for the reduction of exocytotic spots observed when PA metabolizing enzymes are targeted for a long time to secretory granules might be a contribution of this specific PA pool in secretory granule transport. We have established recently that PLD inhibition slowed down directed motion of secretory granules towards the periphery of PC12 cells (Tanguy et al., 2021b). This directed motion has been reported as microtubule dependent (Ñeco et al., 2003). Furthermore, kinesin-1 isoforms have been proposed to bind anionic lipids including PA (Kumar et al., 2024; Manifava et al., 2001; Wang et al., 2017). Thus, secretory granule membrane PA might directly bind to kinesin-1 to promote their initial transport. A final explanation could be that PA regulates the anchoring process of secretory granules at the plasma membrane exocytotic sites. This procedure is believed to be mediated by the formation of actin bundles fasciculated by annexinA2 in chromaffin cells (Gabel et al., 2015). The latter is a protein recruited to specific lipid microdomains and has already been established as a potential PA partner (Gabel et al., 2019).

Hence, PA might be the missing link between annexinA2 and granule membranes, thus promoting secretory granule anchoring to the plasma membrane. Accordingly, our previous observations revealed that monounsaturated PA specifically promoted the docking process of secretory granules by regulating those actin bundles (Tanguy et al., 2021b, 2020). Of note, it was recently shown using TIRF microscopy and a PA sensor that PA slowly accumulates near docked vesicles in PC12 cells (Bills et al., 2024). It now remains to be investigated further whether either one or a combination of these hypothesis explain the contribution of secretory granule PA in exocytosis.

#### *PA degradation on the plasma membrane impaired compensatory endocytosis*

Finally, after uncovering potential specific roles of distinct PA pools in the exocytotic process using this original optogenetic approach, we investigated their potential contribution in compensatory endocytosis. Indeed, after fusion, the secretory granule membrane is transiently incorporated in the plasma membrane. As it has been well established for neuron (Ivanova and Cousin, 2022), to maintain cell surface relatively constant, to preserve the specific lipid and protein composition of the plasma membrane, and finally to recycle the granular components, the secretory granule membrane is preserved within the plasma membrane and selectively recaptured. A functional assay to monitor compensatory endocytosis relies on the use of a probe that would specifically label recycled constituents from the secretory granule membrane (Ceridono et al., 2011; Ory et al., 2013). Hence, we incubated chromaffin cells with an anti-D $\beta$ H antibody which is internalized together with D $\beta$ H upon chase and revealed later with a secondary antibody after fixation and permeabilization of cells. Internalization of D $\beta$ H was evaluated after confocal images acquisition and segmentation of the peripheral area of cells. Recycling of secretory granules membranes starts quickly after they have fused and thus already takes place during the stimulation period. Therefore, we performed control experiments with immediate fixation of transfected cells following 3 min stimulation, corresponding to the time zero of the chase. Hence, we established that during this time, nearly 50% of the D $\beta$ H signal was already internalized without illumination. Importantly, we found no differences in D $\beta$ H internalization for this zero-timepoint between cells transfected with optogenetic PA catabolism enzymes and their catalytically inactive counterparts (Fig. 8-9 A and B). After 5 min of chase following stimulation, the amount of internalized exocytotic spots increases from 50% to nearly 80% in optoLip transfected cells kept in the dark. However, light dependent recruitment of optoLip to the plasma membrane during chase induced a significant decrease in internalization while no differences were noted with the inactive optoLip<sub>PD</sub> (Fig. 8C and D). We obtained similar results for optoPLA1

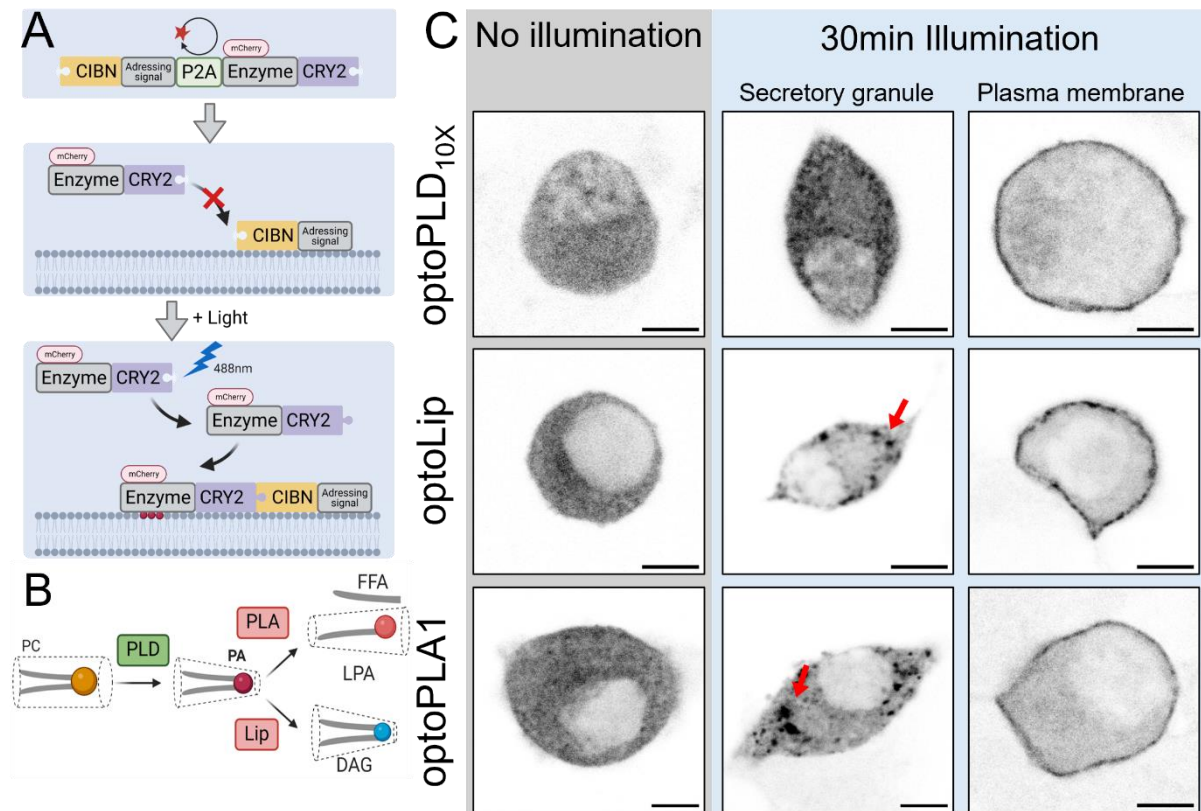


recruitment with only a slight additional difference of internalization when comparing optoPLA1 (non-illuminated) to optoPLA1<sub>LD</sub> (illuminated) (Fig. 9 C and D). Furthermore, we also verified light dependent recruitment of enzymes during these experiments (Supplemental Fig. 3 and 4). More importantly, given the striking effect of plasma membrane PA degradation on exocytosis, an impairment of membrane fusion could have represented a bias when looking at the following compensatory endocytosis. Therefore, we verified that peripheral D $\beta$ H staining was not affected on the zero-timepoint upon stimulation between enzymes and compared the total amount of D $\beta$ H signal in cells between all conditions and only obtained one slight noticeable difference (Supplemental Fig. 5 and 6). Hence, the exocytotic process does not seem to have been impacted during endocytosis experiments. Altogether, these results reinforce the notion that PA is involved in compensatory endocytosis and further indicate a direct involvement of the plasma membrane PA pool. These observations are clearly in line with the previous results obtained with the PLD inhibitor FIPI, which also inhibited compensatory endocytosis (Tanguy et al., 2021b) and with the proposed function of PA produced by DGK in synaptic vesicle cycle (Tu-Sekine et al., 2015). Altogether these results indicate that PA generated by PLD, but also potentially by other sources also contributes to optimal compensatory endocytosis.

## Conclusion

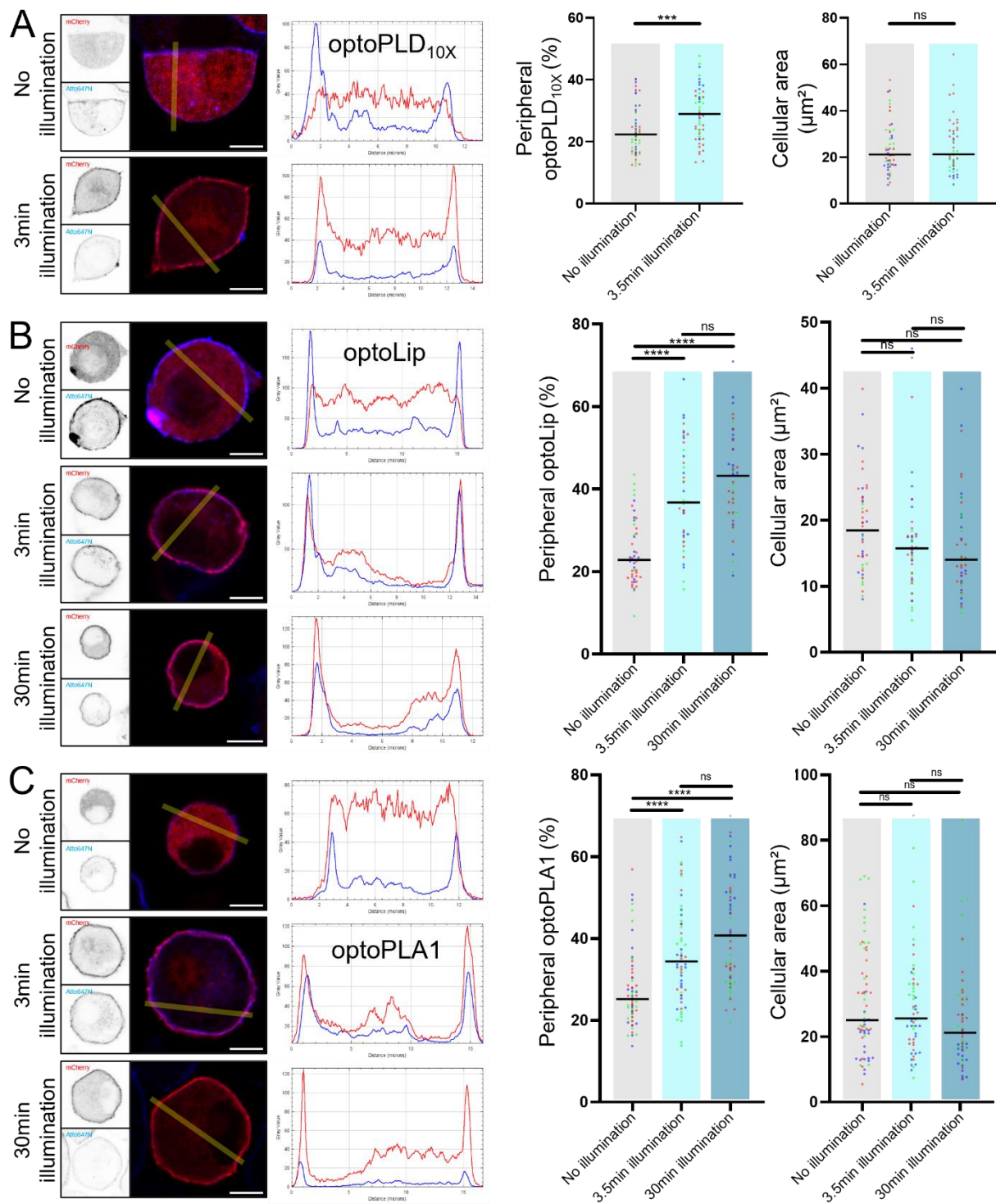
Over the last decades PA has turned out to be a major regulator of numerous cellular processes in numerous experimental paradigms and especially in membrane trafficking. However, as this lipid is present in virtually all membrane compartments, unravelling the contribution of the different PA pools has remained challenging. Therefore, implementation of an optogenetic control of PA production represented an important innovation for understanding the roles of this unique phospholipid with an unseen spatial and temporal control (Tei and Baskin, 2020). Here we used the previously implemented optoPLD<sub>10X</sub> and generated two new light sensible PA degradation enzymes: optoLip and optoPLA1 to explore the function of PA in neurosecretion on primary chromaffin cells. We were able to establish that the granular PA pool is involved in early stages of regulated secretion whereas plasma membrane PA is necessary for late stages and compensatory endocytosis. The usage of three metabolic pathways targeting PA enabled us to confirm that the observed effects were not likely dependent on the subsequent production of DAG, LPA, or free fatty acids. Further work is now needed to expand the number of organelles that can be targeted with PA degradations enzymes, for example to the Golgi apparatus, the endoplasmic reticulum, mitochondria or endosomes and verify their potential contribution to the

overall neurosecretory process. Additionally, targeting other PA degradation enzymes such as PLA2 or cytidine diphosphate synthase and PA synthesizing enzymes such as DGK and LPA-acyl transferase should also prove useful to gain more information on the nature of the PA involved.



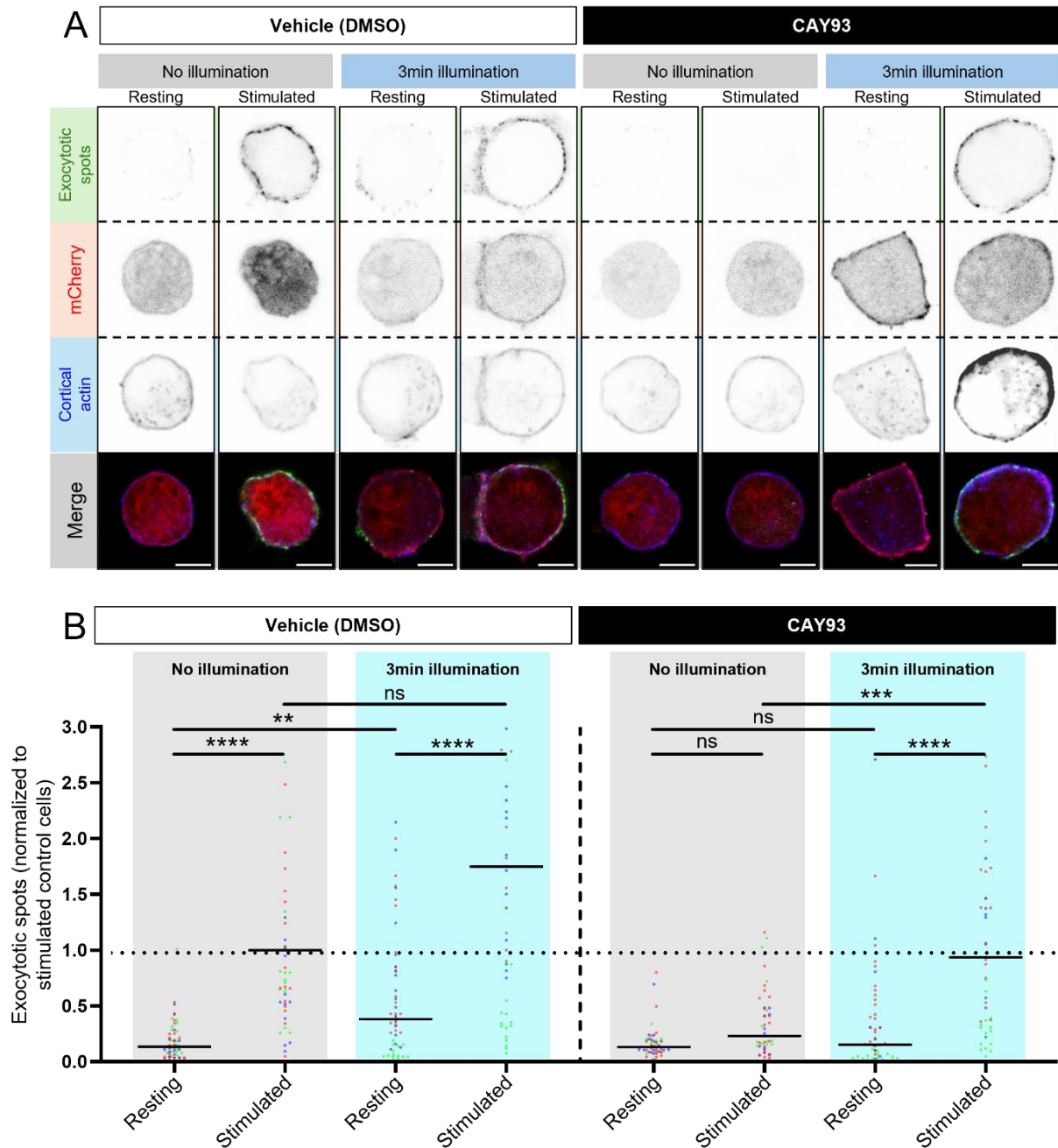
**Figure 1:** An optogenetic approach to target different PA metabolism enzymes in specific membrane compartments.

(A) Schematic representation of the principle of the light inducible targeting system. Both proteins necessary for performing inducible recruitment were expressed through a single plasmid and split thanks to a P2A sequence. Without illumination, enzymes remain in the cytoplasm, however, upon illumination, a conformational change of the CRY2 protein enables its association with CIBN expressed in specific compartments. (B) The three metabolic pathways that were targeted are: 1) PA production mediated by PLD, 2) PLA1-dependent PA degradation and 3) PA-phosphohydrolysis by Lipin. (C) Representative confocal images of bovine chromaffin cells expressing mCherry tagged optoPLD<sub>10X</sub>, optoLip and optoPLA1 without illumination and their recruitment to secretory granules (Rab3a tagged CIBN) and plasma membrane (CaaX tagged CIBN) following 30min illumination. Scale bar: 5µm. Arrows indicate enlarged secretory granules.



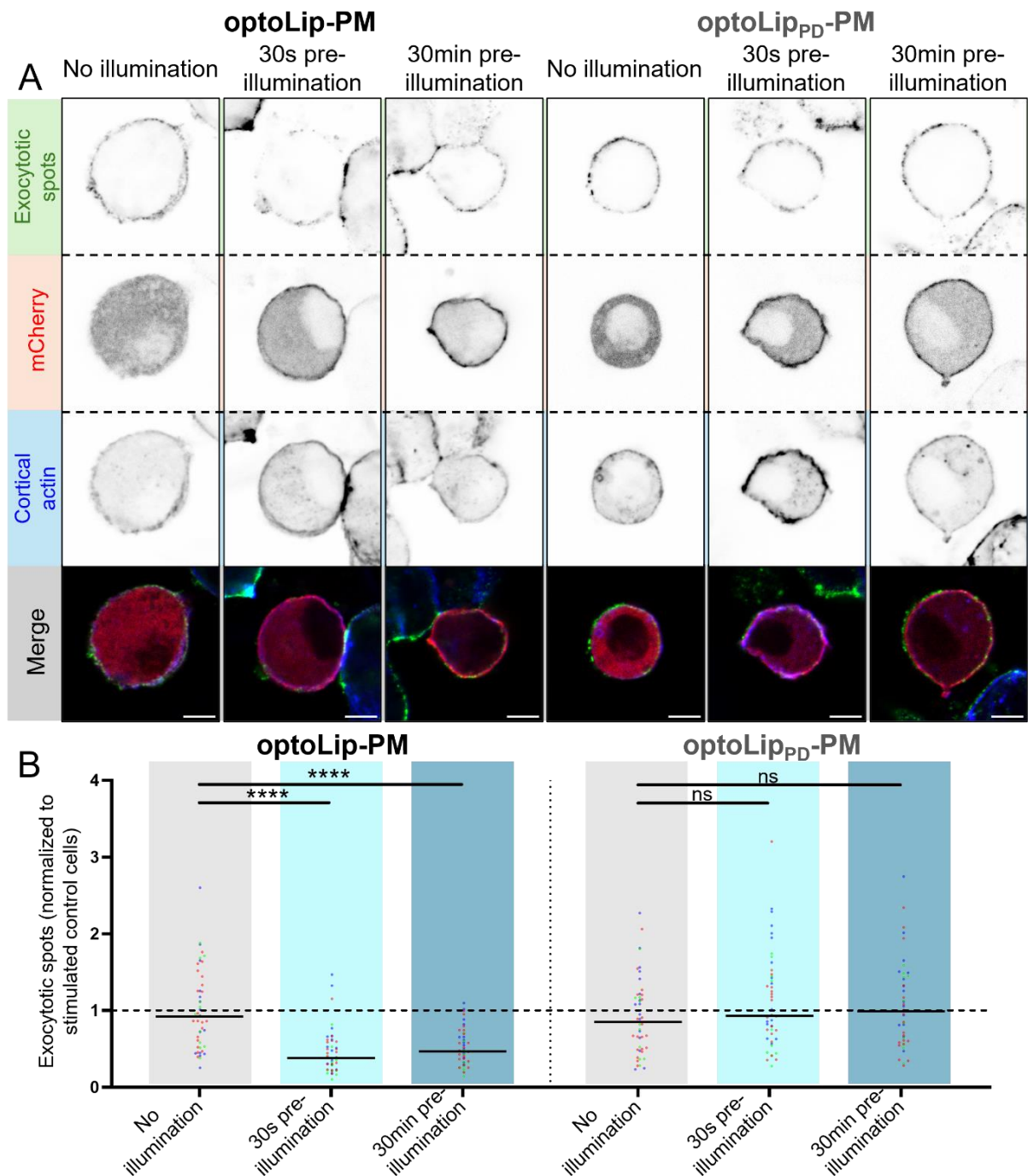
**Figure 2:** Characterization of the recruitment of enzymes involved in PA metabolism to the plasma membrane.

Quantification of (A) optoPLD<sub>10X</sub>, (B) optoLip and (C) optoPLA1 recruitment to the plasma membrane after illumination. Images are represented as inverted grayscale channels and merged (red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5 μm. For each cell, gray value along a 5-pixel wide line traced across the cell is also represented. Graphics represent the quantification of peripheral mCherry signal for each experiment together with the overall cell surface area. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue or green) according to experimental repeat. For PLD<sub>10X</sub>, \*\*\*:  $p < 0.001$  and ns: nonsignificant, Mann-Whitney rank sum test. For Lipin and PLA1: \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and three independent cultures for each condition.



**Figure 3:** PA at the plasma membrane is necessary for exocytosis.

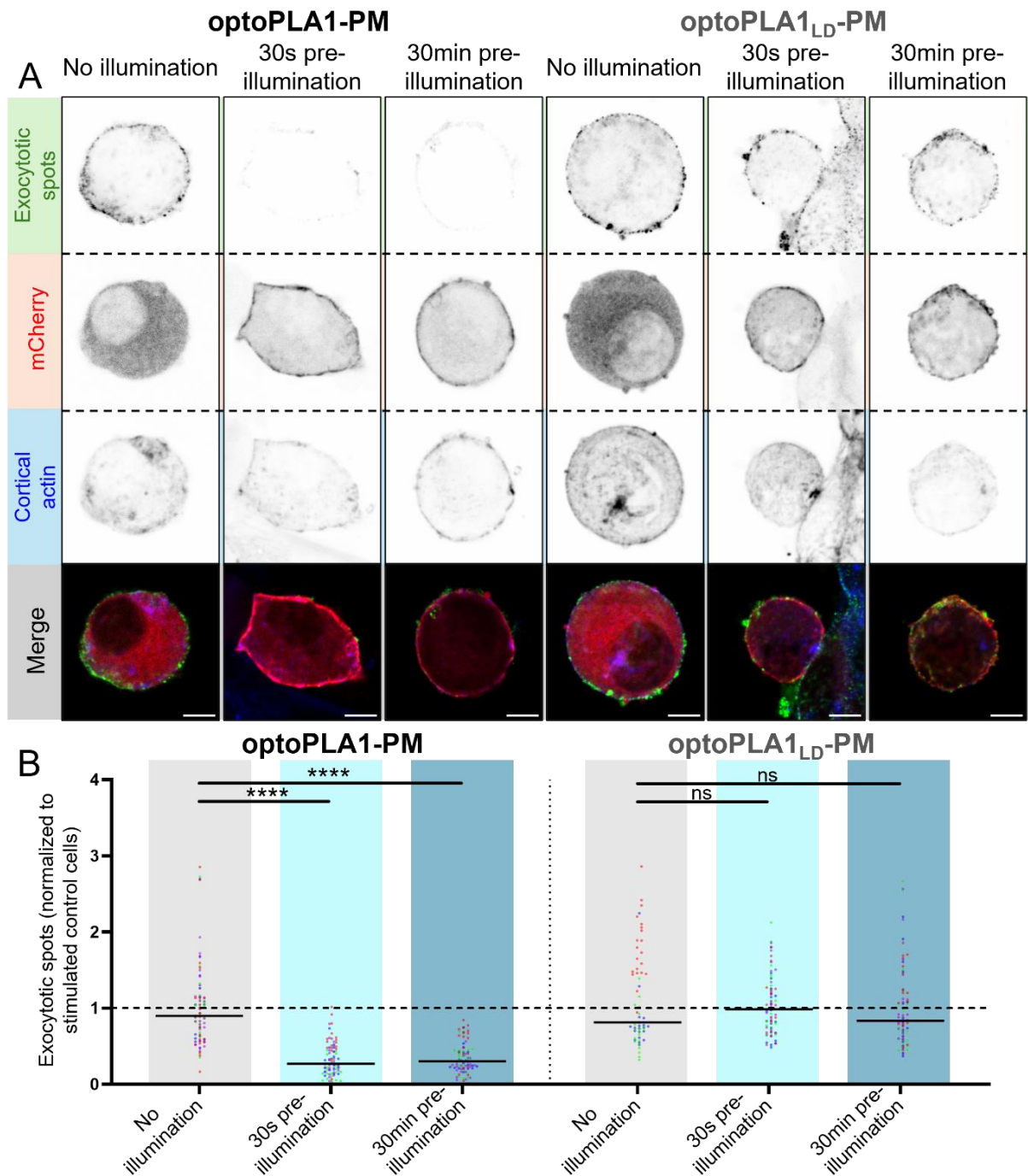
(A) Representative confocal images of exocytotic spot staining of chromaffin cells expressing optoPLD<sub>10x</sub> enabling a recruitment to the plasma membrane upon 3 min illumination. Cells were kept in resting condition or stimulated ([59 mM] K<sup>+</sup>), while PLD1 has been inhibited with CAY93 50 nM when indicated. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5  $\mu$ m. (B) Graphic of the quantification of exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue or green) according to experimental repeat. \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and three independent cultures for each condition.



**Figure 4:** OptoLip recruitment to the plasma membrane decreased exocytosis.

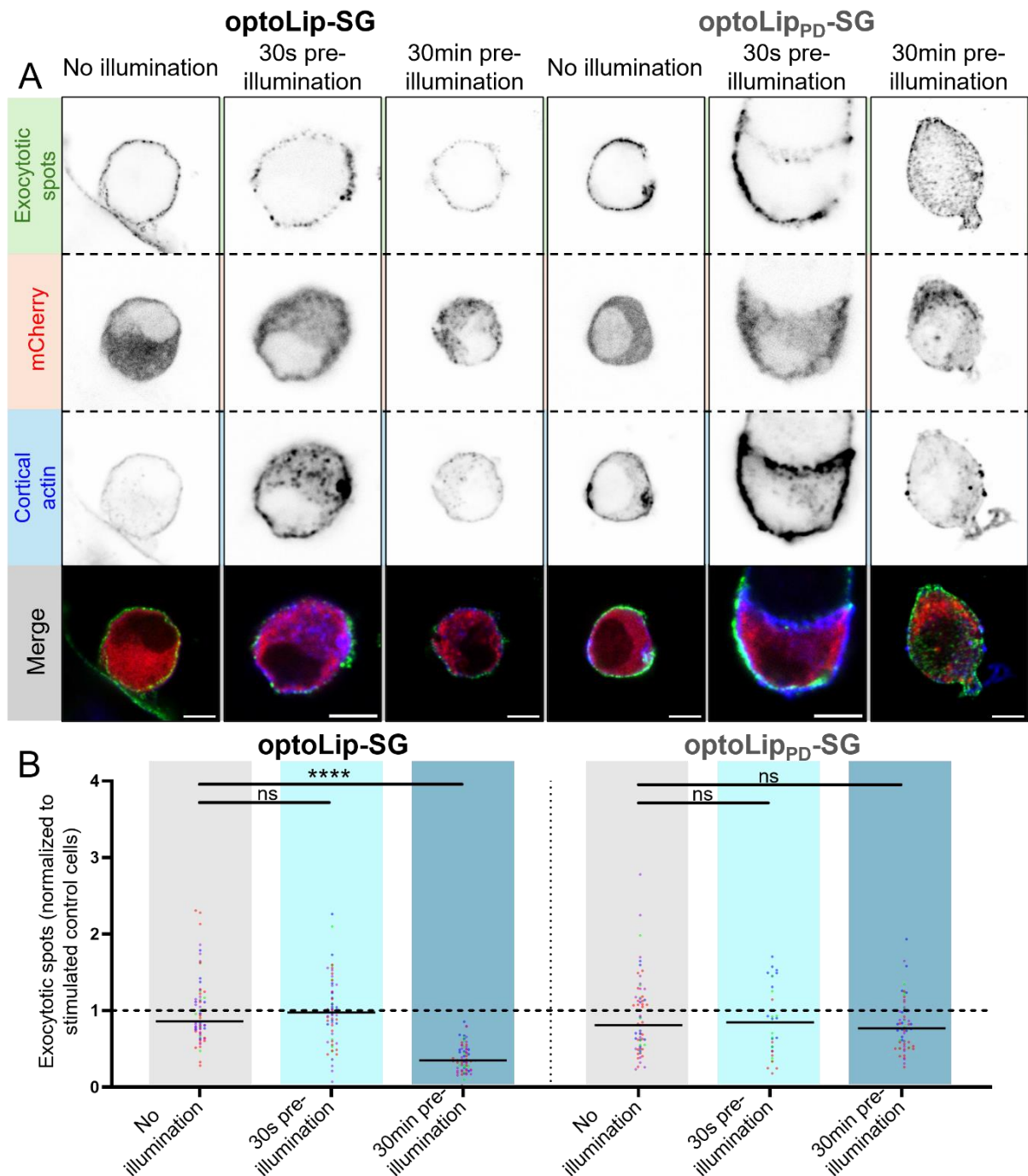
(A) Representative confocal images of exocytotic spot staining of chromaffin cells expressing optoLip or optoLip<sub>PD</sub> enabling a pre-recruitment to the plasma membrane upon 30 s or 30 min illumination. Cells were stimulated with a [59 mM] K<sup>+</sup> solution. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5  $\mu$ m. (B) Graphic of the quantification of exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue or green) according to experimental repeat. \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and three independent cultures for each condition.





**Figure 5:** OptoPLA1 recruitment to the plasma membrane decreased exocytosis.

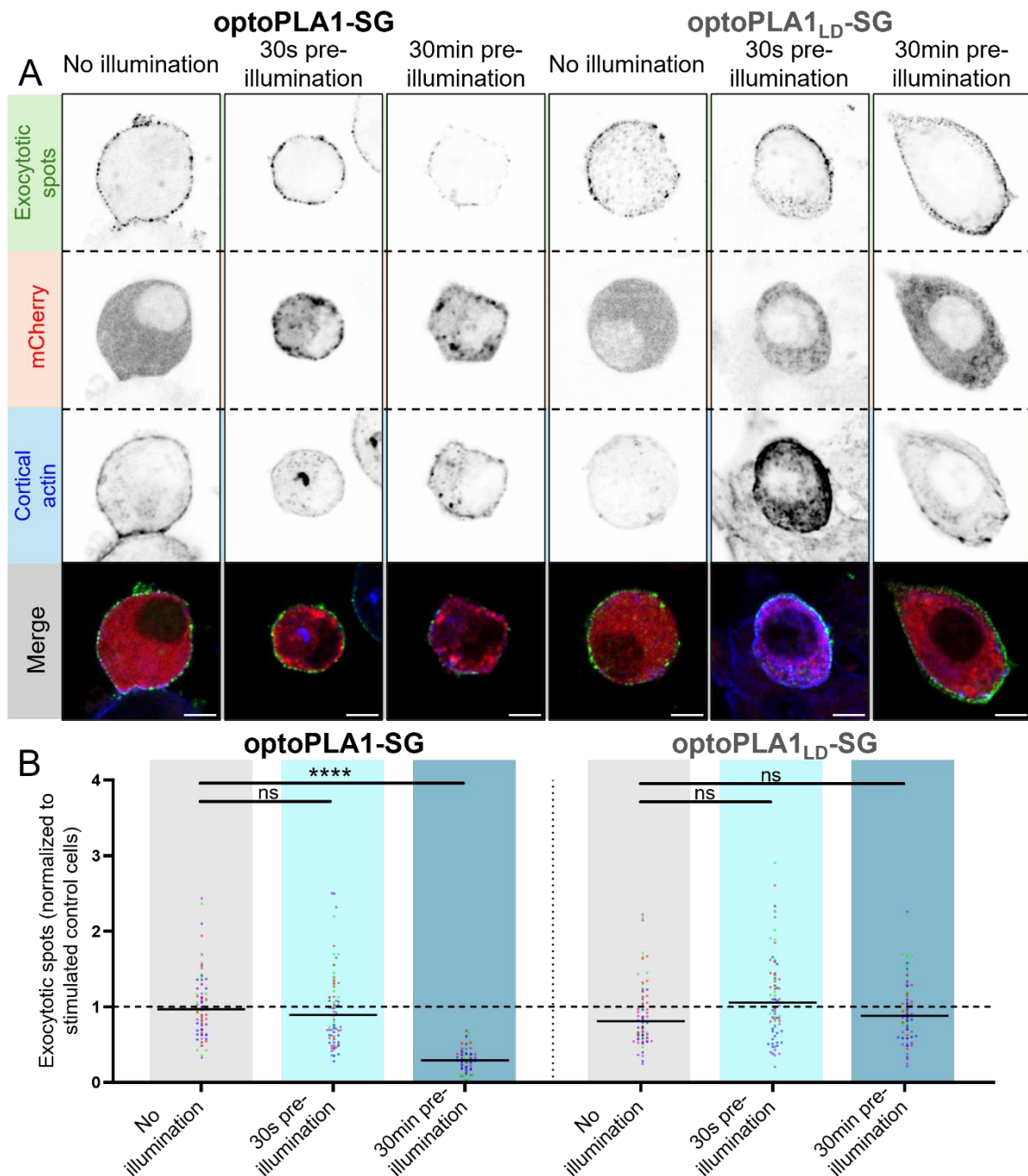
(A) Representative confocal images of exocytotic spot staining of chromaffin cells expressing optoPLA1 or optoPLA1<sub>LD</sub> enabling a pre-recruitment to the plasma membrane upon 30 s or 30 min illumination. Cells were stimulated with a [59 mM] K<sup>+</sup> solution. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5  $\mu$ m. (B) Graphic of the quantification of exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue, green or purple) according to experimental repeat. \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and at least three independent cultures for each condition.



**Figure 6:** A long-term recruitment of optoLip to secretory granule decreased exocytosis.

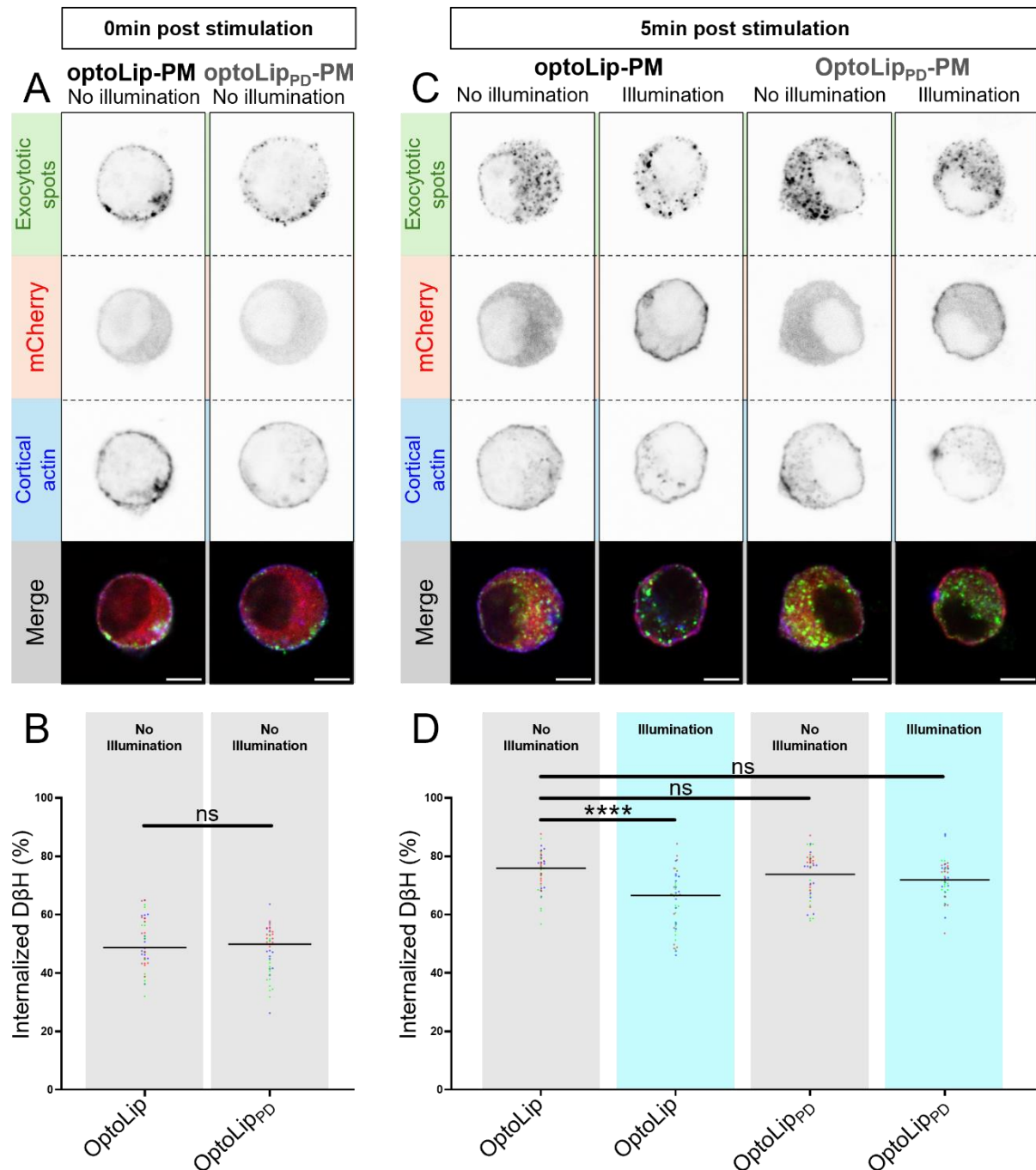
(A) Representative confocal images of exocytotic spot staining of chromaffin cells expressing optoLip or optoLip<sub>PD</sub> enabling a pre-recruitment to secretory granules upon 30 s or 30 min illumination. Cells were stimulated with a [59 mM] K<sup>+</sup> solution. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5  $\mu$ m. (B) Graphic of the quantification of exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue, green or magenta) according to experimental repeat. \*\*\*\*: p<0.0001 and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test. N>40 and at least three independent cultures for each condition.





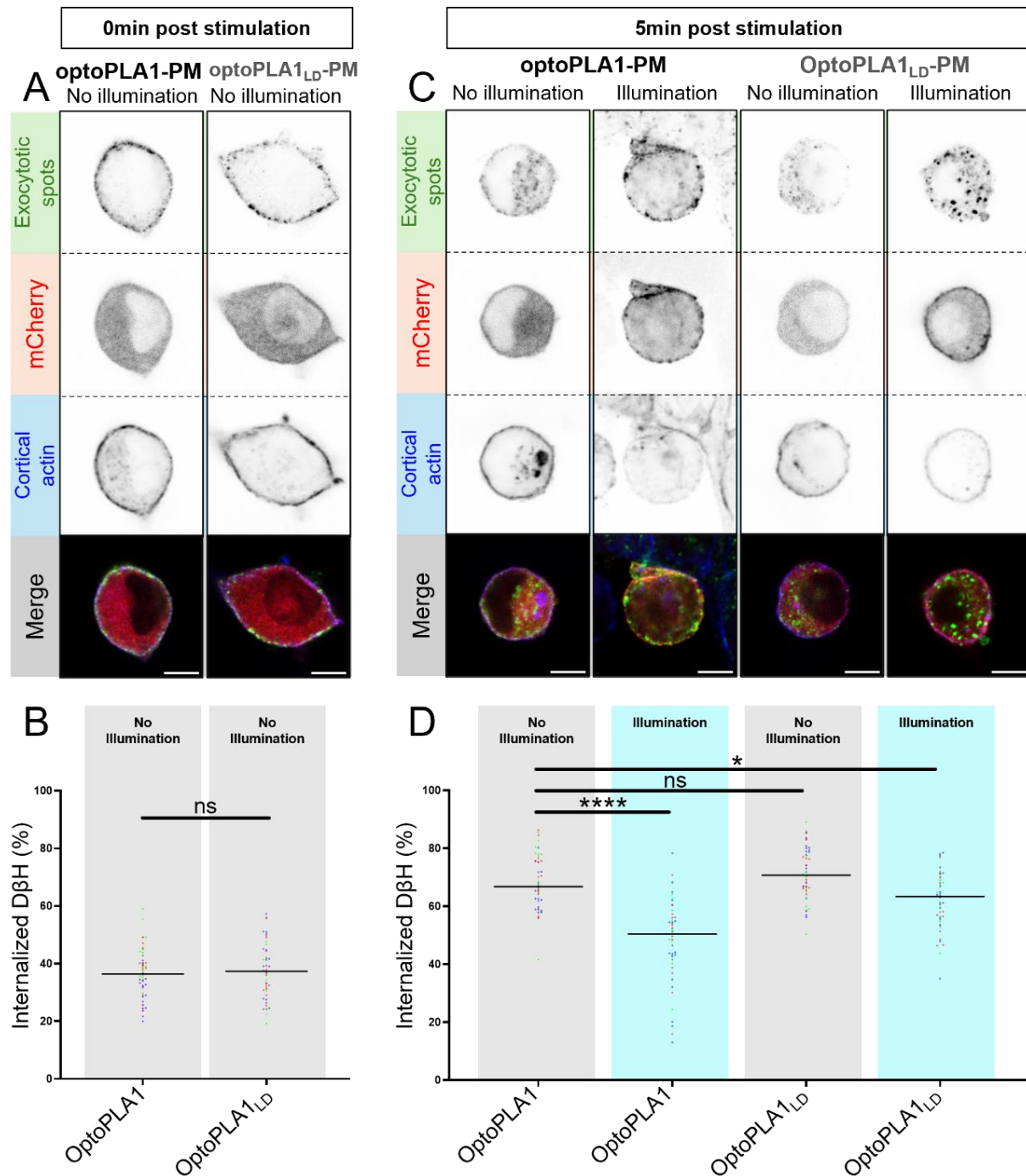
**Figure 7:** A long-term recruitment of optoPLA1 to secretory granule decreased exocytosis.

(A) Representative confocal images of exocytotic spot staining of chromaffin cells expressing optoPLA1 or optoPLA1<sub>LD</sub> enabling a pre-recruitment to secretory granules upon 30 s or 30 min illumination. Cells were stimulated with a [59 mM] K<sup>+</sup> solution. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5  $\mu$ m. (B) Graphic of the quantification of exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue, green or magenta) according to experimental repeat. \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 60$  and four independent cultures for each condition.



**Figure 8:** OptoLip recruitment to the plasma membrane reduced compensatory endocytosis.

(A) and (C) Representative confocal images of internalized exocytotic spot staining of chromaffin cells expressing optoLip or optoLip<sub>PD</sub> enabling a recruitment to the plasma membrane. Cells were stimulated with a [59 mM] K<sup>+</sup> solution and fixed immediately (A) or left for 5min in normal Locke solution (B) and exposed to blue light when indicated. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5 μm. (B) and (D) Graphic representing the amount of internalized exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue or green) according to experimental repeat. For graphic B: ns: non-significant, Mann-Whitney rank sum test. For graphic D: \*\*\*\*: p<0.0001 and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test. N>40 and three independent cultures for each condition.



**Figure 9:** OptoPLA1 recruitment to the plasma membrane reduced compensatory endocytosis.

(A) and (C) Representative confocal images of internalized exocytotic spot staining of chromaffin cells expressing optoPLA1 or optoPLA1<sub>LD</sub> enabling a recruitment to the plasma membrane. Cells were stimulated with a [59 mM] K<sup>+</sup> solution and fixed immediately (A) or left for 5 min in normal Locke solution (B) and exposed to blue light when indicated. Images are represented as inverted grayscale channels and merged (green: exocytotic spots, red: mCherry-enzyme and blue: phalloidin-Atto67N). Scale bar: 5 μm. (B) and (D) Graphic representing the amount of internalized exocytotic spots for each condition. Data are represented as median, and each point is an independent measure from an individual cell, points are also color coded (red, blue or green) according to experimental repeat. For graphic B: ns: non-significant, Mann-Whitney rank sum test. For graphic D: \*: p<0.05, \*\*\*\*: p<0.0001 and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test. N>40 and three independent cultures for each condition.

## Material and methods

### *OptoPLD<sub>10X</sub>-Rab3a, OptoLipin and OptoPLA1 constructs*

OptoPLD<sub>10X</sub> enabling a recruitment to the plasma membrane were gracefully provided by Dr. Jeremy Baskin. Cloning of the OptoLip and optoPLA1 was performed by replacing PLD by Lipin1B or PLA1 using the In-Fusion snap assembly kit (Takara®). Appropriate insertion of the sequences was verified by sequencing using the mCherry sequence (5'-TAAGCGGCCGCGACTC-3'). Rab3a was inserted into optoPLD<sub>10X</sub>, optoLip and optoPLA1 by replacing the CaaX sequence with the same strategy. Phosphatase dead optoLip and lipase dead PLA1 constructs were obtained by site-directed mutagenesis using the Quick-Change II site-directed mutagenesis kit from Agilent® as described previously (Vitale et al., 2000), using the following set of oligonucleotides (optoLip: 5'-CTTCTCCGCCTTGACGAGGAAGTGATTGAAAA-3' and 5'-TTTCAATCACTTCTCCGTGCAAGGCGGAGAAG-3' and optoPLA1 5'-CTCAATAGTATCACATGCCTTGGGATGTGTAA-3' and 5'-TTACACATCCCAAGGCATGTGATACTATTGAG-3') and verified by sequencing using the primer 5'-GGCTACCTGCACTGGGTC-3' and 5'-GTCCACTTTATAGAGATG-3', respectively.

### *Primary bovine chromaffin cell culture and electroporation*

Bovine adrenal glands were obtained at the Hagueneau municipal slaughterhouse and placed in a 4°C calcium-free Krebs solution (NaCl 154 mM, KCl 5.6 mM, NaHCO<sub>3</sub> 3.6 mM, 5.6 mM glucose and 5 mM HEPES, pH adjusted to 7.4) supplemented with the broad-spectrum antibiotic, primocine at 100 µg/mL. Glands were digested twice for 10min at 37°C with a Collagenase A solution (Collagenase A 3.5 mg/mL, BSA 5 mg/mL, resuspended in Krebs solution). The adrenal medulla was separated from the cortex and minced and digested for 5 min with remaining collagenase A solution. Bovine chromaffin cells were passed through a 217 µm sieve, centrifuged for 10 min at 130 g resuspended in Krebs solution and passed through a 70 µm sieve. BCCs were isolated with a Percoll density gradient generated with a 20 min centrifugation at 20,000 g. Cells were resuspended in antibiotic, antimitotic free culture medium (low glucose DMEM supplemented with 1 mM L-glutamine, 10% FBS) and centrifuged at 130 g for 10 min, resuspended and counted.

Bovin chromaffin cell transfection has been performed with the Amaxa basic nucleofector primary neuron kit. For each electroporation, 7,000,000 cells were immediately pelleted by centrifugation for 10 min at 130 g, resuspended in 100 µL of electroporation mix and homogenized

with 5  $\mu$ g of plasmids (OptoPLD<sub>10X</sub>, optoPLA1 and optoLip recruitable to the appropriate compartment). Each mix solution was placed in a cuvette and transfected using the X-001 program of the Amaxa electroporator. After electrical shock, cells were quickly resuspended in 37°C antibiotic/antimitotic free DMEM solution and dispatched into four wells of culture dishes containing bovine fibronectin coated coverslips in an antibiotic/antimitotic free DMEM solution and stored at 37°C, 5% CO<sub>2</sub>. Five hours later, primocin and antimitotics were added to the cells (10  $\mu$ M fluorodeoxyuridine, 10  $\mu$ M cytosine arabinoside and 100  $\mu$ g/mL primocin). The next day, each well was washed twice with culture medium (low glucose DMEM supplemented with 1 mM L-glutamine, 10% FBS, 10  $\mu$ M fluorodeoxyuridine, 10  $\mu$ M cytosine arabinoside and 100  $\mu$ g/mL primocin). At this stage, each culture dish was wrapped in an aluminum foil to prevent early photoactivation and was stored in an incubator at 37°C, 5% CO<sub>2</sub> for 24 h.

#### *Photoactivation and exocytic spots staining*

Well of electroporated cells wrapped in aluminum foil were transferred to a dark room heated at 37°C illuminated with red LED light (Pur Line®) to avoid early activation of the different optogenetic tools. Cells were washed twice with normal Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 11 mM glucose, 570  $\mu$ M ascorbic acid, 10  $\mu$ M EDTA and 15 mM HEPES, pH adjusted to 7.5). When indicated the LED light was switched to blue light and placed on culture dishes to perform illumination for the indicated time. Special care was taken that all solutions that were added to cells before fixation were heated to 37°C. When indicated cells were incubated 45 min in a solution containing 50 nM CAY93, or vehicle (DMSO) at 37°C and stimulated with a 59 mM K<sup>+</sup> solution (85.7 mM NaCl, 59 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 11 mM glucose, 0.56 mM ascorbic acid, 0.01 mM EDTA and 15 mM HEPES, pH adjusted to 7.2) containing either DMSO or CAY93 together with 0.5% anti-D $\beta$ H antibody for 3 min. For resting condition, the 3 min incubation was performed with normal Locke's solution containing either DMSO or CAY93 together with 0.5% of an anti-D $\beta$ H antibody. During this 3min timeframe, cells were illuminated or kept in the dark (No illumination) and fixed after one wash with normal Locke's solution. When indicated cells were illuminated for 30 s or 30 min prior to the stimulation.

For optoLip and optoPLA1 exocytotic spots internalization cells were stimulated during 3 min with a 59mM K<sup>+</sup> solution containing 0.5% anti-D $\beta$ H antibody in the dark and washed once with normal Locke's solution. For 0 min chase conditions, cells were fixed immediately. For 5 min chase condition cells were placed in normal Locke's solution, illuminated or kept in the dark and fixated.

Fixation was performed for 10 min with a cold 4% PBS-paraformaldehyde solution and washed five times with PBS. Special care was taken to not expose any samples to ambient light during fixation, but afterwards, all steps were performed in ambient light.

Samples subject to exocytotic spots internalization experiments were permeabilized for 15 min with a PBS solution containing 0.01% triton X-100 and washed five times with PBS and saturated for 60 min with a 3% PBS-BSA solution containing 10% goat serum at 37°C, washed five times with PBS. Afterwards, cells were incubated for one hour with a PBS-BSA 3% solution containing 0.1% secondary goat anti-mouse alexa555 antibodies at 37°C and washed five times with PBS. Nuclei and cortical actin were stained for 20 min at room temperature with Hoechst33342 and phalloidin-Atto647N, respectively followed by five PBS washes. Finally, coverslips were plunged 10 times in high purity water and mounted on a microscopic slide using Mowiol 4-88 mounting medium, to be left in the dark to polymerize at least 12 h before image acquisition.

#### *Image acquisition*

Images were obtained using a SP5II confocal laser scanning microscope (Leica®) with a 63× oil immersion objective. Acquisition was performed via the LAS AF software in a sequential scanning mode with a pinhole opening of 1 airy unit. The resolution was set at 512×512 pixels with a scanning speed of 400 Hz, a bit rate of 8, no smart offset, a zoom of 6 (pixel size: 80.25 nm) and a line average of 4. Alexa488 (DβH staining) was stimulated with a 490 nm emitting argon laser (10% power). mCherry (transfected optoenzymes) was stimulated with a 561 nm emitting argon laser (10% power). Atto647N (Phalloidin) was stimulated with a 633 nm emitting helium/neon laser (10% power). The gain of photomultiplier was kept constant for the DβH and phalloidin channels, but it was adjusted for each cell according to expression levels for the mCherry channel. All images were saved as LIF files.

#### *Image and statistical analysis*

After acquisition images were converted from LIF files into TIF files by using the standard Bioformat plugin on the FIJI software. Image analysis was performed on the ICY software by using a homemade macro based on the active contour plug-in. We performed segmentation of each analyzed cell by using the typical cortical actin network of chromaffin cells stained with phalloidin. This enabled measurement of the total area of a cell and a 10pixel wide (802.5 nm) peripheral area, together with mean pixel intensities for each channel in peripheral and total area.



Peripheral recruitment of optoPLD<sub>10X</sub>, optoLip and optoPLA1 was calculated using the following formula:

$$\text{Peripheral enzyme (\%)} = \frac{\text{Mean peripheral mCherry pixel intensity} \times \text{number of peripheral pixels}}{\text{Mean total mCherry pixel intensity} \times \text{number of total pixels}}$$

DβH internalization was calculated using the following formula:

$$\text{DβH internalization} = 100 - \frac{\text{Mean peripheral DβH pixel intensity} \times \text{number of peripheral pixels}}{\text{Mean total DβH pixel intensity} \times \text{number of total pixels}}$$

Total amount of DβH in cells was calculated using the following formula:

$$\text{Total amount of DβH} = \text{Mean total DβH pixel intensity} \times \text{number of total pixels}$$

Cellular area was obtained using the following formula:

$$\text{Cellular area (}\mu\text{m}^2\text{)} = \frac{\text{Number of total pixels} \times 80.25}{100.000}$$

Peripheral exocytotic spot intensity or total amount of DβH in cells was normalized with the mean value obtained from the corresponding non illuminated stimulated condition of each experimental repeat.

Statistical analysis was performed on the Graphpad® Prism 8 software by performing Mann-Whitney rank sum test or a Kruskal-Wallis test followed by Dunn's multiple comparison test as indicated in the figure captions. Graphs were produced on the same software.

Shown confocal images were cropped to a size of 250×250 pixels on the FIJI software, each channel, merged image and scale bar was automatically arranged without further modifications by a homemade macro.

## **Fundings**

This work was supported by a grant to N.V. from Agence Nationale pour la Recherche (ANR-19-CE44-0019). The Agence Nationale pour la Recherche (ANR-19-CE44-0019) and the Fondation pour la Recherche Médicale (FDT202304016618) have provided salary to AW, INSERM is providing the salary to NV, SG and SCG.

## **Acknowledgements**

We acknowledge the Plateforme Imagerie In Vitro of ITI Neurostra at CNRS UAR-3256. We wish to thank Dr Jeremy Baskin (Cornell University) for sharing the original opto-PLD constructs.

The authors declare that they have no conflicts of interest with the contents of this article.

Data availability can be requested to the corresponding authors.

## **Contributions**

A.W., M.V., S.Oz., E.T. performed experiments. A.W., E.T., M.V., N.V., S.C-G., S.G., S.Or., S.Oz. designed and analyzed the experiments. N.V. supervised the project. N.V. and A.W. wrote the manuscript. All authors revised the manuscript.

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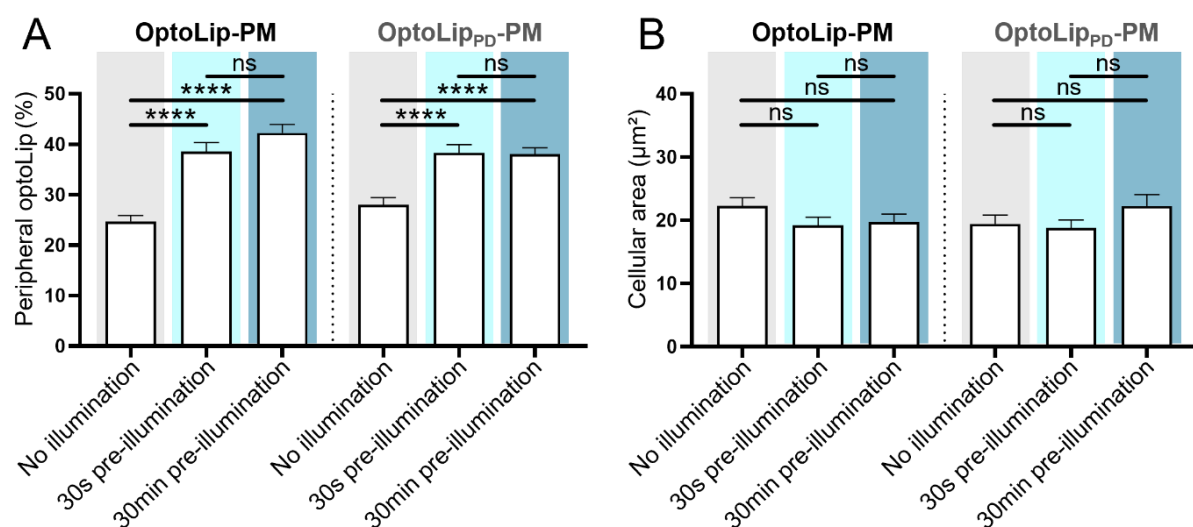
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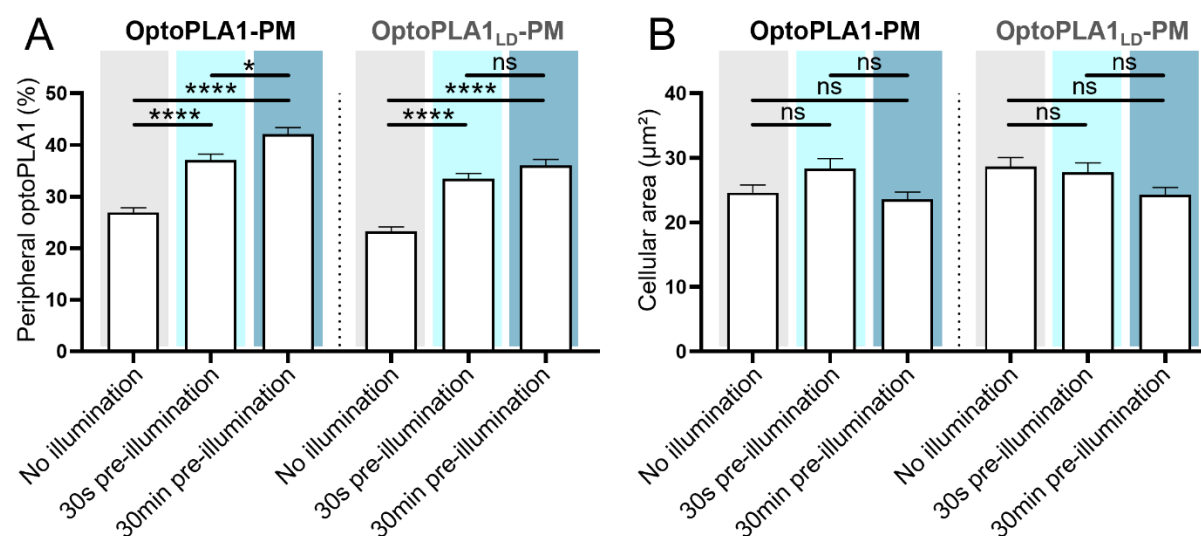


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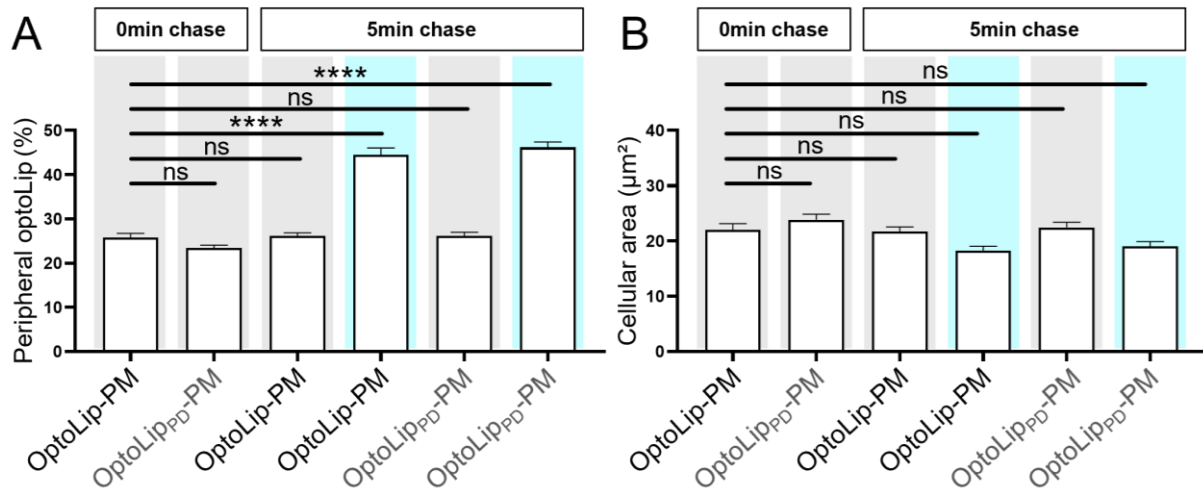
**Supplemental Figure 1:** Validation of optoLip and optoLip<sub>PD</sub> recruitment to the plasma membrane during exocytotic spot staining.

Graphs representing the proportion of peripheral optoLip or optoLip<sub>PD</sub> (A) and cellular areas (B) measured on cells analyzed in the experiments shown in figure 4. Data are represented as mean; error bars represent the standard error to the mean. \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and three independent cultures for each condition.



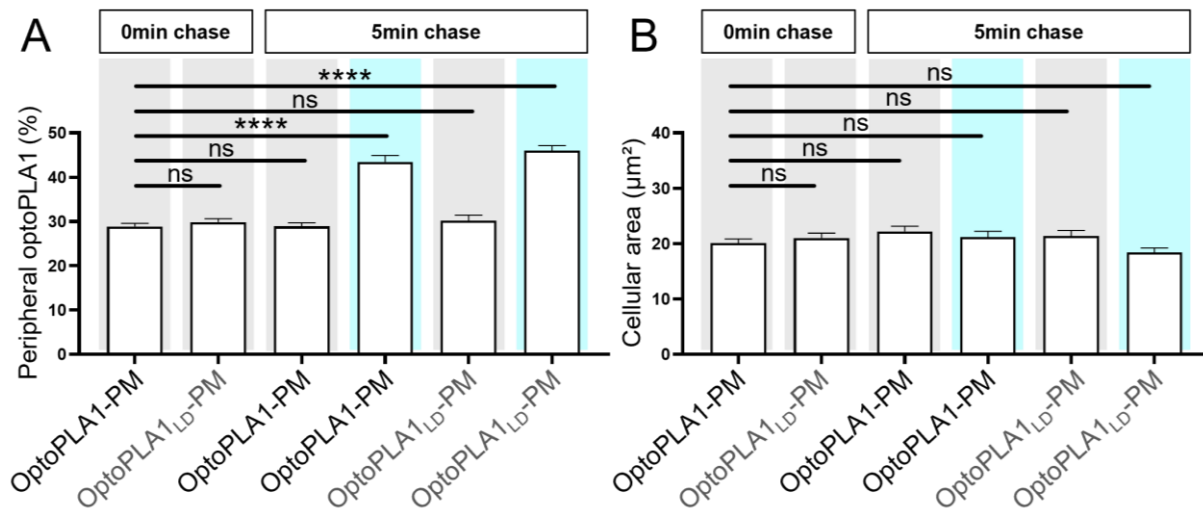
**Supplemental Figure 2:** Validation of optoPLA1 and optoPLA1<sub>LD</sub> recruitment to the plasma membrane during exocytotic spot staining

Graphs representing the proportion of peripheral optoPLA1 or optoPLA1<sub>LD</sub> (A) and cellular areas (B) measured on the same cells as the experiment shown in figure 5. Data are represented as mean; error bars represent the standard error to the mean. \*:  $p < 0.05$ , \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and at least three independent cultures for each condition.



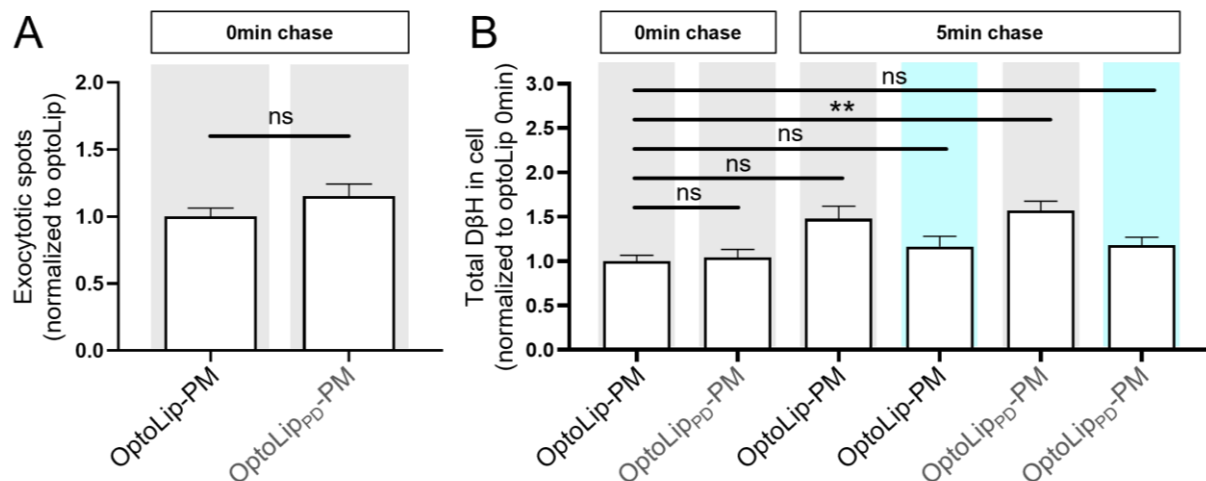
**Supplemental Figure 3:** Validation of optoLip and optoLip<sub>PD</sub> recruitment to the plasma membrane during exocytotic spot internalization.

Graphs representing the proportion of peripheral optoLip or optoLip<sub>PD</sub> (A) and cellular areas (B) measured from cells depicted in figure 8. Illuminated conditions during chase are shown with a blue background. Data are represented as mean; error bars represent the standard error to the mean. \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and three independent cultures for each condition.



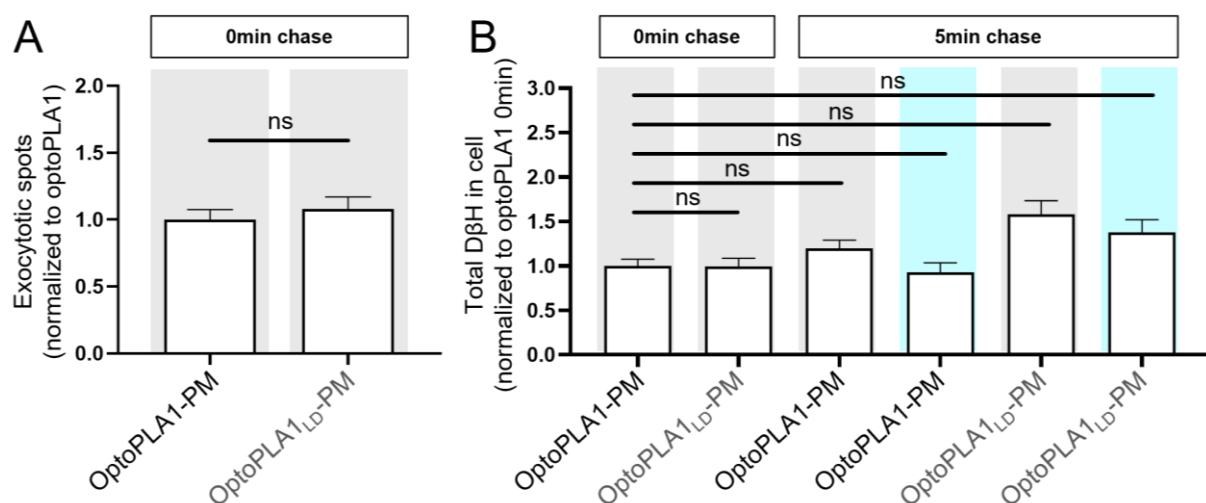
**Supplemental Figure 4:** Validation of optoPLA1 and optoPLA1<sub>LD</sub> recruitment to the plasma membrane during exocytotic spot internalization.

Graphs representing the proportion of peripheral optoPLA1 or optoPLA1<sub>LD</sub> (A) and cellular areas (B) measured from the cells depicted in figure 9. Illuminated conditions during chase are shown with a blue background. Data are represented as mean; error bars represent the standard error to the mean. \*\*\*\*:  $p < 0.0001$  and ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test.  $N > 40$  and three independent cultures for each condition.



**Supplemental Figure 5:** OptoLip and optoLip<sub>PD</sub> does not affect exocytosis without activation during endocytosis experiments

(A) Graphic representing the amount of peripheral exocytotic spots of the experiment presented in figure 8 A and B. ns: nonsignificant, Mann-Whitney rank sum test. (B) Graphic representing the total amount of DβH signal in cells from the experiment in figure 8. Illuminated conditions during chase are shown with a blue background. \*\*:  $p < 0.01$ , ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test. For both graphs, data are represented as mean; error bars represent the standard error to the mean.  $N > 40$  and three independent cultures for each condition.



**Supplemental Figure 6:** OptoPLA1 and optoPLA1<sub>LD</sub> do not affect exocytosis during the endocytosis assay.

(A) Graphic representing the amount of peripheral exocytotic spots of the experiment presented in figure 9 A and B. ns: nonsignificant, Mann-Whitney rank sum test. (B) Graphic representing the total amount of DβH signal in cells from the experiment in figure 9. Illuminated conditions during chase are shown with a blue background. ns: nonsignificant, Kruskal-Wallis test followed by Dunn's post hoc test. For both graphs, data are represented as mean; error bars represent the standard error to the mean.  $N > 40$  and three independent cultures for each condition.

## General discussion and perspectives

During my thesis work, I used two complementary approaches, one based on chemically modified PAs, the other on optogenetic control of PA metabolism. This allowed us to deepen our understanding of the pleiotropic functions of this peculiar signaling lipid along the regulated secretory pathway in primary bovine chromaffin cells. In this final discussion I will consider the data accumulated during the four years of my thesis and focus on three main points to clarify the role of PAs in a general model of neurosecretion:

1. Cooperative roles of mono- and polyunsaturated PAs in SG biogenesis, maturation and transport
2. Only polyunsaturated PA is implicated in late steps of neurosecretion
3. Non vesicular PA transport across the cell

Moreover, I will also present approaches that could be implemented to further explore those functions.

### **I. Cooperative roles of mono- and polyunsaturated PAs in SG biogenesis, maturation and transport**

#### **A. Mono- and polyunsaturated PAs participate in SG budding**

Genesis of SG starts at the TGN, where accumulation of various cargo molecules and membrane phase separation initiate the budding of immature SGs. Binding of CgA to PA has been demonstrated to be one of the main instigators of this process ([Carmon et al., 2020](#)). Here, with our photo crosslinking experiments, we identified CgA as a potent interactor of PAs in primary chromaffin cells. An interaction between PA and CgA was observed for both PA species at apparent similar levels (spectral counts of mass spectrometry analysis), in resting condition, meaning that the acyl chain of PAs does not matter in the initiation of SGs budding. However, upon stimulations, we found a decrease of 50% in the spectral counts for monounsaturated PAs and CgA, whereas this decrease was seemingly less pronounced for polyunsaturated PA, indicating a potential interaction of CgA and polyunsaturated PA in later steps of neurosecretion (Tab. 6). Additionally, we were able to identify two other granins: secretogranin (SCG) 2, also called chromogranin

C, and VGF, as potential binding partners of PAs with a similar pattern upon stimulations (Tab. 6). Both these neuropeptides have been reported to be implicated in cargo selection and budding initiation of SGs in chromaffin cells and other neuroendocrine models (Courel et al., 2010; Fargali et al., 2014). Thus, they might have redundant functions, with CgA to initiate budding of SGs (Fig. 29). Large dense core vesicle formation and secretion have also been observed in CgA depleted neurons (Dominguez et al., 2018). Therefore, such redundant functions could explain formation of SGs in other nervous cell types, in a PA dependent manner. Of note, other granins such as CgB, SCG3, SCG5 and proprotein convertase subtilisin/kexin type 1 inhibitor, have also been fished out, but have been discarded due to their presence in non-illuminated conditions. A more subtle analysis of the fishing results might be interesting to do by selecting candidates that are present at significantly higher levels in the illuminated versus the non-illuminated condition.

A complementary approach that I didn't have time to implement during my thesis would have been to perform multiple hours of optogenetic recruitment of PA degrading/producing enzymes to the TGN. I could then have counted the number of SGs and compared their morphology using electron microscopy. This sort of experiment would demonstrate further a potential implication of PAs present at the TGN in the budding of SGs, which has never been directly demonstrated.

## **B. Polyunsaturated PA selectively participates in exclusion SG maturation**

Optogenetic reduction of PA levels on SGs during 30 min impacted the amount of exocytotic events whereas a 30 s recruitment had no impact. Additionally, preliminary observations indicated that this long recruitment induced less SGs in the cell's periphery and potentially bigger granules, although this fact remains to be confirmed by using electron microscopy as size analysis of individual granules is challenging with classical confocal microscopy.

A potential explanation of this observation would be involvement of PAs on SG membranes in exclusion sorting of undesired molecules from immature SGs following homotopical fusion. This process is believed to be mediated by the recruitment of Arf1 to immature SGs, followed by AP-1, and clathrin coat formation (Austin et al., 2000). Interestingly, Arf1 has already been established as a known interactor of PA (Manifava et

al., 2001). In our fishing experiments, we also detected Arf1 as a polyunsaturated PA binding partner, in resting condition. Furthermore, even if it might not be a direct interaction, AP-1 was also fished for polyunsaturated PAs for the same conditions but not when monounsaturated PAs were used. Finally, spectral counts revealed a potential increase in clathrin signal following the same pattern, even though some signal remained in stimulated condition. Hence, polyunsaturated PA present on immature SGs might be responsible for the recruitment of Arf1, followed by AP-1 and subsequent clathrin recruitment (Fig. 29). Some of the polyunsaturated PAs would therefore be excluded from SGs, while monounsaturated might remain on the granule at this stage. This hypothesis is further supported by the detection of proteins that are known to be left out from SGs during exclusion, with polyunsaturated PAs exclusively. Such proteins include syntaxin-6 (Wendler et al., 2001) and VAMP-4 (Eaton et al., 2000) (Tab. 6). To confirm the hypothesis of a PA dependent Arf1 recruitment on SG, we could evaluate by immunofluorescence experiments the colocalization between Arf1 and SG markers (CgA, D $\beta$ H...) upon recruitment of PA degrading enzymes to SG.

### **C. Mono- and polyunsaturated PAs coordinate transport of SG at different maturation states**

Another explanation for the reduction of exocytotic spots upon 30 min of PA degradation on SG involves a potential binding of PA to molecular motors responsible for microtubule and actin dependent transport (Ñeco et al., 2003). The first phase of this transport is dependent on kinesins. Although, to my knowledge, no kinesin has been reported to perform this transport in chromaffin cells, in other types of secretory cells, such as pancreatic beta cells, kinesin-1 has been reported to modulate transport of SGs (Cui et al., 2010). Moreover, kinesin-1 isoforms have been recently reported to bind anionic lipids (Kumar et al., 2024; Manifava et al., 2001; Wang et al., 2017) and PLD inhibition slowed down specifically motion of SGs towards the periphery of PC12 cells (Tanguy et al., 2021). Interestingly, we have identified all three isoforms of kinesin1: Kif5a, b, and c as potential binding partners of PAs in our fishing experiments. Among those, Kif5a was the only one binding monounsaturated PA, and Kif5b and c interacted with both PA species, but were discarded in our stringent analysis, as they were also present in the non-illuminated controls. Interestingly, Kif5a was only fished in resting cells and modestly



in post stimulated cells, which is the conditions where SGs are expected to undergo the most active transport towards the cell periphery. Hence, monounsaturated PA present on SGs might be responsible for the initial transport of immature SG towards the cortical actin cytoskeleton (Fig. 29). To evaluate this possibility, we could perform live experiments of chromaffin cells with tight optogenetic control of SG PA levels. It would especially be interesting to perform these experiments on cells co-expressing the transmembrane domain of syntaxin6 and CgA that would allow to discriminate between immature and mature SG, respectively. Of note, special care should be taken in the fluorochrome choice as they should resist quenching in acidic medium, but also not interfere with the absorption spectrum of CRY2 to avoid early recruitment.

Polyunsaturated PA on SG seems involved in actin dependent transport. In our fishing experiments, among all myosins known for their role in vesicular transport, that were not present in non-illuminated condition, we have only identified myosin Va as a weak interactant of polyunsaturated PA in stimulated and post stimulation condition. The latter is known for its dual function in SG exclusion maturation and promoting docking of SG to the PM (Desnos et al., 2007; Kögel et al., 2010). On the one hand, this further supports an involvement of polyunsaturated PA in the maturation process, while on the other hand this could also suggest a role of this PA species in the final transport step of SG. Interestingly, myosin Va has an increased ATPase activity, and is thus more mobile, in the presence of high calcium concentrations (Li et al., 2006), potentially explaining why we identified this interactant only upon and after stimulation. However, unlike kinesin-1, this motor protein has not yet been reported to directly bind lipids within vesicles and has only been characterized *in vitro* transporting GFP (Wu et al., 2006). This could explain the low spectral count we obtained for this motor protein as it might not directly interact with PA. In melanocytes and PC12 cells, Rab27a has been reported to recruit myosin Va to SG (Desnos et al., 2003; Wu et al., 2002). However, we have only obtained a weak Rab27a signal for resting cells (Tab. 6). However, Rab3d which was also fished specifically for polyunsaturated PA (Tab. 6), has been proposed to recruit myosin Va to SG in PC12 cells (Kögel et al., 2013; Kögel and Gerdes, 2010). This GTPase might thus represent the missing link between polyunsaturated PA on SG and myosin Va (Fig. 29). As described for Arf1, this hypothesis could be confirmed by evaluating the location of Rab27a in chromaffin cells following PLA1 or lipin recruitment to SG.

The anchoring process of SG is believed to be mediated by the formation of actin bundles linking the PM to the SG. Such structures can be formed by actin fasciculation by annexinA2 in chromaffin cells (Gabel et al., 2015). This protein is recruited to the PM by specific lipid microdomains and has already been established as a potential PA binding partner (Gabel et al., 2019). Moreover, previous observations from the team revealed that monounsaturated PA specifically promoted the docking process of SG by regulating those actin bundles (Tanguy et al., 2021, 2020b). Therefore, an initial model could propose that annexinA2 interacts specifically with this PA species. However, while we successfully isolated this protein upon photo crosslinking, it interacted with both PA species (Tab. 6) (Fig. 29). A possible explanation of this finding could be the method of PA incubation with cells. Indeed, polyunsaturated PA might never be present in the microdomains recruiting annexinA2 in stimulated cells, where exclusively monounsaturated PA could be produced by PLD1 activation. Of note, this control of cortical actin during exocytosis is also dependent on many other proteins, such as scinderin (Zhang et al., 1995), myristoylated alanine rich protein kinase C substrate (MARCKS) (Cuchillo-Ibáñez et al., 2004), Cdc42, and Rac1 (Gasman et al., 1999) which were all identified in the different conditions, including without illumination, suggesting that they may be non-specific interactants. Hence, a different strategy might be useful to clarify the effects of monounsaturated PA on the actin cytoskeleton. For instance, the usage of a fluorescent actin reporter: lifeAct has been successfully used on living primary chromaffin cells (Villanueva et al., 2023). We could therefore use a similar approach while inhibiting PLD1 and probe the effects of mono- and polyunsaturated PA incubation on cortical actin dynamics.

| Function               | Interactant | Monounsaturated PA<br>(18:0-18:1, $\omega$ -9) |            |                  | Polyunsaturated PA<br>(18:0-22:6, $\omega$ -3) |            |                  |
|------------------------|-------------|------------------------------------------------|------------|------------------|------------------------------------------------|------------|------------------|
|                        |             | Resting                                        | Stimulated | Post-stimulation | Resting                                        | Stimulated | Post-stimulation |
| TGN budding            | CgA         | 966                                            | 443        | 934              | 980                                            | 812        | 956              |
|                        | SCG2        | 231                                            | 121        | 227              | 237                                            | 204        | 218              |
|                        | VGF         | 108                                            | 74         | 136              | 100                                            | 76         | 107              |
| Maturation (Exclusion) | Arf1        | ∅                                              | 3          | ∅                | 4                                              | ∅          | 3                |
|                        | AP-1        | ∅                                              | ∅          | ∅                | 6                                              | ∅          | 7                |
|                        | Clathrin    | ∅                                              | 3          | ∅                | 40                                             | 20         | 39               |
|                        | VAMP-4      | ∅                                              | 2          | ∅                | 1                                              | 1          | 2                |
|                        | Syntaxin-6  | ∅                                              | ∅          | ∅                | ∅                                              | ∅          | 1                |
| Transport              | Kif5a       | 31                                             | ∅          | 1                | ∅                                              | ∅          | ∅                |
|                        | Myosin Va   | ∅                                              | ∅          | ∅                | ∅                                              | 1          | 1                |
|                        | Rab27a      | ∅                                              | ∅          | ∅                | 1                                              | ∅          | ∅                |
|                        | Rab3d       | ∅                                              | ∅          | ∅                | 7                                              | 5          | 10               |
| Anchoring              | Annexin-A2  | 9                                              | 18         | 7                | 15                                             | 16         | 18               |

**Table 6:** Potential interactants of mono- and polyunsaturated PA implicated in granulogenesis

Table representing the potential PA partners in SG biogenesis, maturation and transport that we have identified using photo crosslinking. Signal count numbers for each interactant in each condition are indicated.

## **II. Only polyunsaturated PA is implicated in late steps of neurosecretion**

### **A. Polyunsaturated PAs in conjunction with the SNARE complex enables fusion pore formation**

The establishment of the fusion pore is a complex process relying on cooperative functions of the SNARE complex with membrane lipids to form hemifusion intermediates (fusion stalk, inverted micelle and diaphragm) and ultimately the fusion pore. Based on the crystal structure of closed SNARE pins (Stein et al., 2009) and molecular coarse grain simulations (Risselada et al., 2011), zippering of the SNARE complex propagates into the transmembrane domains of VAMP-2 and syntaxin1a. This displaces the membrane lipids in interaction with VAMP-2 and syntaxin1a from a horizontal orientation into a vertical one to form the hemifusion stalk. Interestingly, both VAMP-2 and syntaxin1a have been reported as PA interacting proteins (Lam et al., 2008; Vicogne et al., 2006). Additionally, overexpression of a mutated syntaxin1a, which is unable to bind PA, reduced the number and the kinetics of exocytotic events recorded by carbon fiber amperometry, very similarly to what is observed in PLD1 inhibited or knockout cells (Lam et al., 2008).

These results are in line with a reduction of exocytotic spots when degrading PA at the PM briefly before stimulation and a rescue of PLD1 inhibition induced by quick PM PA production (30 s in both cases). Moreover, we were also able to identify VAMP-2 and Syntaxin1a in our fishing experiments. Of outstanding interest is that both those proteins showed a clear preference for polyunsaturated PA (18:0-22:6,  $\omega$ -9). VAMP-2 interacted preferentially with PA in resting condition and after stimulation, whereas syntaxin-1a count number was massively increased upon stimulation (Tab. 7). In a model synthesizing these data, polyunsaturated PA could be present on granule membrane, interacting with VAMP-2 and be directed toward syntaxin1a upon zippering, explaining the total count number pattern we have obtained during photo crosslinking experiments. In other words, polyunsaturated PAs might be displaced by the transmembrane domain of those two SNARE proteins upon zippering, thus generating the fusion stalk (Fig. 29). Curiously, if this

hypothesis is true, it would mean that formation of other hemifusion intermediates would allow for polyunsaturated PA to also constitute the interior of the inverted micelle, hemifusion diaphragm and ultimately to the fusion pore. Hence, the latter would have both a cytosolic and extracellular leaflet formed by polyunsaturated PA. Therefore, this could represent an original mechanism for trans-bilayer movements of lipids specific to membrane fusion. Alternatively, the transmembrane domain of syntaxin1a and VAMP-2 polyunsaturated PA are interacting with have been reported *in vitro* to enable lipid flip-flop ([Langer and Langosch, 2011](#)). Moreover, the flip-flop rate could be further accentuated by the polyunsaturated acyl chain in *sn*-2 position which accelerates trans bilayer movements compared to monounsaturated PA ([Armstrong et al., 2003](#)). Hence, flip-flop could represent another way for polyunsaturated PA to constitute the interior of the fusion pore.

To further explore the role PA in establishing the fusion pore, we could perform fluorescence lifetime imaging (FLIM) - Förster resonance energy transfer (FRET) experiments to establish in live cells whether different fluorescent PA species interact with ectopically expressed fluorescent partners and establish the kinetics of these interaction relative to the different steps along the secretory pathway. If the fishing data correspond to the actual cycling of interaction of polyunsaturated PA with VAMP-2 and syntaxin1a; depending on SNARE proteins, we expect to obtain results that are specific to fluorescent polyunsaturated PA:

- The fluorescence lifetime of the donor: VAMP-2 on SG should decrease upon fluorescent PA (18:0-22:6,  $\omega$ -3) addition but only in resting condition and upon stimulation it should become like that found in cells without synthetic PAs.
- On the contrary, overexpression of fluorescent syntaxin-1a would not have any impactful change of its lifetime upon fluorescent PA addition. However, it would decrease in the cell's periphery only upon stimulation.

Another interesting consideration is the potential implication of polyunsaturated PA in triggering primed SNARE complexes. Classically, fusion is initiated by calcium binding of synaptotagmin1 to anionic lipids of the PM such as PI(4,5)P2 or PS, displacing complexin and triggering SNARE zippering leading to fusion ([Giraudo et al., 2006](#)). Accordingly, we have identified synaptotagmin-1 in our fishing experiment with polyunsaturated PA in all conditions and with monounsaturated PA only after stimulation

(Tab. 7). Furthermore, polyunsaturated PA addition on resting cells increased the amount of exocytotic sites ([Tanguy et al., 2021](#)). We obtained similar results when characterizing our novel synthetic polyunsaturated PA species together with an increase in secretion when recruiting optoPLD to the PM in resting condition. Hence, addition of PA, allegedly only the polyunsaturated form, to resting chromaffin cells could trigger some SNAREpins by binding synaptotagmin1 explaining the slight increase of exocytotic sites. An approach that could complement these data would be to perform membrane capacitance recordings on resting chromaffin cells and probe whether incubation with polyunsaturated PA favors occurrence of fusion events. This approach would also enable us to distinguish between full-fusion and kiss-and-run types of events in resting condition but also to explore a potential role for polyunsaturated PA in pore expansion upon stimulation (described in the next paragraph).

## **B. Polyunsaturated PAs favor fusion pore expansion**

PLD1 inhibition, knockdown, and knockout reduced the number of fusion events, but also the kinetics of the fusion pore expansion in chromaffin cells upon amperometric recording. This effect is specifically rescued by provision of polyunsaturated PA ([Tanguy et al., 2020b](#)). Additionally, inclusion of lysophosphatidylcholine on the extracellular leaflet of PLD1 knockdown PC12 cells also partially rescued exocytosis inhibition ([Zenou-Meyer et al., 2007](#)). Both these effects have been explained by the induction of negative membrane curvature on the cytosolic side of the fusion pore. This curvature would be generated by PLD1-dependent PA production on the cytosolic leaflet of the PM, which is a conical shaped lipid ([Kooijman et al., 2005b](#)), or by the induction of a positive membrane curvature on the extracellular leaflet by artificially supplementing cells with inverted conical lipid such as lysophosphatidylcholine, that do not flip-flop between leaflet efficiently. Therefore, only polyunsaturated PA, but not monounsaturated PA can promote an efficient expansion of the initial fusion pore. Moreover, in line with this conclusion, molecular simulations demonstrated that polyunsaturated lipids are more prone to adapt to negative curvature ([Pinot et al., 2014](#)). Interestingly, as reported in the previous paragraph, polyunsaturated PA strongly interacted with syntaxin-1a upon stimulation of chromaffin cells (Tab. 7). Moreover, in addition to diminishing the number of exocytotic events, hindering PA binding of this SNARE protein also increases the duration of the

fusion pore in chromaffin cells as seen in PLD1 inhibited or knockout cells ([Lam et al., 2008](#)). Hence, syntaxin-1a binding to polyunsaturated PA might be necessary to confine this lipid at the neck of fusing vesicles enabling expansion. This would also be in line with the increase of spike charge we have observed in cells incubated with our novel synthetic polyunsaturated PA. Indeed, if polyunsaturated PA is implicated in pore expansion, providing it to cells might decrease the low number of kiss-and-run events that could occur upon stimulation in favor of cavicapture or full collapse events, thereby increasing the mean measured amount of released catecholamines per fusing vesicle. Therefore, an original experimental opportunity here would be to confirm this by recording cells using carbon fiber amperometry while performing an optogenetic recruitment of PLA1 or lipin to the PM. This would certainly induce a decrease of the number of fusion events as we have already observed by staining exocytotic spots, but I would also expect to detect an increase in the duration of the fusion pore, while decreasing overall spike charges. We therefore could try to rescue this effect by providing plasmalogen or natural polyunsaturated PA to cells. Indeed, ether lipids are insensitive to PLA1 because this enzyme cannot cleave the vinyl-ether bond linking the acyl chain in *sn*-1 position ([Mawatari et al., 2018](#)). Hence, natural polyunsaturated PA are not expected to rescue the kinetics of fusion pore expansion when combined with a recruitment of PLA1 or lipin, whereas plasmalogen polyunsaturated PA would be able to rescue only the effects of PLA1 recruitment.

Additionally, the role of polyunsaturated PA in the expansion of the fusion pore could be mediated from the internal (extracellular) leaflet of the fusion pore. Indeed, as discussed previously, the specific way the pore is formed might imply a redistribution of polyunsaturated PA on the inside of the channel. From there it could interact with PA binding proteins to regulate fusion pore expansion and avoid closure, which would lead to kiss-and-run fusion. Interestingly, CgA overexpression in chromaffin cells induced an increase in fusion pore duration, by maintaining the  $\Omega$ -shape of the granule ([Abbineni et al., 2019](#)). Moreover, as discussed in the previous chapter, CgA and SCGII bind strongly PA and do not show any preference for these two PA species in resting condition. However, upon stimulation we detected a 50% reduction in the total count number for both CgA and SCGII in the monounsaturated PA condition. This reduction was not seen for polyunsaturated PA, indicating a specific retention of both CgA and SCGII with this PA species during stimulation (Tab. 7). Hence, binding of CgA or SCGII to polyunsaturated PA

could stabilize the fusion pore leading ultimately to its expansion (Fig. 29). This is coherent with our proposed model as only polyunsaturated PA would be present on the extracellular leaflet of the fusion pore. The remaining half of PA interacting neuropeptides that were fished with monounsaturated PA would therefore be in SG that did not fuse during stimulation, immature SG or the budding areas at the TGN. Confirming this hypothesis might however be complicated as knock down of CgA would lead to SG biogenesis defect, therefore mechanically affecting the fusion rate. A potential, but hard to implement approach would be engineering a light sensible CgA, which would be unable to bind PA upon illumination. Using light sensible proteins has been performed with other lipid binding domains ([Glantz et al., 2018](#)). Overexpression of such a photo-CgA should enable to specifically explore the role of CgA/PA binding in pore expansion using carbon fiber amperometry depending on illumination during the experiment.

### **C. Polyunsaturated PAs couple exocytosis to compensatory endocytosis**

In chromaffin cells exocytosis is tightly coupled to compensatory endocytosis in time and space corresponding to the four types of vesicle internalization that follow fusion: ultrafast endocytosis (kiss-and-run), fast endocytosis (cavicapture), slow endocytosis (full collapse fusion) and bulk endocytosis ([Shin et al., 2021](#)). Bulk endocytosis will not be discussed here as it occurs during extreme stimulations of chromaffin cells ([Perez Bay et al., 2011](#)) which has not been performed for the experiments presented in this manuscript.

Previously our team, has explored the role of PA in these endocytic processes after stimulation, by a 30 min incubation with PLD1 inhibitors at 4°C to evaluate internalization of DβH exocytotic spots following re-incubation at 37°C for a given time. We found that PLD1 inhibition moderately reduced the endocytosis levels of DβH at early, but not at later time points. This enabled us to establish that PA might be implicated in the early stages of compensatory endocytosis ([Tanguy et al., 2021](#)). However, this approach posed a few problems. Firstly, PA produced by PLD1 during stimulation may remain present at the PM. Secondly, the 4°C incubation is not an optimal treatment for cells. Thirdly, there was no way to determine what intracellular pool of PA was involved in endocytosis. Therefore, optogenetic recruitment of PLA1 or lipin to the PM has been particularly useful for



studying this underexplored role of PA. Thanks to the recruitment speed offered by a light sensible approach, enzymes were mobilized quickly following stimulation, bypassing the need of a 4°C incubation. Furthermore, we were also able to target PA that has been produced during stimulation.

Given the speed of ultrafast endocytosis and the incomplete opening of the pore it is highly improbable that our exocytotic spots staining protocol allowed to study this process. However, as described in the previous chapter, polyunsaturated PA promotes expansion of the fusion pore, thereby “inhibiting” ultrafast endocytosis in favor of other fusion modes. However, our protocol is probably able to stain exocytotic spots following cavicapture and full collapse fusion events. Given that PM PA degradation after stimulation of cells slowed down entry of exocytotic spots, it is likely that the PM PA pool might have a role to play in coupling exocytosis to endocytosis.

The first protein that comes in mind when talking about compensatory endocytosis is dynamin, the main instigator of membrane scission following cavicapture and full collapse in chromaffin cells ([Fulop et al., 2008](#)), with dynamin-1 specialized in cavicapture fission and dynamin-2 in clathrin mediated endocytosis ([Artalejo et al., 2002](#)). Interestingly, PA has been reported to favor the membrane scission effect of these GTPases through electrostatic interactions and favoring membrane insertion of the dynamin ring ([Burger et al., 2000](#)). Moreover, polyunsaturated lipids also promote membrane curvature and recruitment of dynamin for subsequent scission ([Pinot et al., 2014](#)). Finally, a link between dynamin and PLD1 has been reported ([Lee et al., 2006](#)). Accordingly, while we did not identify any dynamin isoform directly in our fishing experiment, we found a major dynamin regulator: synaptophysin interacting with polyunsaturated PA implying that this specific isoform might be contributing to compensatory endocytosis.

Synaptophysin accompanies membrane fission during cavicapture in chromaffin cells ([Chang et al., 2021](#)) and our fishing experiments indicated that it interacted with polyunsaturated PA during resting and after stimulation. Additionally, we also identified the secretory carrier membrane protein 1 (SCAMP1) as a novel PA interactant mostly specific to polyunsaturated PA. SCAMP1 has been reported to promote cavicapture in PC12 cells thanks to its dual role. Firstly, SCAMP1 promotes dilatation of newly opened fusion pores, which is also in line with a role for polyunsaturated PA in pore expansion described in the previous chapter, and secondly, it regulates fusion pore closure ([Zhang](#)

and Castle, 2011). Hence, CgA and SCAMP1 might cooperate on each side of the fusion pore to maintain polyunsaturated PA and preserve the  $\Omega$  shape of the vesicle, by creating a double decker fusion sandwich (SCAMP1-PA-PA-CgA-pore-CgA-PA-PA-SCAMP1) (Fig. 29), enabling in a second time fission through a recruitment of synaptophysin/dynamin-1. Of interest, another member of the SCAMP family, SCAMP2 was shown to interact with the GTPase Arf6 and PLD1 on SG membrane of PC12 cells (Liu et al., 2005). The latter is also a major regulator of PLD1 activity during exocytosis (Vitale et al., 2002). Additionally, synpatotagmin-1, an already discussed interactant of polyunsaturated PA (Tab. 7), has also been proposed to play a role in compensatory endocytosis in chromaffin cells by recruiting dynamin-1 (McAdam et al., 2015), underlying even more the potential contribution of this specific PA isoform in allowing cavicapture and coupling exocytosis to fast endocytosis.

Involvement of PA in slow clathrin-mediated endocytosis is less clear. The main adaptor implicated in clathrin coat formation for slow endocytosis is AP-2 and its recruitment is classically mediated by PI(4,5)P2 (Gaidarov and Keen, 1999). Moreover, while polyunsaturated PA has been reported to specifically bind AP-180, another adaptor implicated in neuronal compensatory endocytosis, this binding was shown to inhibit subsequent clathrin coat assembly (Hoshino and Sakane, 2022). However, PA production has been reported to accelerate endothelial growth factor receptor endocytosis in different cell lines (Antonescu et al., 2010). Another study reported a direct interaction between AP-2 and PLD1 together with PA to mediate clathrin assembly (Lee et al., 2009). Accordingly, we identified AP-2 as a potential specific polyunsaturated PA interactant together with clathrin for all conditions, with an apparent decrease during stimulation (Tab. 7), which corresponds to the expected pattern for slow endocytosis. But this interaction might not be direct, and as discussed previously, this clathrin signal could also correspond to a role for PA in SG maturation. Furthermore, amphiphysin, a protein recruiting dynamin-2 for vesicle scission following SG full collapse in chromaffin cells (Elhamdani et al., 2006b) also interacted slightly with polyunsaturated PA in all conditions (Tab. 7). Hence, while the involvement of PA in slow compensatory endocytosis is less clear than in its fast counterpart, polyunsaturated PA might also contribute to this phenomenon either by acting on clathrin coat assembly or by favoring amphiphysin/dynamin-2 membrane fission (Fig. 29).

A complementary approach that could be used to clarify the involvement of PA in coupling exocytosis to endocytosis is to perform live STED microscopy experiments as described recently by Shin et al., 2021 (Guo et al., 2022). Incubating chromaffin cells with different fluorescent PA species would enable to establish the dynamic location of this phospholipid in this coupling with a spatial resolution adequate to distinguish individual events and temporal resolution to discriminate between different endocytosis modes. However, such an approach would also require using PAs coupled to Stokes shifted fluorochromes, like Atto490LS, to image an additional STED channel without interference together with the membrane and neurotransmitter staining. Hopefully such novel fluorescent PAs should be easy to produce using SPAAC on our PA-N<sub>3</sub> species.

| Function                      | Interactant     | Monounsaturated PA<br>(18:0-18:1, $\omega$ -9) |            |                  | Polyunsaturated PA<br>(18:0-22:6, $\omega$ -3) |            |                  |
|-------------------------------|-----------------|------------------------------------------------|------------|------------------|------------------------------------------------|------------|------------------|
|                               |                 | Resting                                        | Stimulated | Post-stimulation | Resting                                        | Stimulated | Post-stimulation |
| SNARE proteins                | Syntaxin-1A     | ∅                                              | ∅          | 1                | ∅                                              | 343        | 2                |
|                               | VAMP-2          | 4                                              | 2          | 1                | 18                                             | 7          | 21               |
| Calcium sensor                | Synaptotagmin 1 | ∅                                              | ∅          | 9                | 28                                             | 28         | 17               |
| Pore expansion                | CgA             | 966                                            | 443        | 934              | 980                                            | 812        | 956              |
|                               | SCG2            | 231                                            | 121        | 227              | 237                                            | 204        | 218              |
| Cavicapture                   | Synpatophysin   | ∅                                              | ∅          | ∅                | 6                                              | ∅          | 5                |
|                               | SCAMP1          | ∅                                              | 1          | ∅                | 9                                              | 9          | 14               |
| Clathrin mediated endocytosis | Amphiphysin     | ∅                                              | ∅          | ∅                | 2                                              | 2          | 2                |
|                               | AP-2            | ∅                                              | ∅          | ∅                | 7                                              | 4          | 9                |
|                               | Clathrin        | ∅                                              | ∅          | 9                | 40                                             | 20         | 39               |

**Table 7:** Potential interactants of mono- and polyunsaturated PA implicated in membrane fusion and exocytosis-endocytosis coupling

Table of the potential PA partners in SG fusion with the PM and subsequent endocytosis that we have identified using photo crosslinking. Signal count numbers for each interactant in each condition are indicated.

### III. Non vesicular PA transport

#### A. PA exchange at membrane contact sites

An additional point of interest that will be discussed is the clearance of PA from the microdomains dedicated to exocytosis. Indeed, neurosecretion relies deeply on the control of vesicular trafficking especially in its final step: regulated exocytosis. However, as described multiple times in this manuscript, PA is a potent activator of this process. Hence, chromaffin cells must certainly be equipped with fast and efficient ways to halt these signaling functions. Our team had originally thought that this might be performed by PA catabolism, as overexpression of the PA phosphohydrolase Lipin1 inhibited exocytosis in chromaffin cells. However, Lipin1 knockdown did not increase secretion of

chromaffin cells ([Baneux et al., 2020](#)), implying that PA is required for efficient exocytosis, but that endogenous Lipin1 is likely not stopping his signaling. Hence, an interacting consideration is that lipid metabolism might not be the main mechanism for PA clearance, but that lipid exchange at MCS could evacuate PA from exocytotic sites.

As described previously, fluorescent PA can enter very quickly chromaffin cells to enter intracellular compartments within seconds after integration to the PM in a vesicular independent manner. This fast entry has been reported by other studies ([Kim et al., 2015](#); [Tei and Baskin, 2020](#)). Interestingly, polyunsaturated PA entered cells faster than monounsaturated PA, indicating that the transfer of PA between organelles might use a process that is sensitive to the fatty acyl chain composition of PA providing some kinetics differences. Therefore, we looked at potential LTP candidates in our fishing experiments. Although it is likely that we did not capture all PA LTPs in our experiments, since the signal counts for these proteins are quite low, in agreement with a low affinity interaction of lipids and their LTPs. In resting conditions, the main culprits we have found are VAPs, with VAPb interacting with polyunsaturated PA specifically (Fig. 29). The latter might thus mediate ER-PM polyunsaturated PA clearance from exocytotic sites, in line with the faster cellular upload of fluorescent polyunsaturated over monounsaturated PA. Moreover, the interaction of polyunsaturated PA with VAPb diminished upon stimulation, implying a potential negative regulation of this transport, that could be calcium dependent. This would be coherent with the fact that polyunsaturated PA is required at the PM upon stimulation and should not be cleared immediately. Additionally, VAPb dependent PA transport seems to increase following stimulation, and noticeable at this stage it interacted with both PA species (Fig. 29). Upon reaching the ER, PA would therefore eventually be converted into other structural phospholipids, by entering classical lipid biosynthesis pathways. On another hand, VAPa, was only found in our hits after stimulation (Tab. 8), opening the possibility for potential additional PA translocation following stimulation. Interestingly, this PA clearance could also induce transport of PA towards the TGN, where it could participate in SG biogenesis, replacing some of the vesicles that have fused (Fig. 29).

VAPs are known to be expressed in the ER and need to dimerize with a partner from another membrane compartment harboring a FFAT motif ([Cabukusta et al., 2020](#)). Unfortunately, in our fishing experiment, we did not identify any LTP harboring such a

motif, so we are still missing at least half of the culprits. An efficient strategy which has been used to identify VAP partners, is the fishing of potential interactants using Bio-ID (Cabukusta et al., 2020). We could therefore use the same approach on primary chromaffin cells to gain insight into potential partners of VAPs. This would also help us to refine the specific non vesicular pathways, and traversed membrane compartments PAs are using to enter cells. Moreover, recruitment of PA catabolism enzymes to such organelles (mitochondria, ER...) could also provide information on potential contribution of PM produced PA in SG biogenesis.

Furthermore, VAPs are not the only LTPs were identified in our screen as upon stimulation, we also identified several other proteins which were already known for their ability to transport PA such as PITP $\alpha$  and  $\beta$  (Kim et al., 2024) and other, like E-Syts (Tab. 8) (Fig. 29). Therefore, an interesting experiment to perform would be to follow the dynamics of our synthetic PAs in chromaffin cells upon stimulation. We could also selectively knockdown each of those LTP using siRNAs to see if PA cellular entry is impacted and eventually identify which membrane compartment they are bridging, and which PA species they are transporting (fluorescent PAs would accumulate in specific organelles).

But why do those LTPs preferentially transport specific PA subtypes? The internal cholesterol gradient within cells might be an interesting parameter to consider for answering this question. Indeed, cholesterol can be bound to various lipids, preferentially harboring saturated or monounsaturated acyl chains (Brzustowicz et al., 2002). Hence, depending on the membrane compartment, it might be bound to the membrane, which hinders its ability to be transported at MCS. Lateral segregation mechanisms have therefore been proposed to establish a free cholesterol gradient. The latter favors its non-vesicular transport from its production site in the ER, where lipid unsaturation level are high, toward other compartments where they are low (PM), thus lowering energetic requirements of the counter-gradient transport (Litvinov et al., 2018). In our case, this idea can also be taken the other way around: upon addition to resting chromaffin cells, monounsaturated PA might be less likely to be taken in charge by LTPs at the PM than polyunsaturated PA because of its preferential association with cholesterol. An interesting experiment that could be performed to verify this point is to perform PA entry assays on cholesterol depleted cells, using for example, methyl- $\beta$ -cyclodextrin (M $\beta$ CD).

| LTP           | Monounsaturated PA<br>(18:0-18:1, $\omega$ -9) |            |                  | Polyunsaturated PA<br>(18:0-22:6, $\omega$ -3) |            |                  |
|---------------|------------------------------------------------|------------|------------------|------------------------------------------------|------------|------------------|
|               | Resting                                        | Stimulated | Post-stimulation | Resting                                        | Stimulated | Post-stimulation |
| VAPa          | Ø                                              | Ø          | Ø                | Ø                                              | Ø          | 3                |
| VAPb          | Ø                                              | Ø          | 4                | 6                                              | 2          | 8                |
| PITP $\alpha$ | Ø                                              | 2          | 4                | Ø                                              | Ø          | 4                |
| PITP $\beta$  | Ø                                              | 7          | 6                | Ø                                              | 2          | 6                |
| E-Syt2        | Ø                                              | Ø          | Ø                | 1                                              | 3          | 3                |

**Table 8:** Potential mono- and polyunsaturated PA LTPs in chromaffin cells

Table of the potential PA LTPs we have identified using photo crosslinking. Signal count numbers for each interactant in each condition are indicated.

## B. PA flop on secretory granules

One last problem that arises from the model I propose is a problem of topology. Indeed, if both PA species are present on the luminal leaflet of the TGN and bind aggregating CgA and other neuropeptides, how can they associate with kinesins, APs, VAMPs... to promote transport, maturation or fusion on the cytosolic leaflet of the granule membrane? I would like to propose 3 potential explanations. 1) PA on the luminal membrane leaflet remains associated with proteins such as CgA, while de novo PA synthesis occurs on the outer leaflet of the SG. Indeed, PLD1 has recently been reported to be associated with peripheral SG in PC12 cells, where it produces PA upon calcium dependent activation (Bills et al., 2024). 2) PA could be transferred between secretory granule membrane leaflets by protein dependent lipid scrambling, but to my knowledge, no such proteins are known to be endogenously expressed on SG. 3) PA could flip-flop thanks to its peculiar biophysical properties.

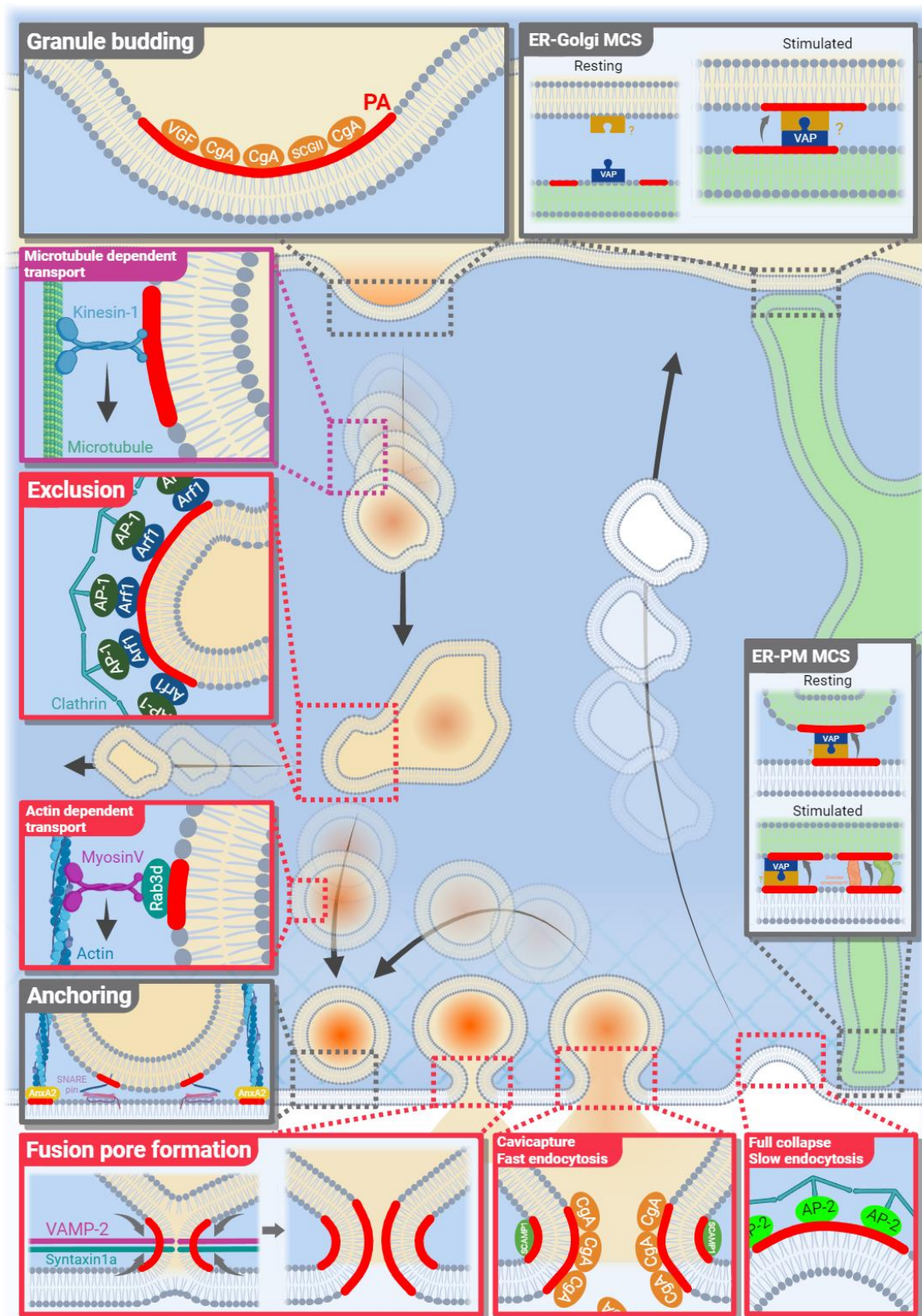
PA itself is clearly present on SG membranes as evidenced by lipidomics performed on purified secretory granules (Tanguy et al., 2020b). The current model proposes that PA at the cytosolic face of the TGN interacts with CgA to promote membrane budding and granule biogenesis (Carmon et al., 2020). However, such PA-CgA interaction should prevent any trans-bilayer movements. Of note, the binding of PA to a PABD is heavily dependent on its ionization state. The latter, in a unique fashion exclusive to PA, is heavily dependent on hydrogen bond formation with interacting proteins and local pH levels (Loew et al., 2013). Interestingly, PA has been proposed for example to form a pH sensor complex when associating with its binding partner Opi1p in yeast. The latter is a transcription factor, which, in normal conditions, is found at the ER bound to PA. However, upon glucose starvation, cytoplasmic acidification releases Opi1p from the ER to induce

transcription of lipid metabolism related genes in the nucleus (Young et al., 2010). Therefore, a similar process might take place inside SG, where upon acidification, some CgA dissociates from PA to form the dense core. Free PA would therefore be able to flop to the outer leaflet to regulate cytoplasmic partners. Hence, PA externalization on SG could represent a signal of granule maturation.

This is especially appealing as PA was also found in our screen to massively interact with proteins of the V-ATPase, which has been also proposed to serve a granule maturation signal through the association/dissociation of the V0 and V1 segments (Poëa-Guyon et al., 2013). Moreover, other factors such as cholesterol, and the local presence of other phospholipids are also able to modulate the ionization state of PA (Kooijman et al., 2005a). Therefore, mono- and polyunsaturated PA might segregate differentially on the inner leaflet of the granule membrane, and they might not be affected the same way by local membrane environment. In consequence different PA species could respond differently to intragranular pH variations, flop at different moments or kinetics and recruit their cytosolic effectors at different maturation stages. In conclusion, the notion of granular PA flop might explain how specific PA species inside SG cooperatively enable sequential recruitment of the right proteins at the right time on SG surface during their journey within chromaffin cells. Additionally, the same process could also represent a cellular detection signal for recycled SG originating from fast compensatory endocytosis which have completed catecholamine refilling and are ready for another round of fusion.

To probe this effect, we can now take advantage of the high intragranular calcium concentrations and use small ratiometric calcium sensors which should be easily clicked on our PA-N<sub>3</sub> specie of choice. Indo-1 derivatives would represent good candidates as they are not very sensitive to pH variations and remain relatively small (Bannwarth et al., 2009). Although Spo20p-GFP recruitment experiment and functional validation would need to be performed to validate those probes. Upon addition of different indo1-PA species to cells, we should be able to determine whether PA is in a high calcium environment (interior of the SG where the emission wavelength is over 480nm) or a low calcium environment (exterior of the SG, with an emission wavelength under 480nm). Thus, we could be able to quantify potential differences in the externalization kinetics between mono- and polyunsaturated PAs overtime on newly formed and reinternalized SG.





**Figure 29:** Model representing the pleiotropic functions of different PA species in the neurosecretory process

Potential functions of PA in the regulated secretory pathway as well as the potential interactants gathered from the work described in my thesis. Membrane areas in red correspond to PA rich domains. Cellular processes are delimited with a red, purple or grey frame depending on the implicated PA subspecies (Red: PA (18:0-22:6), Purple: PA (18:0-18:1) and grey: No identified preference). Created on [biorender.com](https://biorender.com).

## Conclusion

Neurosecretion is a complex and highly choreographed cellular process which relies profoundly on membrane trafficking events. Therefore, neurosecretory cells express proteins to dynamically regulate the succession of these events in space and time. These include, for example, i) cytoskeleton elements serving as rails for vesicle transport but also as scaffold maintaining complex membrane architecture, ii) SNARE pins enabling fusion of two membranes, iii) coat proteins and dynamin which respectively bend membranes and cut them off to produce vesicles. However, neurosecretory cells have also evolved to exploit the intrinsic properties of their membranes to orchestrate all steps of this process. To do this, they control their membrane composition and physical characteristics by locally altering their lipid composition through transport proteins or highly regulated metabolic enzymes. Signaling lipids production and positioning is therefore essential in membrane trafficking to recruit/regulate the appropriate protein complex at the right time and place.

PA produced by PLD1 upon stimulation of chromaffin cells is one of those signaling lipids. It stands out by being the simplest phospholipid, harboring only a phosphate head group. However, this also confers to PA the peculiar properties of being the only cone shaped phospholipid harboring negative charges. Therefore, local PA accumulation on membranes can, in parallel, induce membrane curvature and packing defects, to favor fusion and fission, while also recruiting important protein effectors through electrostatic interactions. Hence, over the last two decades, PA has imposed itself as a pleiotropic regulator of multiple steps of the late neurosecretory pathways, including SG biogenesis, transport, docking, fusion and recycling. To perform this, studies in chromaffin cells have established that multiple key proteins of the neurosecretory process are PA binding partners. This is, for instance, the case for the granulogenic protein CgA, the anchoring protein annexin A2, and the SNARE protein syntaxin1a. Moreover, PLD1-produced PA is also implicated in vesicle transport and endocytosis, suggesting additional partners.

However, how one signaling lipid can perform all those tasks in a coordinated manner remains an unsolved mystery. Moreover, different PA species, depending on their acyl chain composition are present on various membrane compartments during

neurosecretion. In support with the idea that these different PA species have specific contribution along the secretory process, our team has established that on one end monounsaturated PA is implicated in anchoring SG to the periphery, and on the other hand, polyunsaturated PA controls fusion pore formation and expansion. Hence, different PA species might confer their own unique properties to membranes and recruit specific proteins. Unfortunately, probing these effects was very challenging in a dynamic manner at my arrival at the lab. This was primarily due to a lack of adequate tools to quickly modulate subcellular levels of lipids, and an inability to target specific PA species. Hence during my thesis I produced, characterized and used new and original tools enabling the study of PA contributions in neurosecretion under a new light:

- A first approach has been to use clickable PA analogues with an azide group on their glycerol backbone. This enabled us, using click chemistry, to associate two distinct PA species with various molecules such as fluorophore (NBD, Atto647), or photo crosslinking agents. I was able to establish that those PA analogues were able to enter cells and demonstrated that they maintain the original properties of their natural counterpart. Additionally, fishing experiments enabled us for the first time to establish the interactome of mono- and polyunsaturated PA in primary neurosecretory cells under different stimulation states. Different PA species interacted with hundreds of proteins within cells, and most of them can discriminate between the acyl chain of PA state of the cell, potentially explaining the pleiotropic effects of this lipid.
- A second approach was to directly manipulate subcellular levels of PA directly within cells using an elegant optogenetic recruitment of PA metabolism enzymes to specific membrane compartments. This enabled us to determine that a SG pool of PA is implicated in early steps of the regulated secretory pathway, while a PM pool of PA is involved in late steps leading to membrane fusion and compensatory endocytosis. These observations therefore suggest that the pleiotropic function of this lipid can also be explained by its subcellular localization.

By combining the data of those two complementary approaches, I was also able to propose a new potential function of PA in the maturation of SG which may be recruiting Arf1 to immature SG. Additionally, I was able to unravel new molecular partners of various PA species directly within primary chromaffin cells, explaining its contribution to specific

trafficking events such as transport proteins (Kif5a, myosin V), vesicle fusion regulators (SCAMP1) and endocytosis proteins (amphiphysin, endophilin...). Finally, while the focus of my thesis was to explore the role of PA in neurosecretion, this signaling phospholipid is also implicated in multiple other cellular processes such as cell survival, neuronal differentiation. Accordingly, dysregulation of PA levels has been proposed to be involved in numerous disorders including viral infection, stroke, cancer, and neurodegenerative diseases. Hence, those novel tools should enable to explore the role of this unique signaling lipid in other cellular processes and pathologies.

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## Résumé étendu en français

### **Mise au point et utilisation d'une boîte à outils moléculaire pour étudier les fonctions pléiotropiques de l'acide phosphatidique au cours de la neurosécrétion**

#### **I. Introduction**

La neurosécrétion est un processus cellulaire au cours duquel, des molécules responsables de la transmission d'un message nerveux/hormonal sont libérées par des cellules spécialisées. L'équipe « Trafic membranaire dans les systèmes nerveux et neuroendocrines » que j'ai rejoint pour ma thèse cherche à décrypter les mécanismes moléculaires et cellulaires mis en œuvre par les cellules neurosécrétrices afin d'aboutir à une libération contrôlée de neurotransmetteurs ou d'hormones. Ainsi, mes travaux se concentrent sur l'étude du trafic vésiculaire dans les cellules neuroendocrines de la glande médullosurrénale : les cellules chromaffines. Ces dernières sont responsables de la libération d'adrénaline et de noradrénaline dans la circulation sanguine lors de situations de stress. Ce processus appelé exocytose régulée implique une succession d'étapes, permettant la mise en place de multiples points de régulation potentiels. L'exocytose débute par la biogénèse de granules de sécrétion à partir du réseau trans-golgien, qui subissent, par la suite, un processus de maturation leur permettant, entre autres, de se remplir en adrénaline et noradrénaline. Ces granules sont parallèlement transportés vers la périphérie cellulaire, puis arrimés au niveau de sites spécifiques à la membrane plasmique dédiés à l'exocytose. La stimulation des cellules chromaffines par de l'acétylcholine libérée par le nerf splanchnique donne lieu à une élévation des concentrations intracellulaires du calcium libre ce qui conduit à leur amorçage. L'étape finale de l'exocytose permet aux granules de fusionner avec la membrane cellulaire pour libérer leur contenu dans la circulation sanguine. Secondairement, un mécanisme d'endocytose compensatrice assure le recyclage des différents composants de la membrane granulaire.



Au cours des trois dernières décennies, de nombreux acteurs protéiques permettant d'assurer le bon déroulement de chacune de ces étapes ont été identifiés. L'équipe que j'ai rejoint a mis en évidence un rôle prépondérant de la phospholipase D1 (PLD1) dans de multiples étapes de la neurosécrétion. L'action de cette enzyme semble principalement provenir de son produit : l'acide phosphatidique (PA) ; un lipide de signalisation pouvant recruter localement différentes protéines et, soupçonné de moduler la topologie membranaire. En effet, au cours des 20 dernières années, en utilisant des approches variées combinant des knock-down ou knock-out génétiques, des inhibiteurs pharmacologiques ou même des senseurs de PA, l'équipe a pu mettre en évidence les fonctions pléiotropiques de ce lipide de signalisation dans de multiples étapes de la sécrétion neuroendocrine. Cependant, les mécanismes sous-jacents de ces contributions spécifiques restent encore largement incompris et on ne sait toujours pas comment une seule molécule de signalisation peut accompagner autant de fonctions cellulaires distinctes de manière cohérente. Cependant, des analyses lipidomiques sur des membranes obtenues après fractionnements subcellulaires et des expériences de restaurations ont permises de mettre en évidence deux faits importants, i) du PA est présent dans les différents compartiments membranaires intracellulaires et ses niveaux y fluctuent selon l'état de stimulation des cellules, ii) différentes espèces de PA, selon la nature des chaînes d'acide gras qui les composent, sont présentes dans les cellules, et sont impliqués dans des étapes distinctes de la neurosécrétion. Toutefois, à cause d'un manque d'outils adaptés permettant de manipuler rapidement les lipides au niveau subcellulaire ou de cibler différentes sous-espèces de phospholipides, les questions portant sur leurs rôles respectifs au sein de ces compartiments intracellulaires restent difficiles à aborder. Ainsi, lors de ma thèse, j'ai pu produire, caractériser, et utiliser des nouveaux outils innovants qui m'ont permis d'explorer le rôle du PA dans la neurosécrétion sous un nouvel angle. Ainsi, grâce à ces nouveaux outils moléculaires, j'ai pu démontrer que ce lipide de signalisation interagit avec diverses protéines impliquées dans l'exocytose régulée au moment opportun et, de fait impacter les propriétés des membranes de divers organelles afin d'accompagner de nombreuses fonctions cellulaires en parallèle.

## **II. Résultats**

### **A. Développement d'outils optogénétiques permettant de moduler les niveaux subcellulaires de PA**

Les approches actuelles pour manipuler les niveaux d'un lipide cellulaire se basent sur des méthodes génétiques et/ou pharmacologiques touchant les voies métaboliques qui en général nécessitent du temps (au minimum une heure pour cas du PA) tout en affectant l'ensemble de la cellule. Elles ne sont donc pas appropriées pour étudier en détail des processus rapides impliquant différents compartiments membranaires telle que l'exocytose régulée. Au début de ma thèse, j'ai donc mis au point une gamme de nouveaux outils permettant de moduler rapidement et localement les niveaux de PA dans des compartiments membranaires spécifiques (membrane plasmique, granules de sécrétion, vésicules synaptiques, réticulum endoplasmique...). Pour cela, j'ai utilisé des approches de biologie moléculaire afin d'associer des enzymes de dégradation du PA (PLA1 et lipine) à un système de dimérisation optogénétique (CRY2-CIBN), permettant un recrutement vers un compartiment subcellulaire spécifique après illumination. Dans un second temps, j'ai exprimé ces outils dans des cellules chromaffines bovines et évalué le recrutement de ces enzymes après exposition à la lumière dans le compartiment membranaire approprié, validant la spécificité subcellulaire de cette approche. L'utilisation de différents temps d'illuminations (30 sec, 5 min et 30 min) m'a permis de confirmer que le niveau de recrutement est maximal dès 30 sec, et donc de valider la rapidité de cette approche. J'ai également pu constater que des temps de recrutement long (30 min) aux granules de sécrétion impactent leur localisation (moins de granules en périphérie cellulaire), contrairement aux temps de recrutement courts. Je me suis donc concentré sur ce compartiment ainsi que sur la membrane plasmique pour évaluer les conséquences fonctionnelles d'un recrutement court des enzymes PLA1 et lipine (30 sec), par rapport à un recrutement plus long (30 min) avant la stimulation de l'exocytose. Pour cela j'ai utilisé une approche d'immunocytochimie afin de marquer et quantifier les sites d'exocytose après stimulation des cellules. De manière intéressante, dans les deux cas, un recrutement de PLA1 ou de lipine à la membrane plasmique diminue la sécrétion des cellules alors que seul un recrutement prolongé aux granules de sécrétion semble impacter leur fusion. Il est intéressant de noter que l'utilisation de deux enzymes impliquées dans deux voies

métaboliques différentes de dégradation du PA permet de confirmer que les effets observés ne sont probablement pas dû au produit de la dégradation de ce phospholipide (respectivement lyso-PA et DAG), mais bien liés à la disparition du PA. Cela m'a permis de conclure qu'à la surface des granules de sécrétion, le PA est impliqué dans les étapes précoces de la voie sécrétoire (Transport/Arrimage) tandis que le PA à la membrane plasmique est nécessaire aux étapes plus tardives (Amorçage/Fusion). Ces données démontrent, pour la première fois, l'importance fonctionnelle du compartiment membranaire dans lequel se trouve le PA pour accompagner une fonction cellulaire spécifique. Finalement, j'ai également exploré le rôle du PA à la membrane plasmique lors du processus d'endocytose compensatrice, et cette fois en recrutant les enzymes de dégradation de PA, PLA1 ou lipine, après stimulation de l'exocytose. Pour cela, j'ai réalisé des expériences d'immunofluorescence permettant de suivre l'internalisation des sites d'exocytose. Ainsi, j'ai pu observer qu'une dégradation du PA à la membrane plasmique diminue le nombre de vésicules d'endocytose contenant des protéines granulaires internalisées par les cellules. A la membrane plasmique, le PA peut donc accompagner des fonctions cellulaires différentes selon l'état de stimulations des cellules.

## **B. Caractérisation d'analogues synthétiques de PA pour étudier leur dynamique et déterminer leur interactome lors de la neurosécrétion**

D'un autre côté, afin de décrypter les contributions des différentes sous espèces de PA, nous avons travaillé en collaboration avec l'équipe du Dr. Pierre-Yves Renard (COBRA, Rouen, France) sur la production de nouveaux analogues clickables du PA en synthétisant des analogues présentant un groupement azide sur leur noyau glycérol. Cela nous a permis d'associer différentes molécules comme des fluorophores (NBD, Atto647...) ou des groupements photocrosslinker (DDA1) à différentes sous-espèces de PA (molécules brevetées en 2023). Dans un premier temps, j'ai validé leur capacité à intégrer les membranes cellulaires en évaluant le recrutement d'un senseur de PA (Spo20p-GFP) à la périphérie de cellules chromaffines après ajout des différents lipides de synthèse. Ensuite, j'ai évalué la capacité des différents PA de synthèse à restaurer la sécrétion de cellules dont l'activité PLD1 a été bloquée par un inhibiteur spécifique. Ainsi, j'ai pu vérifier que des PA monoinsaturés (18:0-18:1) de synthèse restaurent le nombre d'évènements comme le

font les formes naturelles monoinsaturées du PA. J'ai ensuite validé la capacité des PA de synthèse polyinsaturé (18:0-22:6) à restaurer la cinétique de fusion des granules grâce à l'ampérométrie à fibre de carbone, une technique d'analyse électrochimique des événements unitaire d'exocytose. Ainsi, les différents types de PA de synthèse récapitulent parfaitement les propriétés des PA naturels dans des cellules neurosécrétrices, semblent avoir la même localisation membranaire et, interagissent probablement avec les mêmes cibles protéiques. L'équipe du Dr Maité Montéro (IRIB, Rouen) a également pu observer en utilisant des formes de PA-Atto647 mono- et polyinsaturées la dynamique en temps réel d'entrée de ces PA dans des cellules chromaffines. Malgré une cinétique d'entrée un peu plus rapide pour la forme polyinsaturée, ces PA intègrent les membranes cellulaires en moins d'une minute pour rejoindre des compartiments membranaires intracellulaires (réticulum endoplasmique et appareil de Golgi) visiblement sans passer par des structures de transport endosomales. Ces données suggèrent un transport par d'autres mécanismes de trafic membranaire, comme ceux impliquant des sites de contact entre les membranes des compartiments intracellulaires. De plus, en accord avec l'asymétrie de distribution des lipides dans nos cellules, le PA polyinsaturé semble s'accumuler préférentiellement dans les compartiments intracellulaires, tandis les PA monoinsaturées sont plus enrichies à la membrane plasmique.

Par la suite, je me suis concentré sur l'utilisation de PA présentant un groupement photo-crosslinker, qui, lors d'une exposition aux UVs, est déstabilisé pour réaliser une liaison covalente irréversible avec les molécules environnantes. J'ai ainsi utilisé ces outils pour déterminer l'interactome protéique des PA mono- et polyinsaturés préalablement incubés dans des cellules chromaffines primaires. Les cellules ont été maintenues au repos, stimulées ou remises repos, pour induire l'endocytose compensatrice, lors de l'illumination. Grâce à un second groupement azide sur ces PA, j'ai pu purifier les complexes PA-interactant sur des billes possédant avec un groupement alkyne par la technique de chimie click CuAAC. L'analyse de l'interactome pour ces deux types de PA et dans ces trois conditions de stimulation a été réalisé par spectrométrie de masse (Dr Jean-Marc Strub, LSMBO, Strasbourg). Parmi les plus de 700 différentes protéines qui nous avons identifiées, seulement 20% sont capables d'interagir avec les deux espèces de PA utilisées, impliquant que 80% présente une spécificité pour une forme ou l'autre de PA.

De plus, une analyse bio-informatique nous a permis de retrouver des protéines clefs régulant différentes étapes de la neurosécrétion, comme la CGA impliquée dans la biogénèse des vésicules, plusieurs éléments de la V-ATPase, des kinésines, plusieurs protéines du complexe SNARE, des transporteurs de phospholipides, ou encore des protéines responsables de mécanismes d'endocytose comme les adaptateurs de clathrine. De plus, nous avons également trouvé une grande quantité de protéines de la chaîne respiratoire mitochondriale spécifique des PA monoinsaturé pendant la stimulation révélant potentiellement une implication directe de cet organe dans la neurosécrétion.

### **III. Conclusion**

Lors de ma thèse, j'ai donc pu explorer les multiples fonctions du PA dans le processus de neurosécrétion de cellules neuroendocrines grâce à l'utilisation complémentaire de deux approches innovantes : l'une optogénétique et l'autre de chimie click. Elles m'ont permis de manipuler finement les niveaux de PA directement dans des cellules chromaffines primaires. J'ai ainsi pu démontrer que les fonctions pléiotropiques de ce lipide de signalisation dépendent du compartiment membranaire dans lequel il se situe. De plus, l'utilisation de différents types de PA de synthèse m'a permis d'observer que, selon les chaînes d'acide gras qui constituent ce phospholipide, il est capable d'interagir avec un grand nombre de protéines qui ne sont pas nécessairement exclusives, expliquant donc l'implication du PA dans plusieurs étapes de la neurosécrétion.

## Scientific communications

### **Presented posters:**

**Characterization of novel rapamycin inducible tools to study the pleiotropic functions of phosphatidic acid during neurosecretion.**

Alexander Wolf and Nicolas Vitale

*ED414 Doctoral school days (21/04/2021 - Online)*

**Characterization of novel optic and optogenetic tools to study the pleiotropic functions of phosphatidic acid during neurosecretion.**

Alexander Wolf, Emeline Tanguy, Sebahat Ozkan, Stéphane Ory, Sylvette Chasserot-Golaz, and Nicolas Vitale

*Neurofrance 2021 (21/05/2021 - Online)*

**Molecular tools to modulate phosphatidic acid levels in specific membrane compartments during neurosecretion.**

Alexander Wolf, Emeline Tanguy, Sebahat Ozkan, Cathy Royer, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, and Nicolas Vitale

*Exo-endo club annual meeting (17/06/2022 - Munster, France)*

**Genetically engineered tools to rapidly modulate phosphatidic acid levels in specific membrane compartments: a focus on neurosecretion.**

Alexander Wolf, Emeline Tanguy, Sebahat Ozkan, Cathy Royer, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, and Nicolas Vitale

*17th GERLI Lipidomics meeting (07/10/2022 - St Jean Cap Ferrat, France)*

**Phosphatidic acid regulates multiple steps along the journey of secretory vesicles.**

Alexander Wolf, Emeline Tanguy, Sylvette Chasserot-Golaz, Stéphane Ory, Stéphane Gasman, and Nicolas Vitale.

*BPS Membrane fusion and budding event (25/09/2023 - Estes Park, USA)*

## **Oral communications:**

### **Characterization of a rapamycin- and a light-activable toolbox to modulate PA levels in specific membrane compartments.**

Alexander Wolf, Emeline Tanguy, Sebahat Ozkan, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, and Nicolas Vitale

*Exo-endo annual meeting, (10/06/2021 – Online)*

### **Chemically modified lipids to explore differential roles of various phosphatidic acid species in neurosecretion.**

Alexander Wolf, Emeline Tanguy, Antoine Schlichter, Steven Vertueux, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, Sébastien Balieu, Pierre-Yves Renard and Nicolas Vitale

*18th GERLI lipidomic meeting (17/10/2023 – Paris, France)*

### **Novel light sensible tools to explore differential roles of various phosphatidic acid species in neurosecretion.**

Alexander Wolf, Emeline Tanguy, Antoine Schlichter, Steven Vertueux, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, Sébastien Balieu, Pierre-Yves Renard and Nicolas Vitale

*Exo-endo scientific half day (24/11/2023 – Paris, France)*

### **Illuminating neurosecretion: Optogenetic insights into phosphatidic acid signaling in membrane trafficking.**

Alexander Wolf, Emeline Tanguy, Sebahat Ozkan, Stéphane Ory, Sylvette Chasserot-Golaz, Stéphane Gasman, and Nicolas Vitale

*Exo-endo annual meeting (06/04/2024 – Sète, France)*



# Mediation and student supervision

## **Scientific outreach:**

- 2020: Participation in the DECLIC program to promote science (Lycée Marie Curie, Strasbourg)
- 2021: Participation in the DECLIC program to promote science (Lycée Marie Curie, Strasbourg)
- 09/2022: Doctoneuro back to school event
- 02/2023: Participation in the Lower Rhine regional semifinal of the “My thesis in 180 seconds” contest
- 04/2023: Participation to the “MT180 in your high school” program
- 06/2024: Speaker of the CNRS microconference series: “Lipides: le gras c’est la vie!”.

## **Student supervision:**

- 11/2021: Supervision of Amrita Raja-Ravi-Shankar and Margot Villechalane (First year master) for a technical internship on carbon fiber amperometry
- 01/2022 - 03/2022: Supervision of Léa Fabre (Second year BTS) for an internship on molecular biology
- 11/2022: Supervision of Catherine Bastian and Jeanne Tyrode (First year master) for a technical internship on carbon fiber amperometry
- 03/2023 - 07/2023: Supervision of Micol Vicari (Second year master Erasmus) for an internship on the usage of optoPLD in primary chromaffin cells

# List of publications

## **Original research:**

**Phospholipase D1-generated phosphatidic acid modulates secretory granule trafficking from biogenesis to compensatory endocytosis in neuroendocrine cells.**

Tanguy Emeline, Wolf Alexander, Wang Qili, Chasserot-Golaz Sylvette, Ory Stéphane, Gasman Stéphane, and Vitale Nicolas

*Advances in Biological Regulation* 100844.

<https://doi.org/10.1016/j.jbior.2021.100844>

**V-ATPase modulates exocytosis in neuroendocrine cells through the activation of the ARNO-Arf6-PLD pathway and the synthesis of phosphatidic acid.**

Wang Qili, Wolf Alexander, Ozkan Sebahat, Richert Ludovic, Mely Yves, Chasserot-Golaz Sylvette, Ory Stéphane, Gasman Stéphane, and Vitale Nicolas

*Frontiers in Molecular Biosciences* 10, 1163545.

<https://doi.org/10.3389/fmolb.2023.1163545>

## **Patents:**

**Glycérophospholipides synthétiques comprenant au moins une fonction réactive, leur procédé de préparation et leurs utilisations dans différentes applications**

Balieu Sébastien, Renard Pierre-Yves, Montero-Hadjadje Maité, Vitale Nicolas, Cocq Aurélien, Schlichter Antoine, Ferlet Rémy, Haefele Alexandre, Riachy Lina, and Wolf Alexander

INPI, France, Patent No. FR3133855

<https://data.inpi.fr/brevets/FR3133855?q=#FR3133855>

## **Review:**

**Phospholipase D and cancer metastasis: A focus on exosomes.**

Wolf Alexander, Tanguy Emeline, Wang Qili, Gasman Stéphane, and Vitale Nicolas

*Advances in Biological Regulation* 100924.

<https://doi.org/10.1016/j.jbior.2022.100924>

# A molecular toolbox to study the pleiotropic functions of phosphatidic acid in neurosecretion

## Résumé

La neurosecrétion nécessite une coopération entre différents acteurs protéiques pour orchestrer le transport, la fusion et le recyclage de vésicules sécrétrices. Cependant les lipides, composants majeurs des membranes cellulaires, ont été moins étudiées lors de ce processus, principalement à cause d'un manque d'outils. Ainsi lors de ma thèse, j'ai implémenté deux approches pour étudier les fonctions pléiotropiques d'un lipide de signalisation : l'acide phosphatidique (PA). L'utilisation d'analogues du PA modifié chimiquement m'a permis d'établir leur dynamique ainsi que leurs interactomes directement dans des cellules neurosécrétrices. Une seconde approche optogénétique permet de moduler les niveaux intracellulaires de PA avec une résolution spatiale et temporelle sans précédent pour établir les fonctions non-redondantes de différents pools de PA subcellulaires. Ainsi, grâce à cette nouvelle boîte à outil moléculaire, j'ai pu approfondir notre compréhension des rôles du PA dans la neurosecrétion.

Mots clefs :

Lipide de signalisation – Trafic membranaire – Optogénétique – Chimie click – Neurosecrétion

## Résumé en anglais

Neurosecretion requires multiple proteins acting together to orchestrate transport, fusion and recycling of secretory vesicles. However, contribution of lipids, main components of cellular membranes, have been much less studied in these processes, primarily because of a lack of adequate tools. Hence, during my thesis, I implemented two novel approaches to study the pleiotropic functions of a peculiar signaling lipid: phosphatidic acid (PA). The use of chemically modified PA click-analogues enabled me to establish their dynamics as well as to characterize their interactome directly within neurosecretory cells. A second approach based on the optogenetic targeting of enzymes involved in the PA metabolism modulated its cellular levels with an unprecedented spatial and temporal resolution. This work allowed to unravel the non-redundant functions of various subcellular PA pools. Our novel molecular toolbox for PA, represents a breakthrough in lipid biology that will greatly improve our understanding of the roles of PA in the neurosecretory process.

Key words:

Signaling lipid – Membrane trafficking – Optogenetics – Click chemistry – Neurosecretion