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Induced proximity tools for precise manipulation of lipid signaling

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Abstract

Lipids are highly dynamic molecules that, due to their hydrophobicity, are spatially confined to membrane environments. From these locations, certain privileged lipids serve as signaling molecules. For understanding the biological functions of subcellular pools of signaling lipids, induced proximity tools have been invaluable. These methods involve controlled heterodimerization, by either small-molecule or light triggers, of functional proteins. In the arena of lipid signaling, induced proximity tools can recruit lipid-metabolizing enzymes to manipulate lipid signaling and create artificial tethers between organelle membranes to control lipid trafficking pathways at membrane contact sites. Here, we review recent advances in methodology development and biological application of chemical-induced and light-induced proximity tools for manipulating lipid metabolism, trafficking, and signaling.

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Keywords

Chemical-induced proximity, Light-induced proximity, Optogenetics, Lipid metabolism, Lipid trafficking, Lipid signaling, Membrane contact sites.

Abbreviations

CIBN, N-terminal truncation of CIB1; CIP, chemically induced proximity; CRY2, cryptochrome 2; DAG, diacylglycerol; eDHFR, *E. coli* dihydrofolate reductase; ER, endoplasmic reticulum; FKBP, 12-kDa FK506 binding protein; FRB, FKBP-rapamycin binding domain of the mammalian target of rapamycin (mTOR); IP_3, inositol trisphosphate; LOV2 domain, Light, Oxygen or Voltage-sensing domain; OSBP1, oxysterol binding protein 1; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphoinositide; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; PLD, phospholipase D; PM, plasma membrane; PS, phosphatidylserine; POI, protein of interest; TGN, trans-Golgi network; TMP, trimethoprim; VAP, VAMP-associated protein.

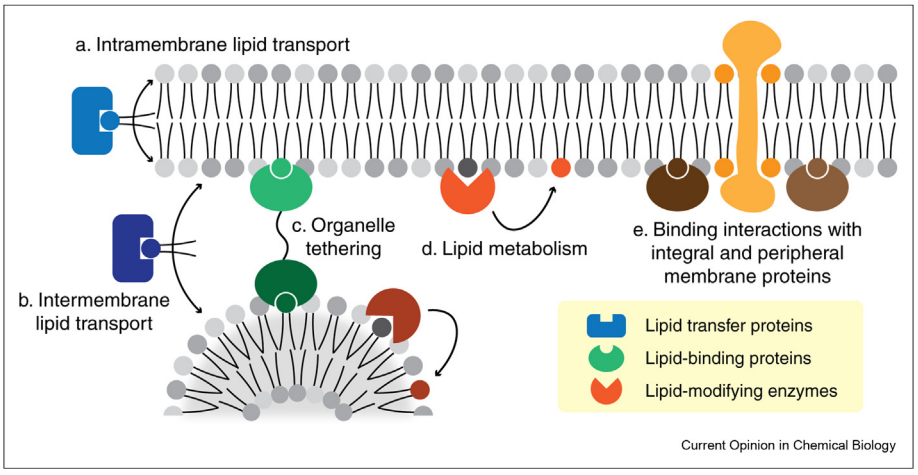
Introduction

Signaling pathways involve complex series of events that allow extracellular molecular cues to be sensed by cells and converted into intracellular information that ultimately triggers appropriate cellular responses. Many extracellular and intracellular signaling molecules are involved in multiple pathways. Cells maintain order amid this chaotic sea of molecular signals by carefully regulating signaling events in space and time. Among the set of privileged molecules that act as intracellular signaling agents are a handful of lipids. Due to their hydrophobicity, these signaling lipids have the critical property that their localization is typically restricted to individual organelle membranes. This spatial confinement is a central component of their cellular signaling functions, which occur via both lipid–lipid and lipid–protein interactions [1]. Lipid-modifying enzymes, lipid transfer proteins, and other membrane-resident proteins work together to regulate the production, transport, and degradation of signaling lipids [2,3]. Rapid conversion between lipid species and transfer between different organelle membranes, as well as between the cytosolic and luminal/extracellular leaflets of membranes, are key processes by which signaling events are turned on or off (Figure 1).

Plasma membrane phosphoinositides

To illustrate such complexity, consider the case of plasma membrane (PM) phosphoinositide (PIP) metabolism [4], which we will revisit throughout this review. This membrane maintains pools of two phosphorylated derivatives of phosphatidylinositol (PI): phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). A major role of PI4P is to engage lipid transfer proteins and ensure directional transport of another phospholipid, phosphatidylserine (PS), from the endoplasmic reticulum (ER) to the PM, at contact sites where these organelle membranes are only tens of nm apart [5]. The presence of PS in the cytosolic leaflet of the PM is critical for cell function, but its externalization to the extracellular leaflet by scramblases, counteracted in healthy cells by ATP-dependent flippases, acts as an apoptotic 'eat-me' signal [6].

Figure 1



Mechanisms by which lipids are regulated by interactions with proteins. (a) Flippases, floppases, and scramblases mediate lipid transport between the cytosolic and luminal/extracellular leaflets of membranes. **(b)** Lipid transfer proteins mediate lipid transport between different organelle membranes. **(c)** Proteins that bind to lipids in two different organelle membranes can act as tethers that help to form membrane contact sites. **(d)** Lipid-modifying enzymes regulate lipid metabolism by catalyzing the production and degradation of lipids. **(e)** Binding interactions between proteins and lipids can stabilize lipids and favor their local enrichment within membrane nanodomains.

$PI(4,5)P_2$, which is produced from PI4P, is a substrate for two enzyme classes activated by cell-surface receptor signaling: PI 3-kinases (PI3Ks) and phospholipase Cs (PLCs). PI3K activation leads to $PI(4,5)P_2$ conversion to $PI(3,4,5)P_3$, a potent, short-lived lipid signaling agent that stimulates mitogenic signaling pathways. PLC activation leads to $PI(4,5)P_2$ conversion to diacylglycerol (DAG), a lipid signaling agent that activates protein kinase Cs and phospholipase D (PLD) enzymes that convert the abundant membrane lipid phosphatidylcholine to the lipid second messenger phosphatidic acid (PA). PLC activation also generates a soluble signal, inositol trisphosphate (IP_3), which causes cytosolic levels of the second messenger Ca^{2+} to rise. These signaling events that deplete $PI(4,5)P_2$ also stimulate its resynthesis, which involves the multistep conversion of DAG to PI, occurring in the ER, not the PM, and relies on several lipid-metabolizing enzymes and transfer proteins.

Overview of tools for visualizing and perturbing lipids

How do the various roles for these metabolically interconnected lipids become established in specific physiological scenarios? Several molecular tools have been central to efforts to unravel these questions. These include genetically encoded fluorescent biosensors (e.g., GFP-tagged PH, C1, and other lipid-binding protein domains) [7] and chemical tools [8,9] that are capable of revealing the subcellular localization of specific lipid classes. Beyond these methods for visualizing lipids, approaches to selectively perturb lipid metabolism are equally important. These include loss-of-function methods such as knockout, knockdown, and pharmacological inhibition of lipid-metabolizing enzymes [10] and lipid transfer proteins [11]. Aside from issues of poor temporal resolution for genetic techniques that can lead to compensation by other metabolic pathways, loss-of-function tools are hugely important for establishing the importance of specific lipids.

An important complement, however, is gain-of-function tools for selectively activating a lipid-modifying enzyme at a precise subcellular location. These tools enable execution of the thought experiment: what if one could rapidly flip a switch and precisely change the lipid composition in a predictable manner in a single organelle? They can enable one to establish causal roles for spatially defined lipid pools in particular signaling contexts. The general approach involves recruitment of a catalytic domain of a lipid-modifying enzyme to a desired organelle membrane to carry out a specific lipid metabolic step, to either create or deplete a specific lipid.

Central to the success of such tools is the use of modular heterodimerization systems, triggered either by small molecules or by light, to induce proximity of the enzyme and the target membrane. Inducible proximity systems have been developed as ways to control protein dynamics in cells. Through dimerization or clustering induced by light or chemical stimuli, they have led to a wide variety of applications [12], including recruiting proteins of interest to specific subcellular locations, triggering protein–protein interactions, and creating signaling scaffolds.

Although induced proximity has been an important part of chemical biology research for decades [13], the first

applications of such systems to the manipulation of lipid metabolism appeared in 2006–2007, where chemically inducible dimerization systems were applied to rapidly dephosphorylate PI(4,5)P_2 at the PM [14–16]. In these landmark studies, a PI(4,5)P_2 5-phosphatase was recruited to the PM based on rapamycin-inducible dimerization of 12-kDa FK506 binding protein (FKBP) and the FKBP-rapamycin binding domain (FRB) of the mammalian target of rapamycin (mTOR). This foundational work set the stage for many technological advances that use induced proximity to manipulate lipid signaling, metabolism, and transfer. In this review, we discuss the most recent developments occurring in the past few years that use induced proximity to manipulate cellular lipid signaling pathways with spatiotemporal precision, highlighting both the innovative aspects of the tool design and the discoveries enabled by these approaches.

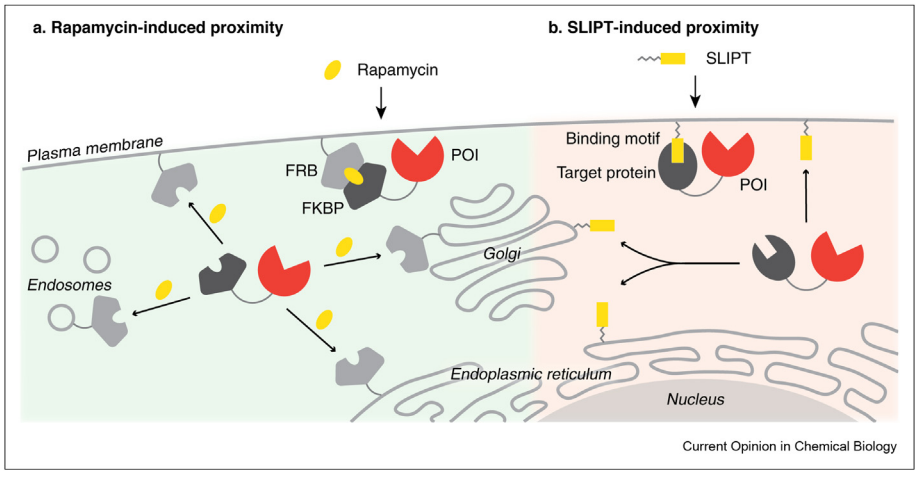
Chemically induced proximity tools

Applying FKBP–FRB systems to study PI metabolism

Most applications of chemically induced proximity (CIP) use the heterodimerization of FKBP and FRB by the chemical stimulus rapamycin or an analog (rapalog) that does not inhibit endogenous mTORC1 (Figure 2a) [17]. Other orthogonal CIP systems exist [18–20], thus enabling the regulation of multiple enzymes within a single cell. In the arena of PIP metabolism at the PM, previous work had established tools for selective degradation of PI4P and/or PI(4,5)P_2 [21], as well as controlled production of PI(3,4,5)P_3 [22,23]. These tools have led to several biological findings, including the regulation of peripheral membrane protein distribution by PIPs [24]. Yet, a major mystery remained, involving the precise localization and requirement for the abundant precursor, PI, in the production of these lipids. In the absence of a suitable imaging probe for PI, Pemberton et al. developed a rapamycin-recruitable PI-specific PLC (PI-PLC) that hydrolyzes PI to DAG and phosphoinositol at designated organelle membranes [25]. Using targeted mutagenesis, they engineered a bacterial PI-PLC to reduce its basal membrane association and tagged the resulting cytosolic PI-PLC with FKBP to make it recruitable to organelle membranes tagged with FRB. Concurrently, Zewe et al. also developed an activatable PI-PLC using a split-enzyme approach [26]. They divided PI-PLC into two parts and fused each of these components to FKBP or FRB. By having one of its components tagged to specific organelle membranes, the split PI-PLC is designed to be activated on target organelle membranes upon rapamycin addition.

By selectively depleting PI on specific membranes using this recruitable PI-PLC and quantitatively monitoring DAG production on the same membrane using a fluorescent DAG biosensor, Pemberton et al. discovered that DAG produced by ER membrane-recruited PI-PLC returned to basal levels rapidly, within 30 min, whereas DAG produced at the Golgi complex persisted much longer [25]. This finding suggested that DAG has different rates of turnover at different organelle membranes, possibly due to different rates of conversion to other metabolites or flip-flop to luminal membrane leaflets. Further, they found that acute depletion of PI at the ER showed similar or greater effects in decreasing PI3P and/or PI4P levels at the PM, Golgi complex, and

Figure 2



Chemical-induced proximity systems for controlling lipid levels on individual organelle membranes. (a) Two-component systems involving rapamycin-induced heterodimerization of FKBP and FRB. The protein of interest (POI), typically a lipid-metabolizing enzyme, is fused to FKBP, and FRB is tethered to a membrane of interest using a genetically encoded tag. The addition of rapamycin causes FKBP–FRB heterodimerization, recruiting the POI to the target membrane. **(b)** Single-component- induced proximity systems using SLIPT. Here, the POI is fused to a target protein (e.g., eDHFR), and its recruitment to the target membrane is induced by the addition of a bifunctional small molecule containing both a ligand to the target protein (e.g., TMP for eDHFR) and a moiety that directs its association in the desired target membrane.

endosomes, where major pools of these lipids exist, than depleting PI on these membranes. Their data suggest that minimal pools of PI exist at steady state on certain membranes such as the PM and endosomes and that a large reservoir of PI in the ER supplies the biosynthesis of PIPs on these membranes via rapid transfer at membrane contact sites.

The study by Zewe et al. also revealed the non-uniform distribution of PI among organelles [26]. Using split PI-PLC, they demonstrated that PI-PLC recruited to the PM failed to increase DAG, which suggested the scarcity of its substrate, PI, in the PM. Moreover, they employed the CIP approach to develop a rapamycin-recruitable PI 4-kinase (PI4K) that can convert PI to PI4P at desired organelle membranes. Using a PI4P biosensor, they observed an increase of PI4P accumulation followed by PI4K recruitment to each organelle but not to the PM, further supporting the surprising notion that there is negligible PI in the PM. Interestingly, a lack of accumulation was also observed for the ER, which was reasoned to be due to SAC1, an ER-resident PI4P phosphatase, as SAC1 inhibition indeed led to significant increases in PI4P accumulation on the ER upon PI4K recruitment.

Three's a crowd: chemically induced trimerization using split FKBP–FRB for exploring membrane junctions

Whereas the classic FKBP–FRB heterodimerization system enables chemical control of dimerization, a recent advance has enabled chemical control of trimerization. Wu et al. designed split versions of both FKBP (sFKBP) and FRB (sFRB) [27]. Each of these split protein pairs can form a trimeric complex with the appropriate full-length counterpart (i.e., FRB for sFKBP and FKBP for sFRB) upon the addition of rapamycin. The authors applied this chemically induced trimerization tool to recruit proteins of interest (POI) to membrane contact sites, which are specialized zones of close apposition between multiple organelle membranes that will be further discussed below. The authors accomplished this goal by expressing each half of the split protein on different organelle membranes (e.g., N- and C-terminal portions of sFRB) and then fusing the POI to the full-length counterpart (e.g., FKBP). They found that trimer formation upon rapamycin addition induced both the formation of the membrane contact site as well as the recruitment of the POI to such junctions from the cytosol. In a control experiment where mCherry was recruited to ER–PM junctions, the sFRB–FKBP complex showed faster trimerization kinetics than the reciprocal sFKBP–FRB (i.e., 12 min vs. 1 h). They also used this chemically induced trimerization approach to form tripartite contact sites between three organelle membranes: PM, ER, and mitochondria. Finally, they functionally modulated ER–PM contact sites by targeting a $PI(4,5)P_2$ 5-phosphatase to them, observing the subsequent depletion of $PI(4,5)P_2$ using a genetically encoded $PI(4,5)P_2$-binding fluorescent probe. This reduction in $PI(4,5)P_2$ levels occurred only 2–5 min after rapamycin addition, demonstrating the rapid perturbation of $PI(4,5)P_2$ metabolism at these specialized membrane locations by this heterotrimerization tool.

SLIPT: a protein recruitment system without protein dimerization

Whereas the FKBP–FRB systems require two or more protein components to induce membrane recruitment of proteins of interest, the self-localizing ligand-induced protein translocation (SLIPT) system enables single-component membrane tethering induced by synthetic self-localizing ligands (Figure 2b). Nakamura et al. developed dual-functional ligands containing a binding motif for a target protein (e.g., trimethoprim (TMP) for *E. coli* dihydrofolate reductase (eDHFR) [28]) and a small-molecule localization motif [29,30]. By screening different localization motifs, they identified a series of lipid tethers that allow targeting of ligands to distinct organelle membranes, including the PM and endomembranes (i.e., ER, Golgi). Using a PM-targeting ligand and eDHFR-tagged PI3K, they demonstrated the activation of the PI3K/Akt pathway by inducing PM recruitment of PI3K upon addition of the ligand. Further, the SLIPT system enables multi-input, stepwise activation by using different combinations of a ligand and a target protein [29], and it also allows for one-time reversibility by the addition of excess free ligand. In particular, this property of reversibility is an important distinction between it and the FKBP–FRB systems.

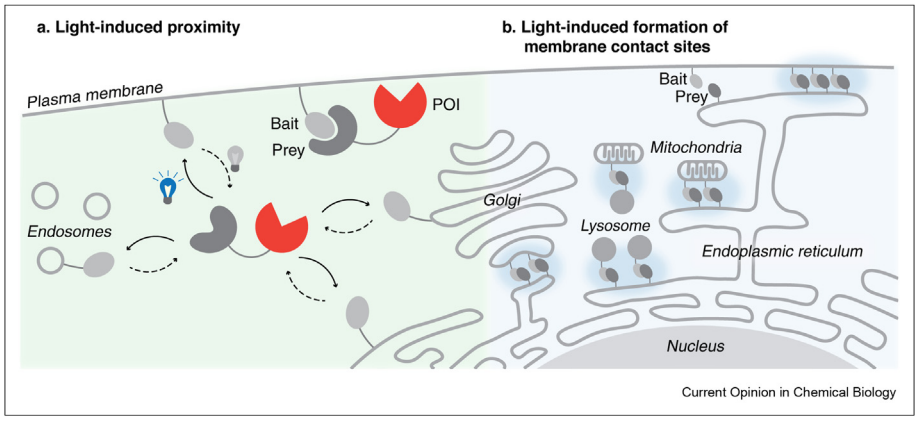
Light-induced proximity, or optogenetic, tools

One drawback of most CIP systems is their lack of reversibility. Another is the necessity for activation to occur only at a population level, i.e., with no spatial control over which cells, or which organelles within a single cell, are activated. Light-induced proximity systems are powerful approaches that enable rapid and reversible control of protein localization with high spatiotemporal control (Figure 3). These tools are based on the optical dimerization of one protein component (the bait) with its complementary partner (the prey). Various optogenetic systems have been developed with different properties, including variations in kinetics, bait/prey size, and required light wavelength [31,32], enabling optimization for distinct applications and, in some cases, multiplexing.

A little light goes a long way with CRY2/CIBN

A popular approach to recruit lipid-metabolizing enzymes to specific membranes with light (Figure 3a) uses CRY2 (Cryptochrome 2) and CIBN (N-terminal truncation of CIB1). This system, which has relatively fast

Figure 3



Light-induced, or optogenetic, proximity tools for controlling lipid levels on individual organelle membranes. (a) Light-induced dimerization systems for controlling the localization of lipid-modifying enzymes. Here, a POI (the enzyme) is fused to the prey, and the bait is tethered constitutively to the target membrane. The light-dependent interaction of the bait and prey leads to the recruitment of POI to the target membrane and subsequent changes to its lipid composition based on the enzymatic activity of the POI. **(b)** Light-induced proximity as an approach to induce the formation of membrane contact sites between two organelle membranes. Here, the bait and prey are targeted to different membranes, and light illumination causes the heterodimerization, leading to induced proximity of the organelle membranes.

turn-on (5−10 s) and slow turn-off (10−15 min) kinetics [31,33], is well suited to applications where sustained dimerization is desirable over many minutes or longer because only intermittent light illumination is needed to keep the two protein components dimerized. By using this approach, Idevall-Hagren et al. developed light-recruitable PI3K to control local $PI(3,4,5)P_3$ synthesis and induce membrane ruffling [22]. Since then, this tool has been applied to control several $PI(3,4,5)P_3$-mediated cell signaling pathways, including insulin-stimulated GLUT4 exocytosis in 3T3-L1 adipocytes [34] and TGF-induced epithelial-mesenchymal transition in A549 human lung cancer cells [35].

More recently, we developed a light-activatable phospholipase D (optoPLD) for controlling the production of PA at different organelles, including PM, ER, Golgi, and endosomal membranes [36]. Key to the success of this approach was the identification of a heterologous PLD, in this case from *Streptomyces* sp. PMF, with little intrinsic membrane affinity. We fused this PLD to CRY2 and used CIBN fusions to a variety of organelle-targeting tags. To ensure equimolar expression and consistent membrane recruitment, we assembled a bicistronic vector encoding these two protein fusions connected via a so-called 'self-cleaving' P2A peptide sequence.

Further, we developed a yeast membrane display-based directed evolution platform using a bioorthogonal labeling method for visualizing PLD activity within live cells previously developed by our lab [37]. Using this approach, we produced a collection of PLD mutants with different catalytic efficiencies, capable of synthesizing PA at a range of levels mimicking the different amplitudes of endogenous PA signaling. In a biological application of these optoPLD tools, we discovered that pools of PA at the PM, but not at other organelles, enable cells to downregulate the Hippo pathway, a major signaling pathway that restricts growth and proliferation and had recently been proposed to be modulated by PA [38]. In particular, using the collection of PLD mutants, we found that the extent of suppression of Hippo signaling, as read out by subcellular localization of the pro-growth transcription factor YAP, was exquisitely sensitive to the dose of PA at the PM. Given the pleiotropic nature of PA, i.e., its implication in modulating diverse biological signaling events from numerous locations within the cell, we envision multiple applications of optoPLD to interrogate PA signaling in the years to come.

Controlling membrane contact site formation with tender LOVing care

Beyond its use to control enzyme activity, inducible proximity has been applied to manipulate the formation of membrane contact sites, which are subcellular structures where two organelle membranes come close to each other, i.e., typically within 10−30 nm (Figure 3b). The first use of induced proximity for studying membrane contact sites involved chemically induced dimerization to form ER−mitochondria contact sites [39,40]. Because of the highly dynamic nature of membrane contact sites, which play important roles in lipid trafficking, calcium homeostasis, and cell signaling [41,42], optogenetic approaches, which have high levels of spatial and temporal control, are most desirable to manipulate them.

To induce the formation of membrane contact sites by light, He et al. employed a LOV2 domain (Light,

Oxygen or Voltage-sensing domain) and developed light-activatable polybasic domains (OptoPB) [43]. The LOV2 domain on OptoPB undergoes a conformational change upon light stimulation [44,45], leading to exposure of the OptoPB polybasic domains and triggering its targeting to the PM via binding to PIPs in this membrane, especially $PI(4,5)P_2$ [46]. By constitutively tagging OptoPB to the ER, ER–PM contact site formation was induced with light. Interestingly, the contact sites introduced by a compact OptoPB construct, which were estimated to permit less than 10 nm of space between the PM and ER membranes, efficiently excluded the PM-resident ORAI1 calcium channel [43]. An increase in the length of OptoPB via the introduction of various flexible peptide linkers prevented the exclusion of ORAI1, presumably by increasing the distance between the two membranes.

The ER forms functionally distinct contact sites with many organelles beyond the PM. Shi et al. developed an optogenetic system to control the formation of ER–mitochondria contact sites [47]. In this study, they tagged one component of a heterodimerization pair to the ER membrane and the other to the outer mitochondrial membrane. After screening six different light-inducible heterodimerization systems, they found that the iLID–SspB dimerization pair caused the least disturbance to ER and mitochondrial morphology and exhibited the lowest levels of background interactions (i.e., dimerization in the absence of light).

Optimizing magnets that are activated by light

The above-mentioned study by Shi et al. highlights the still-empirical element in the design of induced proximity systems and underscores the importance of screening different optogenetic tools, which often have distinct physical properties (i.e., structures and sizes) and chemical properties (i.e., turn-on/off kinetics, basal interactions in the dark). In another systematic study comparing spatially restricted activation of different optogenetic heterodimerization pairs, Benedetti et al. found that Magnets exhibited the highest spatial confinement of induced dimer formation [31]. Magnets are an optogenetic dimerization system engineered from the photoreceptor Vivid by Kawano et al. [48]. They developed two major groups of VVD variants, termed nMags and pMags, which recognize each other based on electrostatic interactions (with n and p standing for negative and positive, respectively). They further engineered a variety of nMags and pMags with different interaction kinetics. In particular, a pair of nMag and pMag was optimized for rapid reversibility (nMagHigh1 and pMagFast2), exhibiting very fast turn-on ($t_{1/2}$ ~1.5 s) and turn-off ($t_{1/2}$ ~6.8 s) kinetics. However, the Magnets system required preincubation at 28 °C to be functional, which has complicated its application in mammalian cells.

A further optimized set of Magnets, termed 'enhanced Magnets (eMags)', was recently reported by Benedetti et al. [49]. The eMags were engineered to have both higher stability, which eliminates the need for preincubation at a lower temperature, and better dimerization efficiency. The authors demonstrated the utility of eMags in several applications, such as the light-controlled degradation and regeneration of $PI(4,5)P_2$ followed by the association and dissociation of an inositol 5-phosphatase with the PM. They also validated the eMag system for use in the formation of various membrane contact sites, including ER–lysosome, ER–mitochondria, and lysosome–mitochondria.

Impressively, they reconstituted ER–Golgi tethering and subsequent PI4P transfer from the Golgi to ER by the proteins VAMP-associated protein (VAP) and oxysterol binding protein 1 (OSBP1). VAP, which localizes to ER membranes, binds to the trans-Golgi network (TGN)-localized OSBP1, inducing it to catalyze the extraction and exchange of PI4P on TGN membranes for cholesterol derived from ER membranes [50]. The authors developed a split version of VAP fused to eMags, where one component of the split pair was expressed in the cytosol and the other was constitutively tagged to the ER. Light-induced heterodimerization of eMags rapidly led to the reconstitution of split-VAP, whose functionality was demonstrated by its interaction with endogenous OSBP1 and subsequent depletion of PI4P from Golgi membranes revealed by fluorescent PI4P biosensors.

Conclusion: keep a close eye on these tools in the future

Chemical-induced and light-induced proximity tools that enable acute manipulation of membrane lipids to study lipid signaling and metabolism have been a major boon to the field. In this review, we have discussed recent developments in such tools and examples of discoveries that have enabled understanding the roles of lipids in mammalian cells. Critically, in virtually all studies, these tools that allow manipulation of lipids are coupled with complementary tools for selective visualization of lipids. In many cases, lipid-binding probes monitor changes caused by manipulation of lipid levels; further, in some cases, the manipulation tools and the visualization tools were integrated into the analysis to draw biological conclusions about lipid trafficking and metabolism. Because of the complementary nature of these tools, the importance of methods for controlling lipid modification and intracellular transport discussed in this review also applies to tools for monitoring lipids [8,9].

We envision that the dimerization tools themselves will continue to be iteratively improved and expanded to regulate different aspects of lipid metabolism, signaling,

and trafficking. A key unsolved issue is whether it will be possible to move beyond empirical screening of different systems for a particular application and develop a framework for being able to streamline the design of future induced proximity tools. Induced proximity has a long and storied history in chemical biology. Its relatively recent application to enable acute manipulation of dynamic pools of lipids is most exciting to this field, and we see a bright future for these tools to reveal new biological functions of lipids.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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