

SPOTLIGHT

Molding a PI(3,5)P₂ biosensor

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The lipid phosphatidylinositol 3,5-bisphosphate—PI(3,5)P₂—is known to be a key regulator of cellular traffic in health and disease, but its cellular localization was somewhat enigmatic until now, with the discovery of a new PI(3,5)P₂ biosensor reported in this issue of JCB by Vines et al. (2023. *J. Cell Biol.* <https://doi.org/10.1083/jcb.202209077>).

The inositol lipid, PI(3,5)P₂, has been known to play a central role in the endocytic pathway for nearly three decades. Recently, it has become the focus of efforts to treat an array of neurodegenerative and viral diseases, including COVID-19. Yet, the details of how this scarce inositol lipid functions in the pathway, and precisely at which membranes, has remained stubbornly inscrutable. That is, until this issue of the *Journal of Cell Biology*, where Vines and colleagues report a new genetically encoded lipid biosensor for this molecule (1). Already, the probe is shedding light on some of the mysteries that still shrouded this key regulator of membrane traffic.

Since its discovery in 1997 (2), PI(3,5)P₂ has become one of the most enigmatic lipid pathways in animal cells but has also shown great promise for clinical exploitation. It is known to regulate the endocytic pathway, principally at the terminal, lysosome-associated steps (3). Recent excitement surrounding research on this pathway has been driven by two discoveries: firstly, that the lipid plays key roles in disease. It is a key host factor for several viruses that hijack the endocytic pathway, including COVID-19. PI(3,5)P₂ synthesis also plays a key role in neurodegenerative diseases. Secondly, a single enzyme, PIKfyve, is responsible for the terminal step in PI(3,5)P₂ synthesis, and this enzyme is provenly drugable. Collectively, therefore, it looks like research into PI(3,5)P₂ function could be rapidly translated into the clinic (4).

The excitement and promise aside, there are still many basic questions about PI(3,5)P₂-driven biology that remain. A prominent example is precisely where along the endocytic pathway does PI(3,5)P₂ operate? The preponderance of evidence, based on the phenotype of PIKfyve loss of activity and the localization of effector proteins, indicates that the lipid plays central roles at the late endosome and lysosome (3, 4). What is less well known is that PIKfyve itself has been repeatedly localized to early endosomes (5, 6). So, does PI(3,5)P₂ function earlier in the pathway, but produce knock-on effects downstream when it is lost? Or does PIKfyve synthesize the lipid at later compartments not reflected by its steady-state localization? Or does it synthesize PI(3,5)P₂ at early compartments, with the lipid continuing through the endocytic pathway to meet its effectors?

The tool that was desperately needed to answer questions like these is a selective probe for PI(3,5)P₂. Most lipid probes take the forms of genetically encoded lipid biosensors, typically derived from the lipid binding domains of effector proteins (7). Identifying such probes is a unique challenge for PI(3,5)P₂ because its levels are so scarce, even relative to most other inositol lipids. This means a biosensor needs to exhibit unusually high affinity and selectivity—even 10% binding to another inositol lipid would ruin any utility when that other lipid is 100-fold more abundant. In a similar vein, the biosensor would need

to lack orthogonal interactions with cellular components that could bias or occlude the lipid's role. To date, candidate biosensors have failed to pass these criteria. The closest was derived from an N-terminal motif of the PI(3,5)P₂ binding mucolipin domain of the lysosomal TRPML1 ion channel (8). However, the probe's specificity did not prove reproducible (9), and it is now known that this motif lacks key tertiary elements of the mucolipin domain structure needed for PI(3,5)P₂ binding (10).

In their paper (1), Vines and colleagues screened the slime mold *Dictyostelium* for proteins containing endocytic lipid binding PX domains that lost localization in PIKfyve null cells. They identified one, DDB_G0289833, which displays PIKfyve-dependent labelling of vesicles and vacuoles. The protein possesses both a coiled-coil and PX domain (Fig. 1 A). Since this is homologous to the known structure of many sorting nexins, and presumably because DDB_G0289833 does not roll off the tongue even by biology's twisting standards, the authors named the protein *Dictyostelium* SnxA (to the uninitiated, this is pronounced “snix-A”). The purified protein exhibits exquisite specificity for PI(3,5)P₂ among the inositol lipids and is suitably high affinity with a dissociation constant of around 200 nM. The isolated PX domain shows very little binding, though a tandem dimer (2xPX_{SnxA}) exhibits similar 200 nM affinity. It seems that the coiled-coil domain mediates

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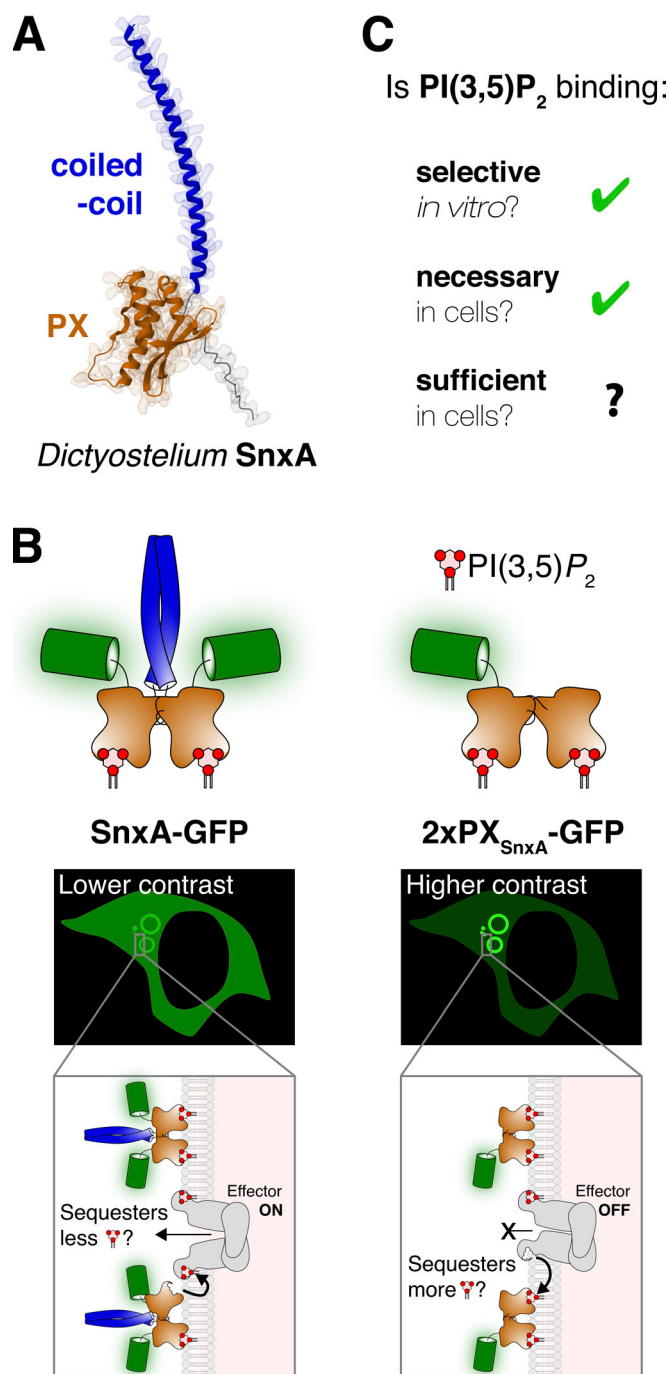


Figure 1. **PI(3,5)P₂ biosensor cheat sheet.** (A) The predicted structure of SnxA from *Dictyostelium* (a.k.a. DDB_G0289833) generated by alphaFold (Q54GY1) and rendered here using Mol*. (B) A comparison of the two SnxA-based PI(3,5)P₂ biosensors, both of which are expected to present two PX domains for lipid binding. (C) An overview of criteria to assess lipid biosensors as applied to SnxA and 2xPX_{SnxA}.

dimerization of the SnxA proteins, enabling a higher avidity interaction by the PX domains required for the high affinities needed for PI(3,5)P₂ sensing.

PIKfyve-dependent localization to vesicles and vacuoles could be seen in both *Dictyostelium* and mammalian cells. Full

length SnxA and 2xPX_{SnxA} behave broadly similarly, though the 2xPX probe often displays improved contrast (Fig. 1B). However, 2xPX also shows a greater tendency to disrupt PI(3,5)P₂ localization and function, potentially because of a slower off rate of the constitutive dimer that leads to more

sequestration of PI(3,5)P₂ away from effector proteins (Fig. 1B). We echo the authors here—both SnxA and 2xPX_{SnxA} have strengths and weaknesses, and the best probe for a particular experiment in a specific system will need to be empirically determined. Be skeptical of voices declaring superiority of one over the other!

We have proposed three stringent criteria for genetically encoded lipid biosensors (7): firstly, the probe should be selective for the lipid, which both SnxA and 2xPX_{SnxA} very clearly are (Fig. 1C). Second is a strict requirement for the lipid to drive localization in cells; this is clearly met by removal of the biosensors' localization after genetic or pharmacologic inhibition of PIKfyve in mold and mammal. The third criterion is trickier to demonstrate: is the lipid alone sufficient to drive localization in the cellular environment—i.e., could the sensor be biased by additional interactions? This is tough to assess because it can only be empirically tested in cells, which have the full repertoire of molecules that could additionally bind the biosensor. It is best tested by driving ectopic synthesis of the lipid in an orthogonal compartment. Unfortunately, a method to do so for PI(3,5)P₂ is not apparent in the literature currently. Even a cursory glance at the currently utilized lipid biosensors (7) shows that relatively few sensors are known to meet this criterion. For now, the reader should bear in mind that localization of a SnxA-based biosensor convincingly shows the presence of PI(3,5)P₂ in that compartment, but its absence cannot indicate absence of the lipid with full confidence.

Many of the larger vacuole-like structures labelled by SnxA in mammalian and *Dictyostelium* were shown to be macropinosomes. Furthermore, accumulation of SnxA on both macropinosomes and phagosomes could be tracked in *Dictyostelium*, albeit more transiently on the former. These longitudinal experiments were able to shed light on the differing cellular localizations of PIKfyve and PI(3,5)P₂. During phagocytosis, PIKfyve associated with the phagosome right before SnxA detected PI(3,5)P₂ synthesis. Unexpectedly, PIKfyve then rapidly dissociates, but PI(3,5)P₂ endures. Therefore, it seems like PI(3,5)P₂ may indeed be made early in the endocytic pathway but endures for longer, meeting effectors at later compartments.

Armed with these new tools, we anticipate that the field has now overcome a

major technical barrier to revealing the pathway's dynamics in cells. It opens the door to better understand PI(3,5)P₂'s ability to activate effectors in different compartments. This could be instrumental in targeting the pathway more precisely—a core requirement, since PIKfyve inhibition has been shown to be both preventative and causative in neurodegenerative diseases, depending on context (3, 4). We predict that this will be a seminal advance in the field, and we can't wait to read the discoveries that will be made with these biosensors.

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