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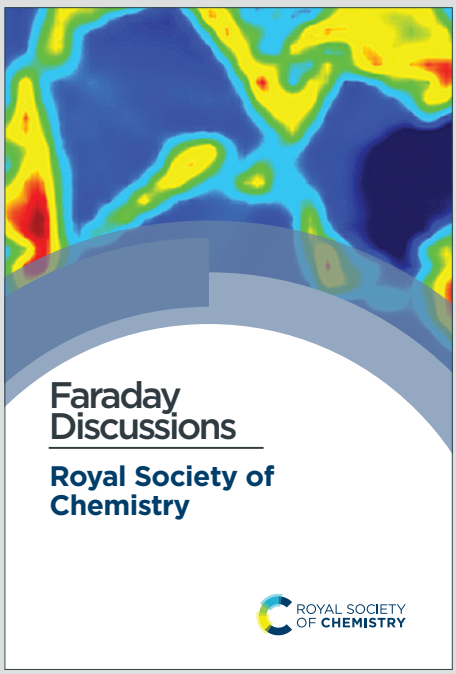

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Does Plasma Membrane Transbilayer Asymmetry Coupled to Lipid Nanodomains Drive Fast Kinetics of FGF2 Membrane Translocation into the Extracellular Space?

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Abstract

Fibroblast Growth Factor 2 (FGF2) is a potent mitogen secreted from mammalian cells through an unconventional secretory pathway. This process is mediated by direct translocation of FGF2 across the plasma membrane into the extracellular space. It requires several components that are asymmetrically distributed between the two leaflets of the plasma membrane. At the inner plasma membrane leaflet, FGF2 undergoes sequential interactions with the Na,K-ATPase, Tec kinase, and the phosphoinositide $PI(4,5)P_2$. While the Na,K-ATPase, and Tec kinase are auxiliary factors, interactions of FGF2 with $PI(4,5)P_2$ trigger the core mechanism of FGF2 membrane translocation, inducing FGF2-oligomerization-dependent formation of lipidic membrane pores. At the outer plasma membrane leaflet, membrane-inserted FGF2 oligomers are captured and disassembled by Glypican-1 (GPC1), resulting in translocation of FGF2 to the cell surface.

In a cellular context, a single FGF2 membrane translocation event occurs within 200 milliseconds. By contrast, *in vitro,* using a fully reconstituted liposomal inside-out system with FGF2 added from the outside and luminal encapsulation of high affinity heparin molecules*,* FGF2 membrane translocation takes several minutes. Here, we hypothesize the observed difference to be, at least in part, due to the asymmetrical membrane lipid distribution and the spatial organization of the FGF2 translocation machinery in native plasma membranes. We suggest the molecular machinery mediating FGF2 membrane translocation to assemble in ordered nanodomains, characterized by sphingomyelin (SM), cholesterol and phosphoinositide $PI(4,5)P_2$ coupled together. The transbilayer asymmetry of these lipids likely plays a crucial role in regulating the thermodynamics and kinetics of FGF2-induced membrane pore formation. Therefore, succeeding in reconstituting the FGF2 translocation machinery in artificial membranes with an asymmetric transbilayer distribution of SM and $PI(4,5)P_2$ and other membrane lipids may reveal a direct impact on pore opening kinetics. Similarly, disrupting lipid asymmetry in cells may significantly impact FGF2 secretion rates, a finding that would underscore the importance of the spatial organization of lipids in membrane dynamics. Testing this hypothesis may advance our understanding of how membrane asymmetry and ordered lipid nanodomains regulate critical biological processes, such as the unconventional secretion of FGF2.

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Introduction

The molecular machinery and mechanism driving unconventional secretion of FGF2

The first pieces of evidence suggesting the existence of ER/Golgi-independent pathways of protein secretion from mammalian cells date back to the late 1980s (Muesch et al. 1990). Along with, for example, Interleukin 1β and members of the galectin family, FGF2 was recognized as one of the first candidates of proteins transported into the extracellular space in a signal peptide-independent manner, suggesting alternative pathways of protein secretion that bypass the classical ER/Golgi route (Pallotta and Nickel 2020). This was experimentally verified by transport of FGF2 into the extracellular space and shown to be insensitive to brefeldin A, an inhibitor of ER/Golgi-dependent protein secretion (Florkiewicz et al. 1995; Trudel et al. 2000; Engling et al. 2002). Another early observation was a possible involvement of the plasma membrane resident Na,K-ATPase. This finding was based on the inhibition of unconventional secretion of FGF2 by ouabain, a cardenolide that was known to inhibit the membrane potential generating function of this ATP-dependent ion pump (Florkiewicz, Anchin, and Baird 1998). However, all of these observations were solely based upon pharmacological evidence and, therefore, not fully conclusive. Due to the lack at the time of advanced techniques such as genome-wide screening systems based upon RNA interference or CRISPR gene knock-outs, as well as the unavailability of sophisticated in vitro reconstitution systems, mechanistic insights into the molecular machinery mediating unconventional secretion of FGF2 became available only much later starting in the mid-2000s.

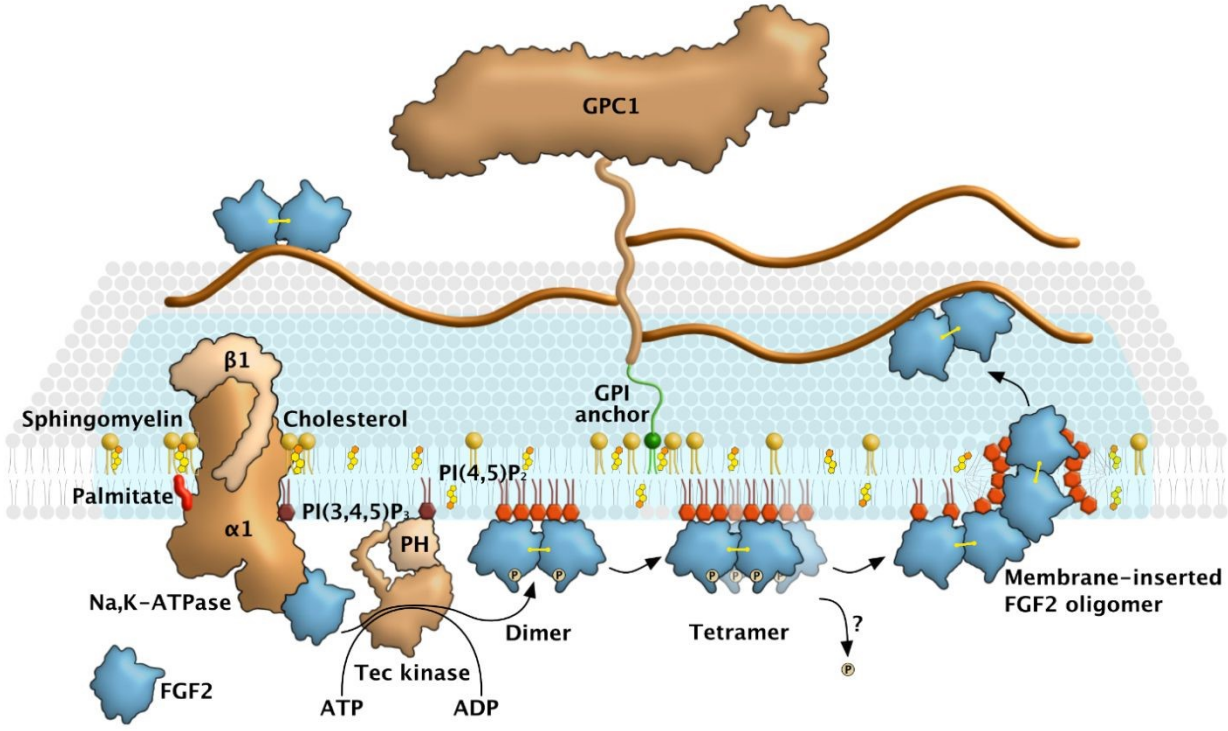

Figure 1. FGF2 unconventional secretion mechanism. *FGF2 secretion follows a unique mechanism involving direct translocation across the plasma membrane. Initially, soluble FGF2 monomers interact directly with the inner leaflet of the plasma membrane via binding to the Na, K-ATPase. Subsequently, FGF2 is transferred to PI(4,5)P_2 within*

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cholesterol-enriched nanodomains, forming FGF2 oligomers. During this process, disulfide-linked FGF2 dimers, mediated by C95, act as intermediates and further self-assemble into tetramer ring structures. The PI(4,5)P_2-binding site in FGF2 also binds an additional four to five PI(4,5)P_2 molecules through weaker interactions, leading to localized clustering of PI(4,5)P_2 molecules under the FGF2 tetramer. Since PI(4,5)P_2 is a non-bilayer lipid, this clustering is thought to compromise the plasma membrane's integrity, facilitating the formation of a toroidal lipidic pore. Finally, the oligomers are captured and disassembled into dimers at the cell surface through interaction with heparan sulfate proteoglycan glypican-1, resulting in the translocation of FGF2 to the cell exterior.

A key observation in elucidating the FGF2 secretion pathway was the ability of FGF2 to physically traverse the membranes of affinity-purified plasma membrane vesicles with an inside-out topology (Schäfer et al. 2004). This suggested unconventional secretion of FGF2 from cells to be mediated by direct translocation across plasma membranes. This conclusion could be confirmed by TIRF imaging of individual FGF2 membrane translocation events at the plasma membrane in real time with single-molecule resolution (Dimou and Nickel 2018; Dimou et al. 2019). Further key insights that led to the unraveling of this secretory mechanism were its dependence on the phosphoinositide PI(4,5)P_2 at the inner leaflet (Temmerman et al. 2008) and heparan sulfate proteoglycans at the outer leaflet of the plasma membrane (Zehe et al. 2006). The interaction of FGF2 with PI(4,5)P_2 was then shown to trigger FGF2 oligomerization, which led to the formation of lipidic membrane pores implicated in FGF2 membrane translocation (Steringer et al. 2012a). Using giant unilamellar vesicles with an inside-out topology containing PI(4,5)P_2 on their surfaces and luminal encapsulation of heparin molecules with high affinity towards FGF2, the minimal machinery required for directional translocation of FGF2 across membranes could be defined (Steringer et al. 2017). These studies revealed PI(4,5)P_2 and heparan sulfates from proteoglycans on opposing sides of the plasma membrane along with the ability of FGF2 to oligomerize and to form lipidic membrane pores to be the minimal machinery driving FGF2 membrane translocation. NMR binding studies of FGF2 towards PI(4,5)P_2 versus heparin established these interactions to be mutually exclusive, providing a compelling data set that explains directionality of FGF2 membrane translocation from the cytoplasm into the extracellular space (Steringer et al. 2017). The functional oligomeric state of FGF2 was found to be highly dynamic, with about 4 to 8 FGF2 subunits required to trigger the formation of a membrane pore (Steringer et al. 2017; Šachl et al. 2020; Singh et al. 2023). The building blocks of pore-forming FGF2 oligomers were found to be disulfide-bridged dimers that form at the inner plasma membrane leaflet, followed by assembly into higher oligomers with a membrane pore-forming activity (Lolicato, Steringer, et al. 2024). It has been hypothesized that FGF2 oligomers cause the local and asymmetric accumulation of the non-bilayer lipid PI(4,5)P_2 at the inner plasma membrane leaflet, which triggers the opening of a lipidic membrane pore with a toroidal architecture. The discovery of PI(4,5)P_2-induced oligomerization triggering the formation of a membrane pore through which FGF2 reaches the extracellular space also explained earlier findings demonstrating that FGF2 membrane translocation does not involve unfolded intermediates of FGF2 (Backhaus et al. 2004; Torrado et al. 2009). With all of these aspects in mind, the unusual secretory pathway of FGF2, schematized in **Figure 1**, can be considered a protein self-translocation mechanism that, depending on PI(4,5)P_2 and heparan sulfates on opposing sides of the plasma membrane, is based upon the ability of FGF2 to trigger a membrane pore through which it is capable of physically traversing the plasma membrane.

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While the early findings on a potential role of the Na,K-ATPase could not be further interpreted in terms of a mechanistic model for quite some time, it gained new attention when the Na,K-ATPase, and Tec kinase were identified as factors whose RNAi-mediated down-regulation limits the efficiency of FGF2 secretion (Ebert et al. 2010). In follow-up studies, a direct interaction of FGF2 with the cytoplasmic domain of the α1 subunit of the Na,K-ATPase could be demonstrated that was found to be sensitive to ouabain (Zacherl et al. 2015). It was then shown that the Na,K-ATPase acts upstream of $PI(4,5)P_2$, accumulating FGF2 at the inner plasma membrane leaflet prior to $PI(4,5)P_2$-dependent FGF2 oligomerization and pore formation (Legrand et al. 2020). Based on these findings, alike Tec kinase (Ebert et al. 2010; La Venuta et al. 2016), the Na,K-ATPase has been proposed to act as an auxiliary factor of the molecular machinery mediating FGF2 membrane translocation into the extracellular space, representing a landing platform for FGF2 at the inner plasma membrane leaflet (Dimou and Nickel 2018; Pallotta and Nickel 2020; Sparn, Meyer, et al. 2022).

The combination of experimental studies in cells and in vitro reconstitution experiments, along with multiscale physics-based computer simulations, proved decisive in elucidating the molecular mechanism by which FGF2 is secreted by direct translocation across the plasma membrane. Even certain discrepancies observed between cell-based data and results from in vitro experiments were informative, spurring new concepts about the spatio-temporal organization of the FGF2 membrane translocation machinery. An example is the kinetics of FGF2 membrane translocation, which is considerably faster in intact cells compared to those observed for artificial membrane systems. While individual events of FGF2 membrane translocation were shown to occur within time intervals of 200 ms (Dimou et al. 2019), This process was found to take place in a time range of minutes when analyzed with giant unilamellar vesicles as model systems (Steringer et al. 2017), as illustrated in **Figure 2**. Several parameters may account for this difference such as the absence of the Na,K-ATPase in the minimal reconstitution system. Similarly, putative redox enzymes, presumably involved in forming disulfide bridges during FGF2 oligomerization in cells, are not included in the in vitro models. Another striking example is the known transbilayer lipid asymmetry of native plasma membranes absent from model membranes used in FGF2 reconstitution experiments. In particular, the exclusive localization of $PI(4,5)P_2$ in the inner leaflet of cellular plasma membranes is strikingly different from the symmetric distribution of $PI(4,5)P_2$ in giant unilamellar vesicles used in in vitro experiments.

Here, we propose the transbilayer asymmetry of membrane lipids in general and $PI(4,5)P_2$, in particular, to play a critical role in lowering the free energy costs of the conversion of a stable lipid bilayer into a lipidic membrane pore with a toroidal architecture. Furthermore, regarding lateral partitioning, $PI(4,5)P_2$ is known to be enriched in ordered nanodomains of plasma membranes (Wen et al., 2021). Along with recent findings demonstrating that (i) cholesterol tunes FGF2 binding to $PI(4,5)P_2$ (Lolicato et al. 2022a), (ii) the Na,K-ATPase has multiple contacts to cholesterol regulating its function (Kanai et al. 2022) and (iii) the predominant heparan sulfate proteoglycan driving unconventional secretion of FGF2, Glypican-1 (GPC1), is localized in cholesterol-rich domains based upon a GPI anchor (Sparn, Dimou, et al. 2022), we propose ordered nanodomains with an asymmetric transbilayer distribution of $PI(4,5)P_2$ to be the organizational principle of the molecular machinery of FGF2 membrane translocation, contributing to fast FGF2 membrane translocation kinetics of about 200 ms as observed in living cells (Dimou et al. 2019).

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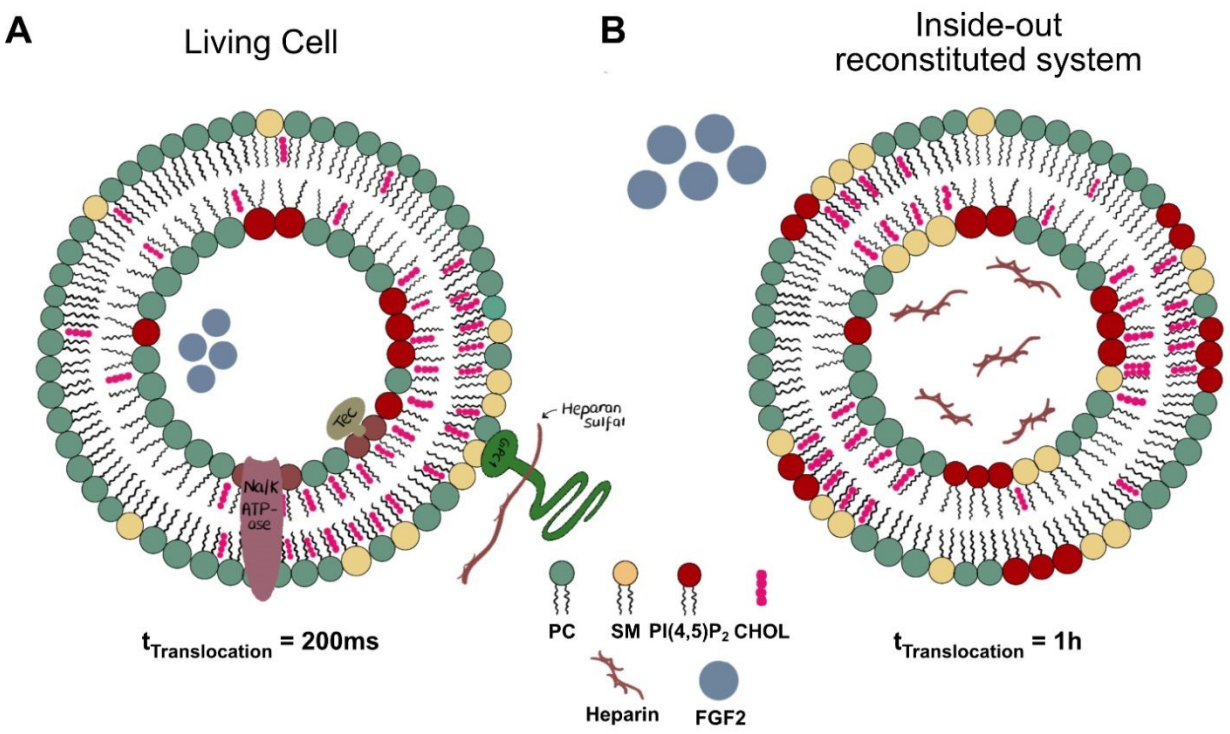

Figure 2. Difference between Living Cells and In Vitro Reconstituted Systems with Purified Components. *FGF2 translocation in (A) Cellular system showcasing nanodomain assembly of the translocation machinery within cholesterol and sphingomyelin-rich regions. A single translocation event is observed with rapid kinetics ≈200 ms; (B) In vitro biochemical reconstitution system utilizing inside-out vesicles highlight multiple nanodomain formations. Heparin long chains are encapsulated inside the vesicles, while FGF2 is added externally. A singular translocation event occurs over ≈1 hour.*

Plasma membrane lipid asymmetry and its relevance

The plasma membrane bilayer's lipid composition is well-established as asymmetrically distributed (Bretscher 1972; Lombard 2014; Lorent et al. 2020). This asymmetry is critical in maintaining cellular health, regulating membrane-dependent processes, and tuning integral membrane protein activity (Pabst and Keller 2024). Specifically, lipids such as phosphatidylcholine (PC) and sphingomyelin (SM) are predominantly localized to the outer leaflet, while phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) are concentrated in the inner leaflet (Bretscher 1972; Lorent et al. 2020). Disruption of this transbilayer asymmetry, particularly through the exposure of cytosolic lipids like PS to the exoplasmic leaflet, profoundly affects cell fate. For instance, the loss of PS asymmetry has been linked to various blood disorders, thrombosis, and metabolic syndromes (Doktorova, Symons, and Levental 2020; Fadeel and Xue 2009). To preserve this essential asymmetry, cells invest significant energy through ATP-dependent flippase and floppase enzymes that actively maintain the lipid distribution within the plasma membrane.

During apoptosis, the global loss of plasma membrane lipid asymmetry serves as a key mechanism for cell clearance, enabling recognition by phagocytes through "eat me" signals such as PS and phosphatidylinositol

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phosphates like PI(3,4,5)P_3 (Fadeel and Xue 2009; Kim et al. 2022). This disruption of asymmetry is also observed in cancer cells, which presents both challenges and opportunities for therapeutic strategies.

The loss of transbilayer asymmetry in cancer cells can be problematic, as it has been linked to reduced permeability to chemotherapeutic agents like cisplatin (Rivel, Ramseyer, and Yesylevskyy 2019). However, it also offers a therapeutic advantage, as the exposure of aminophospholipids such as PE and PS on the outer leaflet creates targets for specific interventions. For instance, small peptides and nanovesicles like OPA and SapC-DOPS exploit this exposure to selectively kill cancer cells, representing a promising strategy for targeted therapy (Patel and Witt 2017; N'Guessan, Patel, and Qi 2020).

Emerging evidence highlights that the temporary loss of plasma membrane lipid asymmetry plays a critical role in maintaining normal physiological functions. For instance, recent studies reveal that plasma membrane SM asymmetry is disrupted during lysosomal damage (Niekamp et al. 2022). Calcium release from damaged lysosomes activates SM scramblase, flipping SM to the cytosolic leaflet, which converts it into ceramide. This ceramide facilitates lysosomal repair through an ESCRT-independent pathway.

Similarly, the loss of PE asymmetry has been observed during cytokinesis (Emoto et al. 1996). Although the mechanism behind this loss is not yet fully understood, but, it is crucial for effective cell division (Emoto and Umeda 2000). During cytokinesis, the two membrane leaflets are coupled, with PE enriched in the outer leaflet of the cleavage furrow, alongside sphingomyelin and cholesterol. In contrast, phosphatidylinositol PI(4,5)P_2 is enriched in the inner leaflet. Disrupting the distribution of any of these lipids impairs cell division (Kunduri, Acharya, and Acharya 2022). The strong coupling of lipids, such as SM, cholesterol, and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) between the two leaflets during cytokinesis shows intriguing similarities to the mechanism of FGF2 membrane translocation during unconventional secretion. These same lipids have been shown to play a critical role in FGF2 membrane binding and the formation of toroidal pores (Sparn, Meyer, et al. 2022; Pallotta and Nickel 2020).

Although it remains unclear why a polyunsaturated lipid-like PI(4,5)P_2 coexists in nanodomains with cholesterol and SM—lipids typically associated with liquid-ordered membrane domains—this arrangement likely creates specialized nanodomains. These nanodomains may involve a complex interplay of membrane curvature and inter-leaflet lipid interactions, increasing membrane tension and thickness (Lolicato et al. 2022b; Griffo et al. 2024). This unique lipid organization could be key to facilitating processes requiring precise mechanical and biochemical membrane properties

Key players in ordered nanodomains

Cholesterol is a key component of ordered (Lo) phase in lipid bilayers, influencing membranes' physical and functional properties, contributing to the formation and stability of nanodomains. The ordering effect arises from cholesterol's ability to interact with acyl chains while simultaneously disrupting their tight packing (Róg et al. 2009). In multi-component lipid mixtures, there is a distinct coexistence between the gel and liquid-disordered (Ld) phases. The addition of cholesterol, which preferentially interacts with long, saturated phospholipids, shifts the gel phase towards a state resembling the Ld phase (Ijäs, Lönnfors, and Nyholm 2013; Martinez-Seara et al. 2008). This is because cholesterol enhances membrane thickness by

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straightening lipid tails in Lo phase bilayers, making them thicker than Ld-phase bilayers (Bleecker et al. 2016). However, the effect of cholesterol on membrane thickness can vary depending on the lipid composition. For instance, in bilayers with saturated 18-carbon chains, cholesterol's shorter structure induces deformation or kinking of the longer phospholipid tails, reducing membrane thickness below the phase transition temperature (McIntosh 1978). Within nanodomains, this difference in thickness generates line tension at domain boundaries, dynamically shaping the size and morphology of ordered regions. Because nanodomains exist in non-equilibrium conditions, mechanisms such as the dimpled morphology of lipids and their "pull-and-push" interactions at domain interfaces, along with the presence of amphipathic peptides and proteins at the interface, might play a key role in stabilizing nanodomain boundaries and temporarily preserve distinct domains (Ursell, Klug, and Phillips 2009; C. Wang, Krause, and Regen 2015).

Furthermore, due to key structural and interactional differences, cholesterol exhibits a lower affinity for unsaturated lipid chains. The rigid, planar structure of cholesterol's steroid ring system is better suited to align with the straight, saturated acyl chains, creating a molecular shape mismatch with the kinked, unsaturated chains. However, the two methyl groups at the steroid also play an important role in cholesterol-lipid interactions by reducing sterol tilt in the bilayer, thereby allowing cholesterol to be oriented optimally (Róg et al. 2007). Additionally, the kinks introduced by double bonds in unsaturated chains hinder tight packing with cholesterol, leading to weaker van der Waals interactions and further reducing affinity (Keller and Heuer 2021). Recent studies indicate that cholesterol is predominantly present in the outer leaflet of the plasma membrane, supporting the hypothesis of an asymmetric distribution (Doktorova et al. 2023). However, this remains a subject of debate, as experimental methods to directly and conclusively address this question are still lacking. In our previous work, we demonstrated that altering cholesterol levels in the plasma membrane directly influences the unconventional secretory machinery of FGF2 by enhancing FGF2 recruitment at the inner plasma membrane leaflet and facilitating its translocation into the extracellular space, primarily through the modulation of $PI(4,5)P_2$ lipid properties (Lolicato et al. 2022b).

Sphingomyelin is a lipid commonly associated with cholesterol-rich domains (Sankaram and Thompson 1990). This association arises from the complementary structural and chemical properties of SM and cholesterol. SM has a straight, saturated sphingosine chain that aligns well with the planar geometry of cholesterol, promoting tight packing and enhancing van der Waals interactions. Additionally, the amide group in the sphingosine chain forms hydrogen bonds with the hydroxyl group of cholesterol, further stabilizing cholesterol-SM complexes. This synergy between SM and cholesterol is functionally significant: cholesterol induces an ordering effect on membranes, while SM provides a scaffold that facilitates cholesterol's interaction with other lipids, creating a mutually stabilizing relationship. SM is asymmetrically distributed in the plasma membrane, with a higher concentration in the outer leaflet. This distribution suggests a coordinated role with cholesterol in membrane organization and function.

$PI(4,5)P_2$ plays an important role in endocytosis, exocytosis, cytokinesis, cytoskeleton activation, vesicle fusion, regulation of other phosphorylated PIs, and many signaling pathways (Lolicato, Nickel, et al. 2024). Most $PI(4,5)P_2$-dependent processes happen at the cytosolic leaflet. So, it is believed to be localized at the inner leaflet in most of the cells, with a few exceptions, such as Hela and epithelial cells (Yoneda et al. 2020). Few reports exist in the literature focusing on $PI(4,5)P_2$ transbilayer plasma membrane asymmetry. Only recently, a flippase enzyme was reported for $PI(4,5)P_2$, the same as PC flippase (Muranaka et al. 2024).

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The preferential localization of SM and cholesterol within Lo phases is well-established (Ahmed, Brown, and London 1997; Sankaram and Thompson 1990; Murata et al. 2022; De Almeida, Fedorov, and Prieto 2003). In contrast, little is known about the involvement of phosphatidylinositol (PI), and its phosphorylated derivatives, in Lo and Ld phases. The most abundant PI(4,5)P_2 species in brain extracts feature 18:0 and 20:4 acyl chains, giving this lipid unique properties (Traynor-Kaplan et al. 2017; Ikhlef et al. 2021). The straight, saturated 18:0 chain closely aligns with cholesterol's structure, promoting tight packing and order within membranes. This is further enhanced by forming hydrogen bonds with cholesterol's hydroxyl group (Wen, Vogt, and Feigenson 2021). Conversely, the highly unsaturated 20:4 chain introduces flexibility and disorder, favoring disordered regions and they might promote interdigitation with the outer plasma membrane leaflet. Detergent-resistant membrane (DRM) experiments revealed that PI(4,5)P_2 is enriched in ordered regions. Notably, cholesterol depletion using methyl-beta-cyclodextrin causes PI(4,5)P_2 to redistribute into disordered regions (Pike and Miller 1998). However, DRM assays are often criticized for potential artifact detergents introduced during the procedure. Alternative studies using Giant Plasma Membrane Vesicles (GPMVs) and Lo/Ld-directed peptides have shown faster PI(4,5)P_2 metabolism kinetic in Lo regions (Myeong et al. 2021). In this study they noted that PI(4,5)P_2 in Lo regions undergoes rapid turnover, being converted to diacylglycerol (DAG) and inositol trisphosphate (IP3) and replenished at a faster rate compared to PI(4,5)P_2 in Ld regions. The reported data suggest that PI(4,5)P_2 in Lo regions functions as a dynamic pool, regulating the concentrations of other PI derivatives through the activity of PI kinases and phosphatases. These enzymes are also reported to localize within Lo regions (Van Den Bogaart et al. 2011; J. Wang and Richards 2012), further supporting the functional interplay between PI(4,5)P_2 and Lo phase.

The asymmetric distribution of these lipid types in the plasma membrane likely plays a role in mediating FGF2 membrane translocation. Cholesterol and SM enrichment in the outer leaflet and PI(4,5)P_2 primarily located in the inner leaflet highlights a spatial organization that supports membrane-associated processes. This asymmetry may create functional lipid environments, such as ordered nanodomains, that facilitate the recruitment and translocation of FGF2 during unconventional secretion. Thus, developing methods to reproduce this lipid asymmetry in vitro reconstitution experiments or to disrupt it in living cell systems selectively could provide insights into how these lipid distributions regulate FGF2 secretion. Such studies will uncover mechanistic details about the secretory mechanism of FGF2 and reveal broader principles about the interplay between lipid organization and protein function in cellular membranes.

Targeting lipid asymmetry to study its role in the unconventional secretion mechanism of FGF2

The asymmetric distribution of lipids in living cell systems is a significant distinction compared to inside-out reconstituted vesicles, which are challenging to produce. While several reports in the literature on generating asymmetric vesicles exist, only a few studies have yet used them to make physiologically relevant plasma membrane-like asymmetric lipid compositions for functional assays.
In our studies on the unconventional secretion mechanism of FGF2, we identified three key lipid species—cholesterol, sphingomyelin (SM), and PI(4,5)P_2—that play crucial roles. Thus, developing methods for generating asymmetric vesicles containing these specific lipids in a PC background and investigating their

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impact on pore formation kinetics could reveal mechanistic insights into this unusual process of protein translocation across a membrane.

Strategies for Cellular Single-Lipid Disturbance

One of the most widely used approaches for manipulating lipid composition at the plasma membrane level is the use of β-cyclodextrin-based methods (Doktorova et al. 2018; Li et al. 2019), as illustrated in Figure 3A. β-Cyclodextrins are cyclic oligosaccharides with a hydrophobic cavity that can encapsulate lipid molecules, making them highly effective for lipid delivery or cholesterol removal at the plasma membrane. For example, β-cyclodextrin can be pre-loaded with a specific lipid of interest, such as cholesterol or sphingomyelin, and subsequently incubated with cells. This process facilitates the targeted delivery of these lipids to the outer leaflet of the plasma membrane, potentially disrupting the asymmetry of lipid species such as $PI(4,5)P_2$, which is exclusively located in the inner leaflet. In addition to lipid delivery, (methyl-)β-cyclodextrin can also be employed to extract cholesterol from the plasma membrane outer leaflet. However, while effective, this lipid extraction approach has limitations that can lead to unintended alterations in membrane composition and induce cytotoxic effects. This method has been employed to manipulate cholesterol content in the plasma membrane. It is well established that altering cholesterol levels positively influences the recruitment and translocation of FGF2 (Lolicato et al. 2022a; Lolicato and Nickel 2022). In contrast, little is known about manipulating SM and $PI(4,5)P_2$ levels in cells influencing the mechanisms underlying FGF2 secretion. Sphingomyelin is predominantly located in the outer leaflet of the plasma membrane, where it plays essential roles in maintaining membrane stability, organizing lipid domains, and mediating signaling processes. SM scramblases can translocate SM across the bilayer. Still, their activity is calcium-dependent, which may expose cytosolic leaflet lipids to the outer leaflet and it is coupled with the enzymatic action of sphingomyelinase in the inner leaflet. This enzyme converts SM into ceramide, introducing metabolic changes that complicate the study of SM dynamics. The single-cell injection method is a precise and promising alternative (Golebiewska et al. 2008), which could deliver SM directly into the inner leaflet. This approach would bypass scramblase activity, offering a controlled environment for investigating lipid redistribution. However, the physical penetration of the cell membrane can cause stress or damage to the cell, potentially affecting its viability or normal function. Furthermore, this approach enables the enrichment of SM in various membrane organelles, which could potentially alter normal cellular functions.

On the other hand, Phosphatidylinositol 4,5-bisphosphate is predominantly located in the inner leaflet of the plasma membrane, where it functions as a critical signaling lipid and regulator of membrane dynamics (Lorent et al. 2020; Zewe et al. 2020). Its intracellular localization presents opportunities for simple external intervention to disrupt lipid asymmetry. One effective strategy involves the targeted delivery of $PI(4,5)P_2$ micelles directly within the cell medium (Golebiewska et al. 2008), as illustrated in Figure 3B. $PI(4,5)P_2$ have a high bilayer partition coefficient and will efficiently incorporate into the outer leaflet of the plasma membrane.

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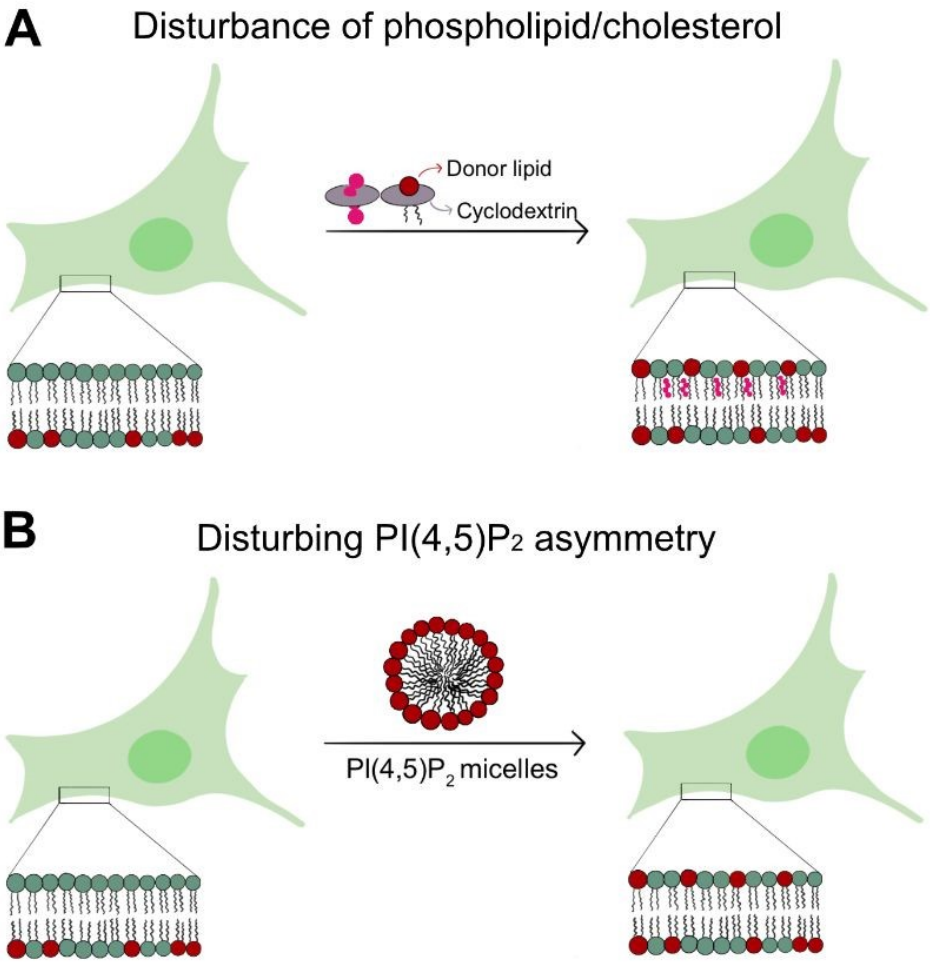

***Figure 3*. Disturbing transbilayer asymmetric distribution.** *(A) Destroying PI(4,5)P_2 transbilayer asymmetry by addition of PI(4,5)P_2 micelles which selectively incorporate PI(4,5)P_2 on the outer leaflet (B) Disrupting phospholipid and cholesterol levels by cyclodextrin, which can selectively substitute lipids from outer leaflet with lipids of choice*

Strategies for asymmetric vesicle generation

There are several methods for creating asymmetric liposomes. Large unilamellar vesicles (LUVs) are usually preferred over giant unilamellar vesicles (GUVs) due to their well-defined size and low susceptibility to high curvature or substrate interactions. In contrast, GUVs, though more fragile, have the advantage of being compatible with confocal microscopy imaging and possess a size range of 5 to 100 μm, closely matching the dimensions of eukaryotic cells (Dimova 2019). This cell-like size makes GUVs valuable as model systems for studying protein-lipid interactions.

Asymmetric unilamellar vesicles have been created using five main strategies: i) The inverted emulsion droplet method uses a lipid-coated oil-water interface to generate GUVs. This approach produces GUVs

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efficiently; however, the size distribution is broad, and residual organic solvents are often present in the hydrophobic core of the bilayer, which may alter the properties of the bilayer and compromise its integrity (Träuble and Grell 1971; Pautot, Frisken, and Weitz 2003; Doktorova et al. 2018). ii) Changes in pH can manipulate the protonation state of limited anionic lipids, such as phosphatidylglycerol (PG) and phosphatidic acid (PA), to flip between bilayer leaflets in their uncharged state. This creates an uneven lipid distribution, with accumulation on the more alkaline side of the bilayer (Hope et al. 1989). iii) Like cellular systems, carrier molecules with hydrophobic cavities, such as methyl-β-cyclodextrin or lipid transfer proteins (Bloj and Zilversmit 1976), are commonly employed to facilitate the exchange of lipids in the outer leaflet of vesicles. Catalyzed lipid exchange is compatible with a wide range of phospholipids; however, separating the two vesicle populations is needed afterward to isolate the desired asymmetric liposomes (Doktorova et al. 2018). iv) The hemifusion method is used to mix the lipid composition of GUVs with a supported lipid bilayer (SLB) by inducing membrane fusion using calcium ions. In the hemifusion state, the two leaflets are connected through a hemifusion diaphragm, promoting lipid exchange between the outer leaflets via lateral diffusion. Further fusion events are blocked by removing ions with calcium chelation, and a mechanical shear is applied to detach the GUVs from the SLB (Enoki 2024). v) The enzymatic method can effectively modify lipid composition in specific bilayer leaflets. For instance, sphingomyelin can be hydrolyzed into ceramide and phosphocholine, while PIP5K1C catalyzes the conversion of PI(4)P to $PI(4,5)P_2$, as illustrated in Figure 4. This approach enables selective modification of the outer leaflet of synthetic liposomes without affecting the inner leaflet, as the enzymes used are typically not membrane-permeable. However, its application is limited by enzyme availability and cost, substrate specificity, and the need for precise reaction conditions.

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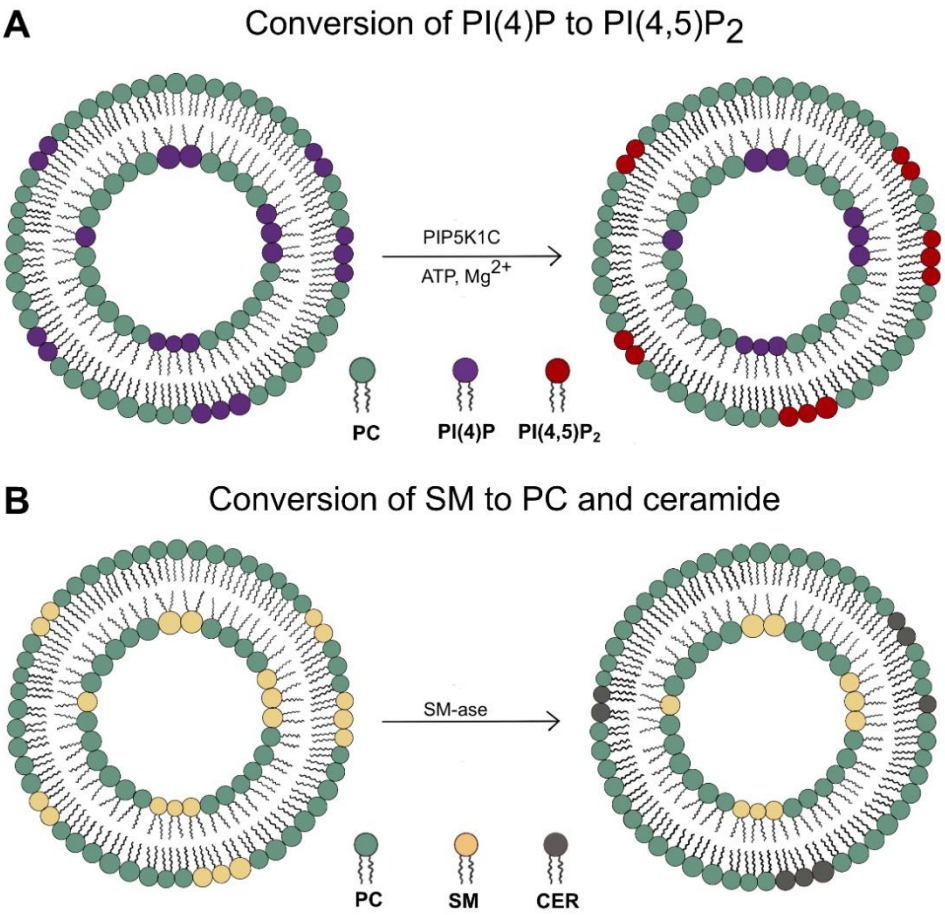

Figure 4. Enzymatic approach for generating asymmetric vesicles using symmetric SM or PI(4,5)P_2 vesicles. *(A) PIP5K1 selectively converts PI(4)P to PI(4,5)P_2 on the outer leaflet; (B) Sphingomyelinase catalyzes sphingomyelin hydrolysis, breaking it down into ceramide and phosphocholine on the outer leaflet.*

Discussion

Several parameters are likely to play key roles in governing the thermodynamics and kinetics of the FGF2 membrane translocation process. The faster kinetics observed in living cells (Dimou et al. 2019), compared to in vitro reconstitution experiments using purified components (Steringer et al. 2012b; 2017), can be attributed to fundamental differences between the two experimental systems. Notably, the absence of auxiliary components in synthetic vesicles such as the Na,K ATPase and Tec kinase. For example, a variant of FGF2 lacking the two key residues responsible for its interaction with the Na,K ATPase exhibits a 30-40% reduction in plasma membrane recruitment and translocation efficiency (Legrand et al. 2020). However, these components are not essential for FGF2 membrane translocation in reconstituted systems. In addition, as a surrogate for the authentic GPI-anchored heparan sulfate proteoglycan GPC1, soluble heparin molecules containing high affinity FGF2 binding sites have been used in in vitro experiments

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(Steringer et al. 2017; Lolicato, Steringer, et al. 2024). Furthermore, we hypothesize that the fast kinetics of this process observed in intact cells is driven by cellular redox systems facilitating the efficient oxidative dimerization of FGF2 disulfide bridges in combination with the asymmetric nature of the plasma membrane. Finally, unlike in living cells, where lipid distribution is asymmetric, artificial vesicles exhibit a symmetric lipid arrangement, introducing another layer of difference between the two experimental systems. All of these and maybe additional factors could potentially slow down the kinetics and efficiency of FGF2 membrane translocation observed in vitro compared to living cells.

Current views describe the plasma membrane as a composite material, where the exoplasmic leaflet primarily ensures functional barrier integrity, while the cytoplasmic leaflet contributes to membrane fluidity (Schütz and Pabst 2023). Due to technical limitations, this duality is often lost in in vitro systems, where synthetic liposomes are typically produced with symmetric membranes. For example, a symmetric distribution of SM—which in living cells is predominantly located in the outer leaflet—can lead to excessive membrane rigidity when interacting with cholesterol. This rigidity disrupts the dual nature of the plasma membrane, and it might create an additional barrier that hinders the FGF2 translocation process. Focusing on $PI(4,5)P_2$, its asymmetric distribution within the plasma membrane can play a fundamental role in the membrane translocation mechanism. Our current model proposes that FGF2 undergoes oligomerization at the inner leaflet of the plasma membrane. Notably, a single FGF2 molecule can recruit up to 5 $PI(4,5)P_2$ molecules through high- and low-affinity binding sites (Steringer et al. 2017). Given that FGF2 is known to oligomerize in a membrane-dependent manner into at least tetramers (Steringer et al. 2017; Šachl et al. 2020; Singh et al. 2023), this would result in the accumulation of approximately 20 $PI(4,5)P_2$ molecules in a spatially confined area. The localized accumulation of $PI(4,5)P_2$ molecules near the protein is expected to result in a local concentration of ~20 mol%. $PI(4,5)P_2$ is generally considered as having a positive intrinsic curvature due to its large, charged hydrophilic head group. However, its contribution to local membrane curvature can vary depending on the cellular context and its molecular interactions. Notably, the most abundant $PI(4,5)P_2$ species in brain extracts contain a polyunsaturated acyl chain (arachidonic acid), which is highly disordered and occupies more space, altering the overall shape of the lipid, as suggested by Lin et al (Lin et al. 2018; Lessen et al. 2022). This characteristic likely shifts $PI(4,5)P_2$'s preference towards negative curvature, which we hypothesize is a critical factor in driving FGF2 pore formation activity. Furthermore, the inner leaflet is already enriched with ~30 mol% of PE lipids (Lorent et al. 2020). PE are also conical, non-bilayer lipids with intrinsic negative curvature properties. We propose that the enrichment of $PI(4,5)P_2$, driven by FGF2 oligomerization, increases the local concentration of non-bilayer lipids beyond a critical threshold. This accumulation induces negative curvature (Figure 5B, left panel), destabilizing the membrane and facilitating the pore formation process (Figure 5C, left panel). Additionally, $PI(4,5)P_2$ carries a highly negative charge under physiological conditions, which would create a charge gradient in spatially confined region across the membrane, resulting in an electric field that could potentially contribute to the pore formation process (Dimova 2011; Gurtovenko and Vattulainen 2005). Contrarily, in symmetric membrane the long acyl chains of $PI(4,5)P_2$ (18:0 and 20:4) could interdigitate from both sides of the membrane, leading to a symmetrical accumulation in both leaflets (Chiantia and London 2012; Chaisson, Heberle, and Doktorova 2024). This would cancel out the charge gradient and

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curvature-inducing effects, as hypothesized in Figure 5B, right panel. Furthermore, heparan sulfate chains, which are also negatively charged, are positioned on the outer leaflet of the plasma membrane. A highly negative lipid such as $PI(4,5)P_2$ in the outer leaflet would reduce the proximity of the heparan sulfate chains to the membrane surface due to electrostatic repulsion, as schematized in the right panels of Figure 5 B,C. Notably, FGF2 translocation depends on membrane-proximal heparan sulfate proteoglycans (HSPGs) on the cell surface, which capture and disassemble FGF2 oligomers. Interestingly, among the diverse sub-classes of HSPGs, including syndecans, perlecans, and glypicans, we unexpectedly discovered that Glypican-1 (GPC1) to represent the principal and rate-limiting factor driving unconventional secretion of FGF2 (Sparn, Dimou, et al. 2022). This is due to its stronger binding affinity for FGF2 and closer proximity to the membrane surface. Therefore, exploring the role of $PI(4,5)P_2$ asymmetry in synthetic and cellular systems is essential to validate the proposed mechanism. Specifically, developing a method to generate $PI(4,5)P_2$-asymmetric vesicles would allow for investigations of FGF2 pore formation and translocation kinetics.

In conclusion, by mimicking the natural asymmetry of $PI(4,5)P_2$ in the inner plasma membrane leaflet, we hypothesize that this approach will accelerate the kinetics of FGF2 oligomerization and translocation, as the spatially constrained accumulation of $PI(4,5)P_2$ and the resulting membrane stress and charge gradient would facilitate membrane curvature and pore opening. Conversely, disrupting $PI(4,5)P_2$ asymmetry in living cell systems could offer deeper insights into its critical role in unconventional FGF2 secretion. We predict that the loss of $PI(4,5)P_2$ asymmetry would neutralize the charge gradient, reduce membrane curvature, and increase the distance of heparan sulfate chains from the membrane surface. This, in turn, would slow down the kinetics of FGF2 secretion.

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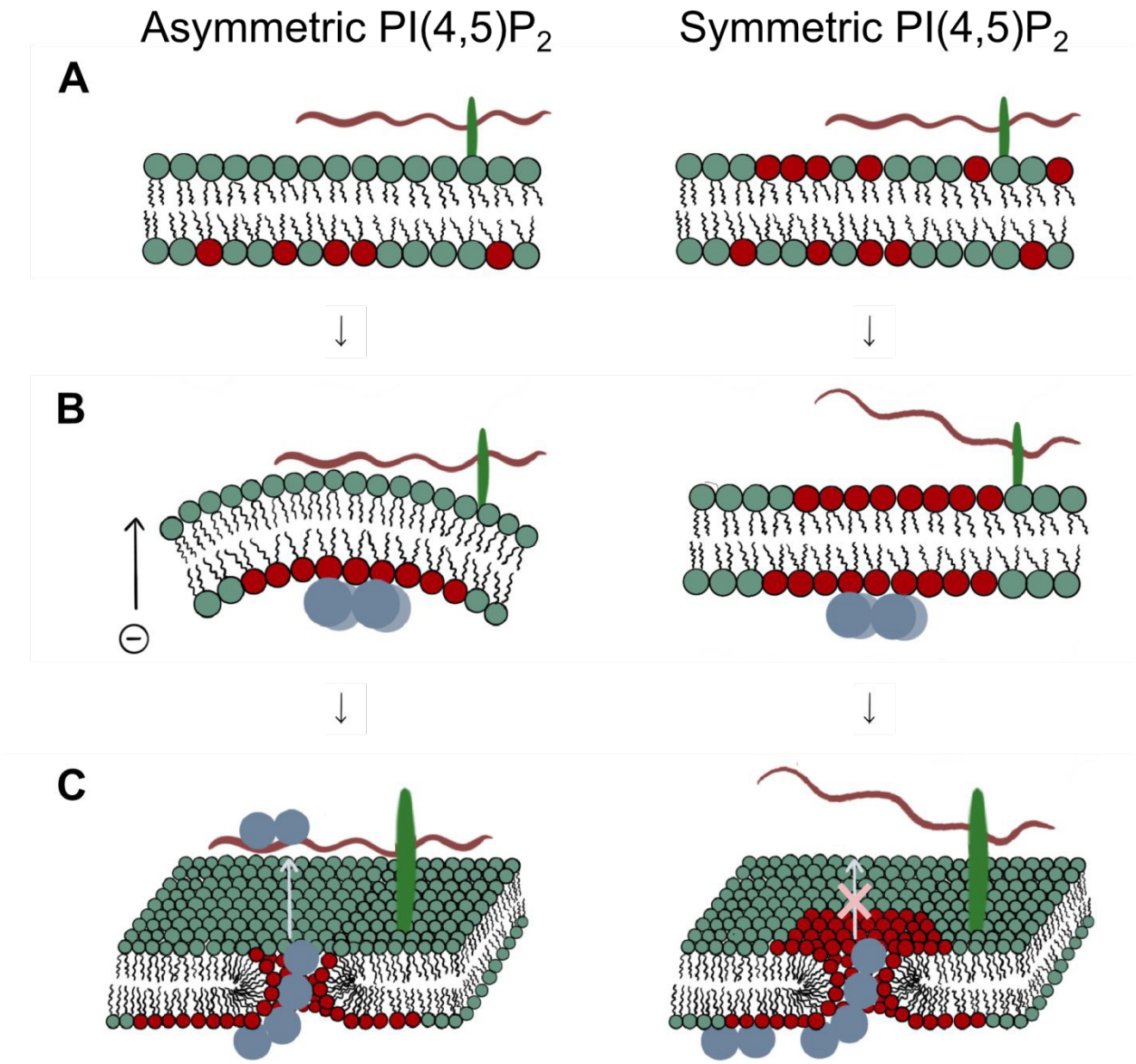

Figure 5. *Role of PI(4,5)P_2 asymmetry in governing FGF2 translocation.* *(A) Membrane with asymmetric and symmetric transbilayer PI(4,5)P_2 distribution; (B) Upon FGF2 oligomerization, accumulation of PI(4,5)P_2 in the inner leaflet causes an extreme charge gradient and curvature-dependent stress in the asymmetric membrane. In the symmetric membrane PI(4,5)P_2 accumulates in both inner and outer leaflet due to interdigitation, due to which the charge gradient and stress are canceled out in the symmetric PI(4,5)P_2 membrane; (C) Presence of symmetric PI(4,5)P_2 in the membrane will cause the repulsion between PI(4,5)P_2 on the outer leaflet and heparan sulfate chains, resulting in the orientation of heparan sulfate chains farther from the membrane, translocating FGF2 presumably slower comparative to the asymmetric system.*

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Data Availability Statement

All data supporting the findings of this study are included within the manuscript. No additional data or materials were generated or used beyond what is provided in the text and figures.

Sincerely,

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