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Review Letter

Lipid lateral diffusion and membrane organization

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It is shown that investigating the lateral motion of lipids in biological membranes can provide useful information on membrane lateral organization. After labeling membranes with extrinsic or intrinsic lipophilic fluorescent probes, fluorescence recovery after photobleaching experiments strongly suggests that specialized cells like spermatozoa, eggs and epithelia exhibit surface membrane regionalization or macrocompartmentation and that lateral microheterogeneities or lipid microdomains exist in the plasma membrane of many cellular systems.

Phospholipid; Photobleaching; Lateral diffusion; Membrane organization; Macrodomain; Microdomain

1. INTRODUCTION

In the Fluid Mosaic Model of membranes, lipids are organized in the form of a bilayer supporting peripheric and integral proteins. This model considers the lipid bilayer as a two-dimensional fluid in which lipids and proteins are free to diffuse. As a direct consequence, one would expect both types of molecules to be randomly distributed within the membrane and the lipid phase to exhibit bulk properties in terms of fluidity.

In fact, membrane organization is certainly much more complex. It is now well established that lipids [1–3] and proteins [3,4] are transversely asymmetrically distributed. Most membrane proteins are not free to diffuse, presumably due to their attachment to cytoskeleton components [5–18]. Studies on membrane model systems show that the lateral motion of lipids strongly depends on their chemical structure and physical state [19,20] and on the lipid-to-cholesterol [21] and lipid-to-protein [22–25] ratios. As shown below, many specialized cells (spermatozoa, eggs, epithelia, etc.) display membrane regionalization or macrocompartmentation and there is increasing evidence suggesting that microheterogeneities exist in biological membranes [13,26–34]. Thinking of membranes in terms of microcompartmentation means that some of their components are not free to laterally (and transversely) diffuse. Conversely, investigating the lateral (and transversal) motion of lipids and proteins can provide useful information on their lateral (and transversal) distribution within the membrane.

This short review presents recent developments in the study of the lateral organization of lipids in membranes through the investigation of their lateral motion.

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Abbreviations: for the sake of clarity, lipid probes are abbreviated as they are in the reference papers. C_{12}NFlu, 5-(*N*-dodecanoyl)-aminofluorescein; C_{14}-Fl, C_{18}-Fl, 5-(*N*-tetradecyl)-, 5-(*N*-octadecyl)-aminofluorescein; HEDAF or C_{16}-Fl or Pam-aminofluorescein, 5-(*N*-hexadecanoyl)-aminofluorescein; C_{18}-Rh, octadecylrhodamine B; C_{14}-, C_{16}-, C_{18}-diI/diO, 1,1′-ditetradecyl-, 1,1′-dihexadecyl-, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine/oxatricarbocyanine; Fl-PE, *N*-fluorescein-*N*′-phosphatidylethanolamine; Rho-PE, rhodamine-phosphatidylethanolamine; ANno, 9-(2-anthryl)-nonanoic acid; ASte, 12-(9-anthroyloxy)-stearic acid; acyl-ANnoGroPCho, *sn*-1-acyl-*sn*-2-[9-(2-anthryl)-nonanoyl]-glycero-3-phosphocholine; NBC-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazol-4-yl-phosphatidylethanolamine; NBD-PC, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazol)-aminocaproyl- (or aminohexanoyl, or aminododecanoyl)-phosphatidylcholine; NBD-PtdCho, -PtdEtn, -PtdSer, 1-acyl-2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-aminododecanoyl-phosphatidylcholine, -phosphatidylethanolamine, phosphatidylserine

2. MEASUREMENT OF THE LATERAL MOTION OF LIPIDS IN MEMBRANES

Most investigators use a fluorescence recovery after photobleaching (FRAP) technique [19]. After labeling the membrane with an appropriate lipophilic fluorescent probe, a concentration gradient of the fluorophore is induced photochemically in a small area of the membrane (photobleaching step) and then, the time course of its diffusion-mediated redistribution in the membrane plane is monitored (fluorescence recovery step). Experiments are carried out through a fluorescence microscope. This method has the advantage of being non-invasive and being applicable to living intact cells.

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Two parameters can be evaluated: the lateral diffusion coefficient D and the mobile fraction M of the probe. If the radius r_i of the illuminated area (around 1–2 μm in most experiments) is small compared to the R of the circular domain equivalent to the diffusion area ($R/r_i > 5$, conditions of an infinite reservoir) as is the case for most eukaryotic cells (diameter > 10 μm), and if the particles are free to diffuse, then M must be equal to 100% [35]. In contrast, partial recoveries indicate that some of the probe molecules are immobile, at least within the time scale of FRAP experiments.

An 'apparent' immobile fraction can be observed when r_i and R tend to be similar ($R/r_i < 2$, conditions of a finite reservoir) [35]. A numerical approach has been proposed to calculate D, M and the ratio R/r_i [35]. As shown for vascular endothelial cells [57], this enables membranes to be described in terms of macrodomains.

In most cases, FRAP experiments are carried out on single cells. It should be stressed that within a given (in principle homogeneous) cell population, the measured D and M values are not constant but vary from one cell to another. From a strict statistical point of view, these values have to be averaged over a rather large number of cells (at least 30) in order to represent the cell population [35]. Since in general this requirement is not often met, a direct comparison among D and M values obtained by different groups and for different cellular systems can be risky. In contrast, FRAP experiments performed by a given group on a given cellular system provide a relative scale of D and M values from which conclusions can be drawn with more safety. In what follows, we will restrict our analysis of FRAP results to this type of comparison.

For the sake of clarity, all D values are expressed in the unity u = 10^{-9} cm$^2 \cdot$s^{-1}.

3. FLUORESCENT LIPID PROBES

As for any probe approach, the validity of the FRAP technique relies on the ability of the lipophilic probes to correctly report on the host membrane lipids. Many fluorophores (anthracene, oxa- and indo-carbocyanine, fluorescein, rhodamine, NBD) are used. They are attached either to long polymethylenic (alkyl/acyl) chains or to phospholipids (for chemical structure, see [19,36]). All these molecules share the same characteristic of being extrinsic probes which are supposed to randomly distribute within the constitutive membrane lipids. This is a crucial point which is not necessarily met. These probes can label different parts of the membrane depending on both their chemical structure [36] and the physical state (gel/liquid) of the host lipids [36,37].

Alternatively, a very attractive and promising way is to work with constitutive (intrinsic) lipids. The fluorophore is now introduced into the membrane lipids through the regular lipid metabolic pathway. Such an approach is being developed in our laboratory, using 9-(2-anthryl)-nonanoic acid [38]. This fluorescent and photoactivatable fatty acid (anthracene photodimerization) metabolically incorporates into the lipids of prokaryotic [39] and eukaryotic cells [40], and the anthracene-labeled lipids can be used for investigating both the lateral distribution [41–43] and the lateral motion [35,44,45] of lipids in membranes.

4. MEMBRANE REGIONALIZATION AND MACROCOMPARTMENTATION

During a number of important processes of cellular differentiation: sperm maturation and capacitation, early embryogenesis, tight junction formation in epithelia, highly polarized cells are able to overcome the randomizing effect of lateral diffusion and to localize certain membrane components to specific regions of the cell surface [26,32,33].

Thus, spermatozoa exhibit membrane regionalization, with different lipid and protein composition between the anterior and posterior head, midpiece and tail regions [26,33,46]. Correlatively, the lateral motion of the lipid probe C_{16}-diI inserted into the plasma membrane of ejaculated ram sperm vary from one cell surface region to the other with respective D and M values of 6.3 u and 58% in the anterior head, 5.9 u and 71% in the posterior head, 9.3 u and 44% in the midpiece and 4.8 u and 58% in the tail [47]. D and M also differ between testicular and ejaculated cells [47] and they vary during cell capacitation [48]. Nevertheless, bleaching experiments carried out on large areas of these various domains indicate that the lipid probes are free to exchange between head and midpiece and midpiece and tail [47].

Interestingly, D and M vary during sperm maturation. In mouse round pachytene spermatocytes and spermatids, the probe C_{16}-diI has diffusion rates of 1.77 u and 2.17 u with nearly complete recoveries of 85% and 80%, respectively. In contrast, stage 10–11 condensing spermatids and testicular spermatozoa exhibit slightly higher diffusion coefficients and large non-diffusing fractions both in the anterior (2.39 u, 59%; 2.85 u, 65%) and posterior (3.26 u, 58%; 3.31 u, 65%) head regions [46]. This progressive surface regionalization with changes in the diffusibility properties of lipids during spermatogenesis reflect changes in the plasma membrane organization. This can presumably be related to the membrane protein regionalization which can be inferred from ultrastructural studies carried out on mature cells [26,32,33].

As another example, embryos of the mollusc *Nassarius reticulatus* exhibit regional and temporal differences in the lateral motion of their plasma membrane lipids during the first three cleavage cycles [49]. In the uncleaved fertilized eggs, the probe C_{14}-diI

diffuses at similar rates in the animal and vegetal poles ($D \sim 4.5$ u). At the time when the polar lobe preceding first cleavage is being formed, D in the vegetal polar lobe area starts to increase. A significant difference (on average 30%) between the animal and vegetal zones of the egg is then observed. In all cases, the mobile fraction remains relatively constant at around 76%. At third cleavage, this animal-vegetal polarity becomes even more pronounced with D and M values of respectively 2.9 u and 51% in the four animal micromers and of 5 u and 78% in the four vegetal macromers. Superimposed upon this animal-vegetal membrane regionalization, D in the polar lobe membrane area shows a cell-cycle dependent modulation, with maximum (7–7.8 u) and minimum (5–5.9 u) values during the S phase and early mitosis, respectively. These observations are in agreement with previously observed ultrastructural heterogeneities in the *Nassarius* egg plasma membrane [49].

Xenopus eggs also exhibit an animal-vegetal plasma membrane polarity in terms of lipid mobility [50]. The probe HEDAF has D and M values of respectively 15 u and 49% in the animal pole and of 76 u and 44% in the vegetal pole of unfertilized eggs. This polarity is strongly ($>100\times$) enhanced upon fertilization. Two distinct macrodomains can now be discriminated: the animal half where the probe molecules are nearly completely immobilized ($D \ll 0.1$ u, $M \ll 5\%$); the vegetal half where D and M are only slightly affected with values of 44 u and 66%, respectively. The transition between the animal and vegetal domains is sharp and seems to coincide with the boundary between the presumptive ecto- and endoderm. The immobilization of lipids in the animal plasma membrane might arise from the fusion of cortical granules which are more numerous in this part of the cell. The large immobile fractions measured in these experiments also suggest the existence of lipid microdomains in the animal and vegetal membrane macrodomains [50].

Epithelia are composed of highly polarized cells having a surface membrane which is separated by tight junctions into apical and basolateral domains. These two membrane domains differ in terms of morphology, transport processes, ionic permeability, distribution of glycolipids and proteins and sensitivity to hormones and drugs ([51] and references cited therein). In accord with cell-to-cell phospholipid [52] and glycolipid [53–55] transfer experiments, FRAP data [57,58] show that for probe molecules located in the exoplasmic leaflet of the plasma membrane, confluent cell monolayers can be described as a mosaic of closed diffusion areas. In these macrodomains, which are clearly restricted to the apical and basolateral areas, lipids are free to diffuse ($M \sim 100\%$) but they do not flow from one cell to the next or from the apical to the basolateral membrane. They also diffuse faster in the apical than in the basolateral domain [57,58]. In contrast, FRAP [56] and lipid transfer [52] data suggest that lipids can exchange between the apical and basal domains of the plasma membrane in the cytoplasmic leaflet.

Tight junctions are believed to be the barrier responsible for such a membrane macrocompartmentation [52–57]. After disruption of cell contacts, probes located in the exoplasmic leaflet can move from the apical to the basolateral pole of the membrane [57]. In vascular endothelium, cell junctions have also been shown to reversibly affect overall plasma membrane organization [57]. When cell junctions are disrupted, the lateral diffusion coefficient of HEDAF is the same in both apical and basal domains (2 u). It regains its initial higher value (2.7 u) in the apical pole when cell contacts are restored [57].

The two monolayers of the membrane lipid bilayer provide another kind of membrane macrocompartmentation. Each layer displays different lipid composition [1–3] and spontaneous transfer (flip-flop) of lipid molecules from one leaflet to the other occurs at a very slow rate, unless the membrane is equipped with specific 'phospholipid flipases' [59]. All these notions are well illustrated by the membrane of erythrocytes. In human red blood cells, FRAP experiments carried out at 20°C indicate that lipid probes specifically inserted into the inner leaflet (NBD-PtdEtn, NBD-PtdSer) diffuse about 5-fold faster ($D \sim 5$ u) than those which stay in the outer leaflet (NBD-PtdCho, $D \sim 1$ u) [60]. In contrast, similar D_{in} and D_{out} values around 4 u were found for the NBD-PE probe in the membrane of turkey erythrocytes [61]. But, in this case, a mobile fraction of 80% was measured on both sides of the membrane at 20°C, which reduced to 70% in the outer leaflet and to 30% in the inner leaflet at 10°C [61]. Fluorescence polarization measurements with *cis* and *trans* parinaric acids did not indicate significant formation of gel phase in these membranes at low temperature [61]. In human erythrocytes, decreasing the temperature also resulted in a decrease in M [62] and in D [62,63].

Even though these results are not fully consistent with each other, they strongly suggest that the inner and outer lipid layers of the erythrocyte membrane have distinct dynamical properties. It is tempting to relate these differences to the well known transverse asymmetry of distribution of phospholipid head groups in this membrane [1–3] and to the larger cholesterol content [64,65] and higher viscosity [66–68] which exist in the outer layer as compared to the inner layer.

Similar results were obtained in the parasite *Schitosoma mansoni* [69]. This worm has a surface membrane composed of two tightly apposed bilayers. Fluorescence quenching and FRAP experiments indicate that different lipid probes (Fl-PE, C_{14}-dil, C_{18}-dil, C_{14}-Fl, C_{16}-Fl, C_{18}-Fl, C_{18}-Rh) partition differently between this tetra-layer system and that some of them can diffuse freely while the others exhibit

restricted lateral diffusion. The lipid probes which are free to diffuse (C_{18}-Fl: D = 3.6 u, M = 79%; C_{18}-Rh: D = 0.4 u, M = 79%) reside mainly in the external monolayer of the outer membrane. Lipids with restricted diffusion (C_{18}-dil: D = 4 u, M = 34%; Fl-PE: D = 0.4 u, M = 29%) are located mainly in the inner monolayers. Interestingly, changes in membrane organization occur as the parasite develops. In juvenile worms, the probes C_{18}-dil and C_{18}-Fl are mainly located in the outer layer of the external bilayer. They diffuse about 10-fold more slowly ($D \sim 0.3$ u) than in mature worms and both display large immobile fractions ($M \sim 30$–50%). This indicates lateral lipid asymmetry in this monolayer in the juvenile stage of the parasite as opposed to transverse asymmetry in the adult. Such lateral asymmetry might correspond to the coexistence of lipids in gel and liquid phases in that monolayer while the transverse asymmetry might reflect interaction of the internal monolayer with cytoskeleton components [69].

Finally, focal contacts, where the cell plasma membrane is in close contact with the substratum on which the cells grow, represent another type of membrane regionalization. In chicken fibroblasts, lipid and protein molecules diffuse slightly more slowly in that membrane area than in non-attached parts of the membrane. The mobile fraction of lipids remains unchanged (90–100%) while that of proteins considerably reduces (from 90% to 47%) [70].

5. LIPID MICRODOMAINS IN MEMBRANES

The cellular systems described above not only display surface membrane regionalization but probably membrane microcompartmentation since in the various delineated surface areas, mobile fractions of less than 100% are measured. Occurrence of lipid immobile fraction is not to be restricted to these examples but seems to be a common feature to many eukaryotic cells [71–84]. In many reports, these fractional recoveries are not discussed [71–74] and in what follows, we will restrict our analysis to those [75–84] which are subjected to interpretation. They illustrate various aspects of cell biology and all suggest the occurrence of lateral heterogeneities in membranes.

Fertilization is one of the early events in developmental biology. D and M for varoius lipid probes (NBD-PC, C_n-dil n = 10, 12, 14, 16 and 18, C_{18}-diO) inserted into the plasma membrane of mouse [75,76] and sea urchin [77,78] eggs appear to decrease upon egg fertilization. Thus, C_{18}-diO exhibits D and M values of 6 u and 90%, respectively, in unfertilized sea urchin eggs which reduce to 2.7 u and 80% in fertilized cells [78]. D and M vary from one probe to the other but in a non-correlative way, suggesting that depending on their chemical structure and chain length, these probes are located in different parts of the membrane [76,77].

FRAP studies on soybean protoplasts afford an interesting example of the effect of temperature on membrane organization [79]. D and M values for various lipid probes (NBD-PtdCho, NBD-PtdEtn, C_{18}-dil, C_{14}-dil, C_{12}NFlu) inserted into the plasma membrane were found to depend strongly on their chemical structure and concentration and on temperature. For example, at 18°C and for a probe concentration of 100 μg/ml, the lateral motion of NBD-PtdCho and NBD-PtdEtn is correctly described with two diffusion components, one fast ($D \sim 2$–3 u, $M \sim 30$–60%) and one slow ($D \sim 0.45$ u, $M \sim 20$–30%). Increasing the temperature to 37°C reduces the recoveries to a single fast component ($D \sim 10$ u, $M \sim 60$%) suggesting that two different diffusing species represent the partitioning of the lipid probes between two distinct lipid domains, one fluid-like and another gel-like [79].

Phorbol esters are well known tumor-promoting agents. In the plasma membrane of Madin-Darby canine kidney cells treated with such molecules, the phospholipid-like probe collarein reports the induction of a new lipid environment which is characterized by higher mobility ($D \sim 5$ u) and reduced mobile fraction ($M \sim 50$%) than in control cells (D = 2.88 u, M = 98.5%) [80]. Similar changes can be mimicked by modifying the cell shape by perturbation of the microfilamentous organization with cytochalasin B, or by alterating the substratum on which the cells adhere and grow. Phorbol esters, by themselves, have no effect on lipid diffusibility in membrane model systems. These results might reflect a membrane reorganization originating from a redistribution of peripheric and integral membrane proteins [80].

Rat heart myocytes afford an interesting cellular system which undergoes age-dependent changes in its lipid composition [81]. Thus, young cultures have 0.6, 10 and 0.42 nmol of sphingomyelin, phosphatidylcholine and cholesterol per μg of DNA, respectively. Old cultures display 100% more sphingomyelin, 20% less phosphatidylcholine and 50% more cholesterol. For the probe NBD-PE in the plasma membrane, aging is accompanied by a decrease in D from 4.9 u to 3.3 u and an increase in M from 57% to 74%. These changes in D and M values can be reversed by treatment of the cells with phosphatidylcholine liposomes which results in an alteration of both the phosphatidylcholine/sphingomyelin ratio and cholesterol content. This strongly suggests the existence of lipid microdomains, whose amount and possible composition would depend on the membrane lipid composition [81].

Neurons have a characteristic shape specialized for their integration functions. Concurrent with morphological regionalization, the cells of the nervous system display regionalization at the molecular level (localization of ionic currents, acetylcholine receptors, Na^+ channels) and a modulation of their functions in response to alteration of the lipid composition of the

plasma membrane ([82] and references cited therein). In *Aplysia* neurons, the probe NBD-PC exhibits similar D (3.7 u) and M (74%) values in different regions of the cell (cell body, hillock, axon). In contrast, fluidizing agents like ethanol and butanol selectively affect the diffusibility of the probes NBD-PC and Rho-PE. Increasing the temperature t from 4°C to 25°C did not significantly alter D for NBD-PC (3 u) but significantly increased its mobile fraction from 34% to 60%. This non-conventional relationship between D and t was even more pronounced for Rho-PE. As t increased, D decreased while M increased. As above, these observations suggest that membrane lipids are organized into domains of localized composition and viscosity. As t increases, immobile viscous lipid molecules are recruited into a mobile fraction, resulting in the maintenance of a nearly constant diffusibility [82].

Taking advantage of the possibility of metabolically incorporating 9-(2-anthryl)-nonanoic acid (see section 3) into the lipids of Chinese hamster ovary cells, the mobility of intrinsic lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were anthracene-labeled [40]) was investigated in comparison to that of extrinsic probes (Pam-aminofluorescein, ANno, ASte, acylANnoGroP-Cho). At 20°C, the diffusion coefficients for the extrinsic and intrinsic probes in the plasma membrane ranged over 1–2 u. Small but significant differences were observed between these various molecules reflecting the differences they exhibit in size and polarity. All the extrinsic probes were free to diffuse ($M \sim 100\%$). In contrast, a fractional recovery of only 75% was observed for the intrinsic anthracene-labeled phospholipids, suggesting that the anthracene fatty acid was metabolically incorporated into plasma membrane lipid regions, which were inaccessible to the extrinsic probes [44].

The data reported above indicate that the idea of 'bulk' or 'average' membrane fluidity is not valid for the various cellular systems studied. In addition to the fact that different lipid analogues probe different lipid microenvironments [36,37], they suggest that these plasma membranes are composed of lipid microdomains differing in composition and/or physical state. These microdomains might correspond to the coexistence of lipids in the gel and liquid states [76,78]. In this respect, most of the non-diffusing fraction which was observed in the plasma membrane of spermatozoa [46–48] was retained in lipid bilayers prepared from lipids extracted from these membranes [83]. As another possibility, lipid microdomains might originate from a protein lattice trapping lipid molecules and acting like a fence preventing lipids from laterally diffusing [13,44,80].

On account of the micrometric size of the illuminated spots in FRAP experiments, these lipid microdomains have been postulated to be sub- or micro-metric in scale [44,81]. In an attempt to elucidate further the nature and size of such lipid domains, Yechiel and Edidin have proposed an approach consisting in measuring D and M for lipids and proteins in membranes, as a function of the radius r_i of the illuminated area [84]. Although very interesting in principle, this method can be difficult to use due to various sources of experimental artifacts. In particular, it should be remembered that the larger the beam radius and the lower the numerical aperture of the objective used, the larger the depth of focus of the microscope [44,85,86] and therefore the greater the risk of integrating, in the bleaching step, a fluorescence signal from intracytoplasmic particles and organelles which may not participate in the recovery process [44]. In any case, in the plasma membrane of human fibroblasts, the mobile fractions measured for the lipid probe NBD-PC and for labeled membrane proteins decreased monotonically from 90% to 27% and from 66% to 22%, respectively, upon increasing the beam radius r_i from 0.35 to 5.0 μm. For NBD-PC, diffusion coefficients slightly increased from 0.9 u (r_i: 0.35 μm) to 2 u ($r_i > 2.5$ μm). When measured with a small beam radius of 0.35 μm, these D values distributed into two distinct populations suggesting that these small spots can sample different regions of the membrane with different diffusibility properties. Consequently, the fibroblast surface membrane would consist of protein-rich domains about 1 μm in diameter characterized by a low lipid diffusion coefficient, embedded in a relatively protein-poor and more mobile lipid continuum [84].

6. CONCLUSION

All the data reported above, which concern a large variety of cellular systems, clearly indicate that the FRAP approach can be of great help in elucidating membrane organization and dynamics. Many of the data presented correlate with ultrastructural studies (when they exist) to demonstrate the occurrence of membrane regionalization and macrocompartmentation in differentiated cells. These data, in themselves, enable such a conclusion to be reached.

Occurrence of large lipid immobile fractions in membranes, differential probe partitioning and diffusibility properties, temperature effects on lipid motion, differences between extrinsic and intrinsic probes strongly support the concept of a lateral asymmetry of distribution of lipids in these biological membranes. Despite many efforts, these membrane lipid microdomains are still poorly understood at a molecular level. Various structural models are proposed but all of them require further investigation before being clearly established. The use of intrinsic lipid probes and a more systematic study of membrane lipid composition in connection with lipid lateral and transverse distribution and dynamics should be of great help.

Investigating the topology and dynamics of membrane components is one aspect of the more general problem of how a living cell spatially controls the biogenesis of its various membrane fractions and temporally maintains their spatial organization in relation with cell polarity and functions. This remains a challenge of prime interest in cell biology.

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