CLATHRIN- AND NON-CLATHRIN-MEDIATED ENDOCYTIC REGULATION OF CELL SIGNALLING

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Abstract | The internalization of various cargo proteins and lipids from the mammalian cell surface occurs through the clathrin and lipid-raft endocytic pathways. Protein–lipid and protein–protein interactions control the targeting of signalling molecules and their partners to various specialized membrane compartments in these pathways. This functions to control the activity of signalling cascades and the termination of signalling events, and therefore has a key role in defining how a cell responds to its environment.

LIPID RAFTS
Rich in cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins and signalling molecules, these membrane microdomains, which are distinct from clathrin-coated pits, function as signalling platforms.

MORPHOGEN SYSTEM
In morphogen systems, signalling molecules are produced at a localized region and spread away from their source. In embryonic tissue, morphogens influence the movement and organization of cells by forming a concentration gradient.

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Proteins are organized into discrete regions within a cell and this has a crucial role in controlling the flow of information through signal-transduction pathways. Wide-ranging mechanisms regulate the localization of proteins, for example, peptide motifs, domains and post-translational modifications that control protein–protein and protein–lipid interactions. Furthermore, cell membranes are organized into a mosaic of assorted lipid molecules that include phosphoinositides and membrane microdomains that are known as LIPID RAFTS, which are particularly important for trafficking events. In this review, we focus on how the cell uses endocytosis and membrane composition — in particular, lipid rafts — to control cell signalling, with an emphasis on the epidermal growth factor (EGF) receptor (EGFR) and transforming growth factor-β (TGFβ) receptor (TGFβR) pathways. We then summarize some signalling pathways that are compartmentalized in specific membrane microdomains and discuss the potential role of endocytic pathways in two other MORPHOGEN SYSTEMS (Hedgehog (Hh) and Wingless/Int-1 (Wnt)).

The mosaic of cellular membranes

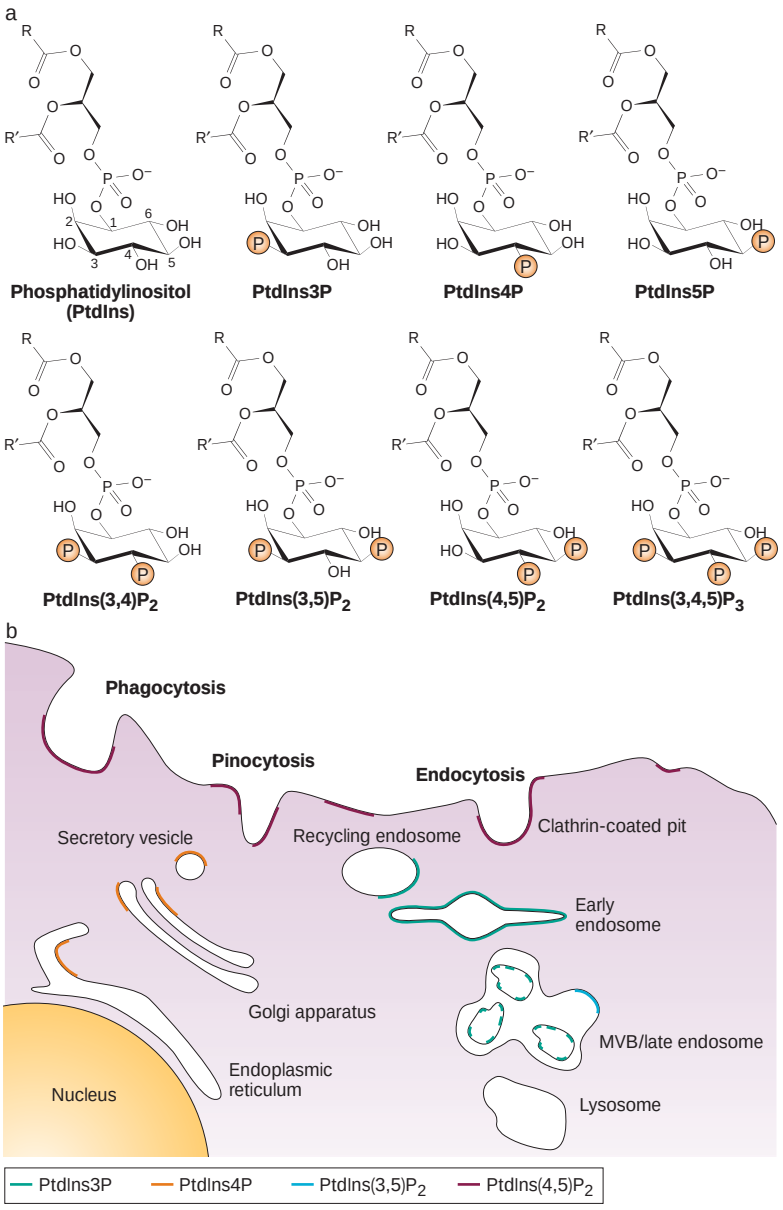
Phosphoinositides. Inositol phospholipids or phosphoinositides represent a small proportion of the phospholipids that are found in cell membranes, but they are involved in numerous intracellular events, such as cell proliferation, cell metabolism, cell death, cell motility, cytoskeletal rearrangement and membrane trafficking. Phosphoinositides can specifically interact with, and control the localization of, proteins that contain lipid-binding domains[1,2] (BOX 1; TABLE 1; for a fully referenced version of TABLE 1, please see online supplementary information S1 (table)). Moreover, the generation of certain phosphoinositides through the regulation of lipid-kinase and lipid-phosphatase activity — for example, PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) and PTEN (phosphatase and tensin homologue) activity, respectively — is under dynamic control, so phosphoinositides have crucial roles in assembling and controlling cell-signalling pathways (for a review, see REF. 3).

Lipid-raft microdomains. Lipid-raft microdomains are another type of membrane domain that compartmentalizes the membranes of living cells. Lipid rafts consist of a dynamic assembly of cholesterol and GLYCOSPHINGOLIPIDS that form liquid-ordered domains that float in the less-ordered liquid domains of the surrounding membrane[4]. Although lipid rafts of some form or another almost certainly exist, their size, lifetime, biogenesis, and lipid and protein composition remain indefinable and are outstanding questions in the field (BOX 2). Nevertheless, it is thought that lipid rafts probably cluster into large platforms that can segregate membrane components and, owing to their ability to recruit as well as exclude specific lipids and proteins, they have been implicated

Box 1 | Phosphoinositides

In vivo, there are seven distinct phosphoinositide species[1] (see figure, part **a**; phosphatidylinositol (PtdIns) can be phosphorylated at the 3, 4 and 5 positions, which gives rise to seven different phosphoinositides). This group of phosphoinositides fulfils its range of functions by interacting with specific protein domains that include the 'Fab1, YOTB, Vac1, EEA1' (FYVE) domain, the Phox-homology (PX) domain, the pleckstrin-homology (PH) domain, and the epsin-N-terminal-homology (ENTH) domain (TABLE 1). The FYVE domain has a high affinity for PtdIns-3-phosphate (PtdIns3P), and PX domains also bind to PtdIns3P, as well as to other phosphoinositides. PH domains interact with several phosphoinositide species with various affinities and specificities, whereas ENTH domains have a high affinity for PtdIns-4,5-bisphosphate (PtdIns(4,5)P_2; TABLE 1)[1]. The C2 (protein-kinase-C conserved region-2) domain is another phosphoinositide-binding motif that can interact with phospholipids in either a Ca^{2+}-dependent or -independent manner[124].

Through their restricted distributions, phosphoinositides can control the localization of phosphoinositide-binding proteins by targeting them to specific organelles. For example, PdtIns3P, which is abundant in the early endosome, binds FYVE-domain-containing proteins, such as early endosome antigen-1 (EEA1), and targets them to this compartment (TABLE 1). Part **b** of the figure shows phosphoinositides and membrane domains, and the localization of the various phosphoinositides is highlighted by different colours. PtdIns(4,5)P_2 is mainly distributed at the plasma membrane and has a role in endocytosis, PHAGOCYTOSIS and MACROPINOCYTOSIS. PtdIns3P is enriched in early endosomes and is apparently excluded from the plasma membrane. PtdIns-4-phosphate (PtdIns4P) is particularly abundant in the Golgi complex and in the endoplasmic reticulum, where it is important for the regulation of secretory trafficking and actin-cytoskeleton organization[2]. Finally PtdIns-3,5-bisphosphate (PtdIns(3,5)P_2) is present in multivesicular bodies (MVBs)/late endosomes, and in *Saccharomyces cerevisiae*, it is important for maintaining vacuole-membrane integrity, as well as membrane recycling and turnover[2].



in the regulation of various physiological processes[5], such as lipid sorting, protein trafficking, cell polarization[6] and signal transduction[4,7,8].

Partitioning proteins and signalling in lipid rafts. An important question is how are membrane proteins partitioned between lipid-raft and non-lipid-raft domains? Various studies have shown that the partitioning of proteins into lipid rafts is dependent on direct interactions of glycosylphosphatidylinositol (GPI)-anchored proteins, lipidated proteins and transmembrane proteins with raft lipids, as well as on protein–protein interactions with lipid-raft-resident components (BOX 3). Partitioning could help the nucleation of specific signalling networks in lipid rafts, and there is considerable evidence to indicate that lipid rafts can function as platforms for signalling receptors and their downstream targets[4,9]. Indeed, lipid rafts are crucial for the effective activation of T-cell-receptor-dependent signalling cascades[10], for B-cell-receptor signalling[11], and for the insulin-mediated translocation of the glucose transporter GLUT4 (REF. 12).

Another example is the glial-cell-derived neurotrophic factor (GDNF), a distant member of the TGFβ family that signals through the transmembrane tyrosine kinase RET and the GPI-anchored co-receptor GFRα1 (GDNF-family receptor-α1), which is present in lipid rafts. GDNF binding to GFRα1 induces RET translocation to lipid rafts, which facilitates GDNF-induced intracellular signalling[13]. Interestingly, GFRα1 also interacts with a transmembrane isoform of neuronal-cell-adhesion molecule ($p140^{NCAM}$) and

PHOSPHATIDYLINOSITOL 3-KINASES (PI3Ks). Type-I and -II PI3Ks phosphorylate the D-3 position of PtdIns4P and PtdIns(4,5)P_2, respectively. Type III PI3Ks phosphorylate the D-3 position of PtdIns. Type-I PI3Ks are thought to be involved in numerous signal-transduction and membrane-trafficking systems.

Table 1 | Examples of phosphoinositides and their binding partners*

Phosphoinositide	Phosphoinositide-binding protein domain	Example proteins containing a phosphoinositide-binding domain	Membrane localization
PtdIns3P	FYVE	EEA1	Early endosomes
		HRS	Early endosomes
		SARA	Early endosomes
	PX	SNX3	Early endosomes
PtdIns(4,5)P_2	PH	PLCδ1	ND
	PH	Oxysterol-binding protein	Golgi apparatus
	ENTH	Epsin	Clathrin-coated pits
	ANTH	AP180	Clathrin-coated pits
	α-subunit	AP2	Clathrin-coated pits
	PH	Dynamin	ND
PtdIns(3,4,5)P_3	PX	CISK	Early endosomes
	PH	Oxysterol-binding protein	Golgi apparatus
	PH	AKT/PKB	ND
PtdIns(3,4)P_2	PH	AKT/PKB	ND
PtdIns(3,5)P_2	PX	CISK	Early endosomes
PtdIns4P	PH	FAPP1	ND

*For a fully referenced version of this table, please see online supplementary information S1 (table). ANTH domain, AP180 N-terminal-homology domain; AP2, adaptor protein-2; AP180, adaptor protein-180; CISK, cytokine-independent survival kinase; EEA1, early endosome antigen-1; ENTH domain, epsin N-terminal-homology domain; FAPP1, phosphatidylinositol-4-phosphate adaptor protein-1; FYVE domain, 'Fab1, YOTB, Vac1, EEA1' domain; HRS, hepatocyte-growth-factor-regulated tyrosine-kinase substrate; ND, not determined; PH domain, pleckstrin-homology domain; PKB, protein kinase B; PLCδ1, phospholipase Cδ1; PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(3,4)P_2, phosphatidylinositol-3,4-diphosphate; PtdIns(3,5)P_2, phosphatidylinositol-3,5-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; PX domain, Phox-homology domain; SARA, SMAD anchor for receptor activation; SNX3, sorting nexin-3.

induces its accumulation in lipid rafts. As GFRα1 is required for GDNF-induced p140^{NCAM} signalling through Fyn and FAK (focal adhesion kinase), which are also found in lipid rafts, these data indicate that GFRα1 promotes signalling by controlling the raft partitioning of p140^{NCAM} (REF. 14). Numerous reports have also uncovered important roles for lipid rafts in pathways such as: H-Ras-mediated Raf activation[7,8]; the tumour necrosis factor-α (TNFα)-mediated nuclear factor (NF)-κB activation that is induced by TNF receptor-1 (TNFR1) and other downstream components[15]; the CD3/CD28-co-stimulation-induced NF-κB activation that is induced through CARMA1 and other proteins[16]; and interleukin-6 (IL-6)- and interferon-γ (IFN-γ)-induced signal transducer and activator of transcription (STAT) signalling[17]. Taken together, these data clearly show that membrane microdomain lipid rafts, in which various signalling molecules are localized, represent important sites for organizing and transducing responses to extracellular signals.

Endocytosis

Endocytosis or uptake is characterized by the internalization of molecules from the cell surface into internal membrane compartments, and vesicular trafficking can be divided into two main pathways — the classic, clathrin-mediated endocytic pathway (BOX 4) and the non-classic, clathrin-independent, but lipid-raft-dependent route (BOX 5).

The clathrin-dependent pathway. Clathrin-mediated endocytosis (BOX 4), which is responsible for the internalization of nutrients, pathogens, antigens, growth factors and receptors, is the most well-characterized mechanism for the entry of molecules into cells. Adaptor-protein complexes are key components of this pathway that bind directly to clathrin, other endocytic regulatory proteins and cargo to stimulate the formation of the clathrin coat. Adaptor protein-2 (AP2) is one such adaptor that mediates the constitutive endocytosis of cargo proteins that contain tyrosine- or di-leucine-based motifs[18], for example, the TRANSFERRIN RECEPTOR[19]. By contrast, EGFR internalization is more dependent on the clathrin-interacting adaptor, epsin, and its partner proteins EGFR-pathway substrate-15 (EPS15) and EPS15-related protein (EPS15R)[18]. These adaptors assemble to form a complex that is regulated by the EGFR-dependent phosphorylation of EPS15, as well as by the ligand-dependent induction of epsin and EPS15 MONOUBIQUITYLATION[20]. Monoubiquitylation, which was initially found to be important in *Saccharomyces cerevisiae* trafficking[21], has emerged as an important signal in mammalian endocytic pathways[22]. The monoubiquitylation of epsin and EPS15, which can be mediated by the NEDD4 ubiquitin ligase, requires the ubiquitin-interacting motif (UIM) of epsin and EPS15. The UIM also binds to monoubiquitin, which possibly restricts ubiquitin-chain elongation[20]. Furthermore, the UIM also facilitates binding to monoubiquitylated cargo and cooperates with other protein-interaction domains in the endocytic machinery

PTEN
(phosphatase and tensin homologue). PTEN dephosphorylates PtdIns(3,4,5)P_3 and PtdsIns(3,4)P_2 at the D-3 position, and is a negative regulator of PI3K signalling.

GLYCOSPHINGOLIPIDS
Lipids that contain at least one monosaccharide residue and either a sphingolipid or a ceramide (*N*-acylated sphingoid).

PHAGOCYTOSIS
Phagocytosis is a process that is used by cells to internalize large particles such as debris, apoptotic cells and pathogens. The internalized particles can be stored or degraded by cells.

MACROPINOCYTOSIS
Macropinocytosis is a form of regulated endocytosis that involves the formation of large endocytic vesicles after the closure of cell-surface membrane ruffles.

TRANSFERRIN RECEPTOR
The transferrin receptor is the archetypical cargo for internalization through clathrin-mediated endocytosis. At the cell surface, it binds its ligand (ferrotransferrin) and is internalized into early endosomes, where it releases the iron and then recycles back to the plasma membrane.

(MONO)UBIQUITYLATION
A highly conserved 76-amino-acid protein is covalently attached to a lysine residue in the target protein. Ubiquitylation requires ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes that are responsible for selecting targets for ubiquitin modification. Two families of E3s are the HECT-domain-containing enzymes (such as SMURFs and NEDD4) and RING-domain-containing enzymes (such as Cbl).

NEDD4
(neuronal-precursor-cell-expressed developmentally downregulated-4). The prototypical protein in a family of E3 ubiquitin ligases that contain a HECT (homologous to E6-AP C terminus) catalytic C-terminal domain and WW and C2 domains that are involved in substrate recognition and cellular localization.

Box 2 | Outstanding questions in the lipid-raft field

Lipid rafts and their associated proteins have been revealed by fluorescence resonance energy transfer[123,125], chemical crosslinking[126], immunofluorescence[127] and biochemical isolation in a low-buoyant-density, detergent-resistant membrane fraction[4]. Analysis of detergent-resistant membranes must be interpreted cautiously, as their lipid and protein composition might be dependent on the type of detergent that is used for the extraction[128]. Therefore, it would seem that the outstanding questions surrounding lipid rafts should not focus on whether they exist, but rather on understanding their biogenesis, size, stability, and lipid and protein composition[8]. Many models for lipid rafts have been put forward. One model proposes that lipid rafts arise owing to the spontaneous association of cholesterol and sphingolipids to form membrane platforms that isolate proteins[4]. A second model proposes that lipid rafts are constructed of shells of just a few lipids that assemble around proteins to form a molecular 'address', which targets the protein–lipid assembly to liquid-ordered lipid-raft domains[129]. Assembly into larger domains is proposed to be dependent on the propensity of such shells to assemble into higher-order structures, and could therefore account for both the clustered and non-clustered distributions of lipid-raft-bound proteins[129]. In a third model, lipid rafts are proposed to be small, unstable membrane complexes that contain at least three molecules. In this model, the lipid rafts can be merged into large-scale stabilized structures[9]. In a fourth model, lipid rafts are small, dynamic, pre-existing lipid assemblies in which protein monomers exist, and these rafts can give rise to large-scale stable lipid rafts[5]. The different datasets that have given rise to these models might reflect the fundamental dynamics of raft size, assembly, stability and composition that would accommodate all of these models, so the models might not be mutually exclusive.

to drive the assembly of multiprotein endocytic complexes that promote clathrin-coated-pit formation. The latter activity is also dependent on interactions with clathrin and AP2, as well as on interactions with the phospholipid bilayer through the phosphatidylinositol (PtdIns)-4,5-bisphosphate (PtdIns(4,5)P_2)-binding ENTH (epsin-N-terminal-homology) domain of epsin[23], which can facilitate vesicle formation by actually bending the phospholipid bilayer.

After endocytosis, receptors enter the early endosome where they are sorted, and they are either recycled back to the plasma membrane or are degraded in the late endosome/LYSOSOME. Recycling through RAB11-positive endosomes can be facilitated by the dissociation of ligand–receptor complexes in the early endosome[24], or can be directly regulated — as in the case of the protein kinase C (PKC)-dependent phosphorylation of EGFR, which promotes recycling[25]. With respect to degradative sorting, sustained MULTIUBIQUITYLATION of receptors in the endosome is a key signal that is recognized by the UIM-containing protein HRS (hepatocyte-growth-factor-regulated tyrosine-kinase substrate; also known as HGS), which is required to sort cargo to ESCRT-I (endosomal sorting complex required for transport-I). ESCRT-I, through ESCRT-II and -III, subsequently directs cargo into the intralumenal vesicles of MULTIVESICULAR BODIES (MVBs)[26], which results in signal termination and receptor degradation in the lysosome. HRS functions in a ternary complex with EPS15 and STAM (signal transduction adapter molecule)[27], has a PtdIns-3-phosphate (PtdIns3P)-binding 'Fab1, YOTB, Vac1, EEA1' (FYVE) domain that is important for its early endosomal localization, and can recruit clathrin to early endosomes[28]. HRS is also phosphorylated after receptor tyrosine kinase (RTK) activation and it stimulates the assembly of the ESCRT-I complex through interactions with the tumour-susceptibility gene-101 (TSG101) subunit of this complex[29,30]. Although TSG101 is predominantly localized on the limiting membrane of MVBs, overexpression of HRS can lead to the accumulation of TSG101 on early endosomes[30], which indicates that ESCRT-I complexes might assemble early in the endocytic pathway. Furthermore, MVB formation is dependent on ESCRT-I (REF. 29), which supports the idea that HRS and ESCRT-I mediate the biogenesis, from the early endosome, of an intermediary early multivesicular endosome that matures into an MVB[30,31]. By linking MVB biogenesis to receptor sorting into intralumenal vesicles, HRS therefore provides a rapid way to terminate signalling from endosomes. From this synopsis, it is clear that numerous components of the endocytic machinery are directly regulated by receptor-dependent phosphorylation and ubiquitylation, and that phospholipids such

UIM
(ubiquitin-interacting motif). A module of 20 amino acids that specifically recognizes ubiquitin. It is present in endocytic proteins (such as EPS15 and HRS) and in proteasomal components.

LYSOSOME
A type of organelle that is characterized by a low internal pH, contains hydrolytic enzymes and is involved in the post-translational maturation of proteins, the degradation of receptors and the extracellular release of active enzymes.

RAB PROTEINS
Ras-like small G-proteins that control trafficking, exocytosis, endocytosis and endosome fusion. They can be modified by geranylgeranyl groups, are tightly associated with membranes and can be specifically localized to different compartments (for example, Rab5 is localized to early endosomes).

MULTIUBIQUITYLATION
The addition of several monoubiquitin molecules to a target protein. (As opposed to polyubiquitylation, in which a single chain of several ubiquitin molecules is appended to the target protein.)

Box 3 | The partitioning and targeting of proteins in lipid rafts

An interesting area in the study of lipid-raft function in cell signalling is elucidating and defining how proteins are targeted to lipid rafts. Among these proteins are cell-surface glycosylphosphatidylinositol (GPI)-anchored proteins[4], which are tethered to lipid rafts through interactions between the long, saturated ACYL CHAIN on the GPI anchor and sphingolipids and cholesterol in the lipid rafts[130]. Intracellular lipidated proteins are also found in lipid rafts — for example, non-receptor tyrosine kinases, heterotrimeric G proteins and Ras[105,123]. However, lipid modifications alone might not be sufficient to direct all proteins to lipid rafts, and can work in conjunction with protein conformation[8] and protein–protein interactions[131]. Binding to lipid-raft-resident proteins such as caveolin, FLOTILLIN and ANNEXIN is another targeting mechanism. For example: caveolin, through its scaffolding domain[105], targets proteins to lipid rafts; flotillin binds to the sorbin-homology (SoHo) domain in Cbl-associated protein (CAP) and vinexin-α, and recruits proteins into flotillin-containing lipid rafts[132]; annexin-II has been implicated in the apical targeting of sucrase isomaltase[6]; and annexin-VIIIb mediates the lipid-raft apical-membrane localization of the C2 (protein-kinase-C conserved region-2)-domain-containing ubiquitin ligase NEDD4 (REF. 133). Finally, raft targeting might also be intrinsically encoded in the protein, as is the case for the transmembrane domain of the influenza virus haemagglutinin protein and the extracellular juxtamembrane region of the epidermal growth factor receptor[52]. Combining all of these observations with emerging models of dynamic lipid-raft assembly (BOX 2) indicates a possible model in which lipid-raft coalescence and stability is dependent not only on the inherent propensity of raft lipids to cluster, but also on specific protein–protein interactions between lipid-raft-associated proteins. This cooperativity between lipid and protein interactions could function to assemble cell-signalling networks into ordered structures with defined components that are poised to transmit extracellular signals.

ESCRT
(endosomal sorting complex required for transport). The multiprotein ESCRT machinery (ESCRT-I, -II and -III) promotes inward vesiculation at the limiting membrane of the sorting endosome, and selects cargo proteins for delivery to the intralumenal vesicles of multivesicular bodies.

MULTIVESICULAR BODIES
Endocytic intermediate organelles in the lysosomal degradative pathway that contain small vesicles and are surrounded by a limiting membrane.

EEA1
(early endosome antigen-1). EEA1 is a membrane-bound, FYVE-domain-containing protein that binds PtdIns3P. It is an effector of the small GTPase Rab5 that controls early-endosome fusion dynamics.

ACYL CHAIN
An acyl chain is a carbonyl group with an alkyl group attached.

FLOTILLIN
Flotillins are integral membrane proteins and constituents of lipid rafts. Flotillin-1 and -2 were originally discovered in neuronal cells as Reggie-2 and -1, respectively, and they are thought to be involved in insulin-receptor and T-cell-receptor signalling.

ANNEXIN
A family of Ca^{2+}- and phospholipid-binding proteins that are found in lipid rafts. They are involved in membrane-trafficking events and in the organization of membrane compartments and the plasma membrane.

CAVEOLIN
These integral membrane proteins have a central hydrophobic domain that forms a hairpin loop inside the membrane to leave both C- and N-terminal domains facing the cytosol.

SIMIAN VIRUS 40
A member of the papilloma-, polyoma- and vacuolating-virus family of non-enveloped DNA viruses. At the cell surface, the virus probably binds the major histocompatibility (MHC) class-I antigen receptor, but whether this mediates its endocytosis into caveolae is unknown.

Box 4 | The classic clathrin-dependent endocytic pathway

The central defining feature of the classic clathrin-dependent endocytic pathway is the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. The clathrin triskelia assemble into a polygonal lattice at the plasma membrane to form coated pits that bud and pinch off from the membrane in a dynamin-dependent manner and give rise to clathrin-coated vesicles[134,135]. Clathrin-binding adaptors, such as adaptor protein-2 (AP2), bind to clathrin directly to initiate this process, and they also bind to cargo proteins and thereby mediate their endocytosis. In addition, phospholipids, such as phosphatidylinositol-4,5-bisphosphate, are also found in coated pits and they facilitate vesicle formation and budding by binding to clathrin adaptors such as epsins. Clathrin-coated vesicles are uncoated after endocytosis and then fuse with the early endosome. The early endosome is highly enriched in phosphatidylinositol-3-phosphate (PtdIns3P) and has unique protein constituents, such as the PtdIns3P-binding 'Fab1, YOTB, Vac1, EEA1' (FYVE)-domain proteins, which can bind to and control the activity and destination of proteins in the compartment. The early endosome is therefore a key control point for sorting receptors, which can be directed to Rab11-positive recycling endosomes and back to the cell surface, or are directed to the intralumenal vesicle of multivesicular endosomes, and therefore to the multivesicular body, late endosome and lysosome for degradation.

Two theories have been proposed for how this pathway is organized. In the 'maturation model', vesicles that are derived from the plasma membrane give rise to a *de novo*, temporary early endosome that matures to become a transient late endosome and then a degradative lysosome[136]. In the 'pre-existing-compartment model', the early endosome and late endosome are stable compartments that are connected by vesicular traffic that moves cargo through the pathway[137]. Importantly, non-clathrin endocytic pathways can also deliver certain molecules to endosomes and lysosomes[46,138], and clathrin pathways can target cargo molecules to other compartments, such as the Golgi, too[31].

as PtdIns(4,5)P_2 might be generated locally to promote clathrin-coated-pit formation. So, protein–protein and protein–lipid interactions that are coupled to the distinct lipid composition of different endocytic compartments are important for sorting cargo molecules in the clathrin-mediated endocytic pathway and intimately link cell signalling to the endocytic machinery.

Non-classic endocytosis pathways. Although their existence has been controversial for a long time, efforts at present are focused on elucidating the molecular machinery that is involved in non-classic endocytic pathways (BOX 5). These internalization routes are not well characterized, but they are exquisitely sensitive to cholesterol depletion[32]. Furthermore, the depletion of rafts in clathrin-coated pits[33,34], together with the general observation that rafts and raft-associated proteins internalize through non-clathrin pathways[34,35], has led to the idea that these alternative endocytic pathways are raft-dependent, although this has not been formally shown. One important non-clathrin endocytic route involves the raft-resident protein CAVEOLIN, which induces the formation of caveolae at the cell surface (BOX 6). Motile caveolin-positive vesicles have been identified and the rate of caveolar endocytosis has been shown to be dependent on a balance of caveolin-1 and the raft lipids (that is, cholesterol and glycosphingolipids)[36] — overexpressing caveolin-1 reduces caveolar endocytic rates[37], whereas increasing raft-lipid levels accelerates internalization rates[36]. Interference with the actin cytoskeleton also impairs the caveolae-dependent internalization of GPI-anchored alkaline phosphatase[38] and SIMIAN VIRUS 40 (SV40)[39], and a study of the dynamics of the caveolar system has shown that the relatively low motility of caveolae at the cell surface is dependent on cortical actin filaments, whereas the rapid movement of caveolin-positive vesicles (cavicles; also known as caveosomes) in the cytoplasm is dependent on the microtubule network[40]. Most caveolin-positive vesicles are segregated from the classic endosomal compartments[41], and the trafficking of components through this pathway is regulated by non-RTK activity and is dependent on PKCα activity[36,42]. Furthermore, a recent report indicates that EGF stimulates the caveolin-dependent internalization of E-cadherin, which is consistent with the idea that the activity of this pathway is regulated[43]. However, it is important to point out that the caveolin pathway is one division of the non-clathrin endocytic pathways and that in the absence of caveolin, lipid-raft-dependent trafficking occurs[44]. Furthermore, these caveolin-independent pathways have been shown to mediate the internalization of interleukin-2 receptor-β (IL2Rβ)[45] (see below) and can direct some GPI-anchored proteins to the Rab11-positive recycling endosome[46]. Therefore, non-clathrin endocytic pathways can crosstalk with components of the classic endosomal system. Indeed, recent data have shown that caveolin-positive vesicles can interact with early endosomes in a Rab5-dependent process[41]. However, the biogenesis of non-clathrin endocytic vesicles, the identity of the proteins that modulate their trafficking and how the activity of these systems is controlled, are key unanswered questions in the field.

Trafficking and the control of signalling

It has long been assumed that clathrin-dependent endocytosis is the main pathway that is involved in the down-regulation of cell-surface receptors such as EGFR[47]. However, studies on several cell-surface receptors have made it clear that many receptor systems are endocytosed through non-clathrin pathways. Moreover, both types of pathway are used to internalize TGFβRs. Cells therefore use various internalization pathways to control cell-surface receptors, and this is crucial for regulating cell signalling, receptor turnover and the magnitude, duration and nature of signalling events.

Box 5 | Non-classic endocytic pathways

The recent development of new techniques, reagents and markers has provided new insights into non-clathrin-mediated internalization pathways. For example, the uptake of Simian virus 40 (REF. 110), cholera-toxin B subunit, glycosylphosphatidylinositol–green-fluorescent-protein fusion proteins[34,111,112], transforming-growth-factor-β receptors[94], interleukin-2 receptor-β (REF. 45) and autocrine-motility-factor receptor[108] from the cell surface is not inhibited by interfering with clathrin-mediated endocytosis (FIG. 3). Clathrin-independent internalization routes are sensitive to cholesterol depletion, which has led to the idea that they are lipid-raft dependent — a concept that is further supported by the fact that many lipid-raft-bound components seem to be endocytosed through non-clathrin pathways. Non-clathrin pathways seem to be further subdivided between those that are dynamin-GTPase dependent and those that are dynamin-GTPase independent. In contrast with clathrin-mediated endocytosis, almost nothing is known about the machinery that regulates the biogenesis of vesicles in these non-classic routes. However, the lipid-raft-resident protein caveolin might have an important role in a subset of these pathways — as indicated by the identification of motile caveolin-1-positive vesicles (caveosomes) that do not accumulate transferrin or FLUID-PHASE MARKERS and do not contain markers of endosomes, lysosomes, the Golgi apparatus or the endoplasmic reticulum (ER)[34,110–112]. Non-clathrin endocytic pathways can deliver molecules to various intracellular compartments that include the Golgi apparatus and the ER[34,110–112], as well as to classic endocytic compartments, such as the recycling endosome[46].

EGFR

The RTK family controls the proliferation, differentiation, cell survival, migration and adhesion of a wide range of cell types. The mechanisms that underlie, and functions that are fulfilled by, the trafficking of this class of receptor are most well understood for the EGFR system. In this system, EGF binding induces the dimerization of EGFR[48] and the *trans*-autophosphorylation of tyrosine residues in the cytoplasmic domain of EGFR, which then form docking sites for Src-homology-2 (SH2)- or phosphotyrosine-binding-domain-containing downstream effector proteins. The latter proteins function to assemble a signalling network that regulates the biological response to the ligand[18].

EGFR and lipid rafts. EGFRs have been found to localize in caveolae and GM1-containing lipid rafts[49,50]. A range of reports have been published on the proportion of EGFR that is found in rafts, with levels that range from 60% (REF. 49) to just a few percent[51]. Given the reported dynamics of lipid-raft size and lifetime, coupled to a lack of understanding regarding how the extraction methods affect protein associations with lipid rafts, these disparities are perhaps not surprising. The localization of EGFRs within or outside lipid rafts is an important regulator of ligand binding — cholesterol depletion increases EGF binding, whereas cholesterol loading decreases it, and this difference is caused by a change in the number of EGFRs that are available for ligand binding, rather than by a change in their ligand-binding affinity[50]. The localization of EGFR to rafts is mediated by a specific extracellular sequence in EGFR[52] and once stimulated with ligand, the level of EGFR in rafts decreases[49]. Interestingly, this trafficking event has been shown to be dependent on RTK activity, which indicates that it is coupled to signal initiation, and it is modulated by Src kinase and PKC activities[49], which are important in caveolar trafficking. These data indicate a model in which EGF signal initiation induces EGFR exit from rafts[49,53] and clathrin-dependent internalization (FIG. 1). However, the recent elucidation of a non-clathrin-dependent trafficking route for EGFR indicates that further endocytic pathways might also be used (S. Polo and P. P. Di Fiore, personal communication).

EGFR sorting and clathrin-mediated endocytosis. The EGFR is an extensively studied model of cell-signalling-receptor internalization through clathrin-mediated endocytosis that occurs within minutes of ligand stimulation. The internalization of EGFR is mediated by Cbl, a RING-finger E3 ubiquitin ligase that persistently binds to activated EGFR, as well as to many other receptors, to promote sustained multiubiquitylation of the receptor[22,54]. This multiubiquitylation targets EGFR for endocytosis and subsequent sorting to the MVB[22]. Cbl can also promote endocytosis together with CIN85 (Cbl-interacting protein of 85 kDa) and endophilins that possess BAR ('Bin, amphiphysin, Rvs') domains, which induce membrane curvature and help in the fission of clathrin-coated buds from the membrane[55,56] (FIG. 1). Cbl is therefore an important component in EGFR trafficking, although EGFR is by no means passive cargo and actively participates in its own trafficking by directly regulating the endocytic machinery. Indeed, clathrin is phosphorylated by EGFR and this induces the redistribution of clathrin at the cell periphery[57]. Furthermore, the phosphorylation of EPS15 by the receptor is essential for EGFR internalization[58], and the monoubiquitylation of both EPS15 and epsin is also regulated by EGFR[20]. As both EPS15 and epsin are required for EGFR internalization, they probably function as a complex during EGFR internalization[59]. However, a further twist to the story is that monoubiquitylated epsin-1 loses its capacity to bind to PtdIns(4,5)P_2 and to interact with AP2 and clathrin, which indicates that ubiquitylation might also promote the release of internalized EGFR

Box 6 | Caveolae

Caveolae, which are a morphologically identifiable type of lipid raft[105], are flask-like invaginations of the plasma membrane that contain sphingolipids, cholesterol and the caveolin proteins[139]. Caveolin proteins bind fatty acids and are modified by palmitoylation, which is required for cholesterol binding and for the proper transport of caveolin proteins to the plasma membrane[105]. Moreover, caveolin-1 has been shown to be important for the formation of caveolae, as caveolin-1-null mice lack caveolar structures[105,140]. Furthermore, in cells that are deficient in caveolin-1, the re-introduction of caveolin-1 leads to the creation of caveolae[141]. The formation of caveolae is also dependent on cholesterol, which indicates that caveolin induces lipid rafts to assemble into these membrane invaginations[105]. It is also important to note that structures that are morphologically similar to caveolae have been observed in some cells that lack caveolin proteins[53,142], which indicates that the formation of caveolae is not exclusively regulated by caveolin.

FLUID-PHASE MARKERS
Markers of fluid-phase endocytosis, which presumably does not involve receptor-mediated trafficking. These markers include proteins such as horseradish peroxidase and dextran.

from clathrin adaptors[18]. It would be interesting to precisely define where and when the monoubiquitylation of specific proteins takes place during EGFR trafficking.

After internalization into the early endosomes, receptors can be sorted to the recycling endosome, from which they travel back to the cell surface (FIG. 1). Alternatively, together with EPS15, they can form a ternary complex with STAM and HRS[27] that directs them to TSG101 and the ESCRT complexes and then into the intralumenal vesicles of multivesicular endosomes, which results in signal termination[18,60] (FIG. 1). Ubiquitylated HRS is also phosphorylated in response to the activation of EGFR and other RTKs, although the function of this phosphorylation has not been well defined[61]. All of these data indicate that clathrin-mediated endocytosis together with phosphorylation, multiubiquitylation and the specialized composition of endocytic membranes, function cooperatively to control the fate of EGFR and many other receptor systems that traffic through the clathrin pathway.

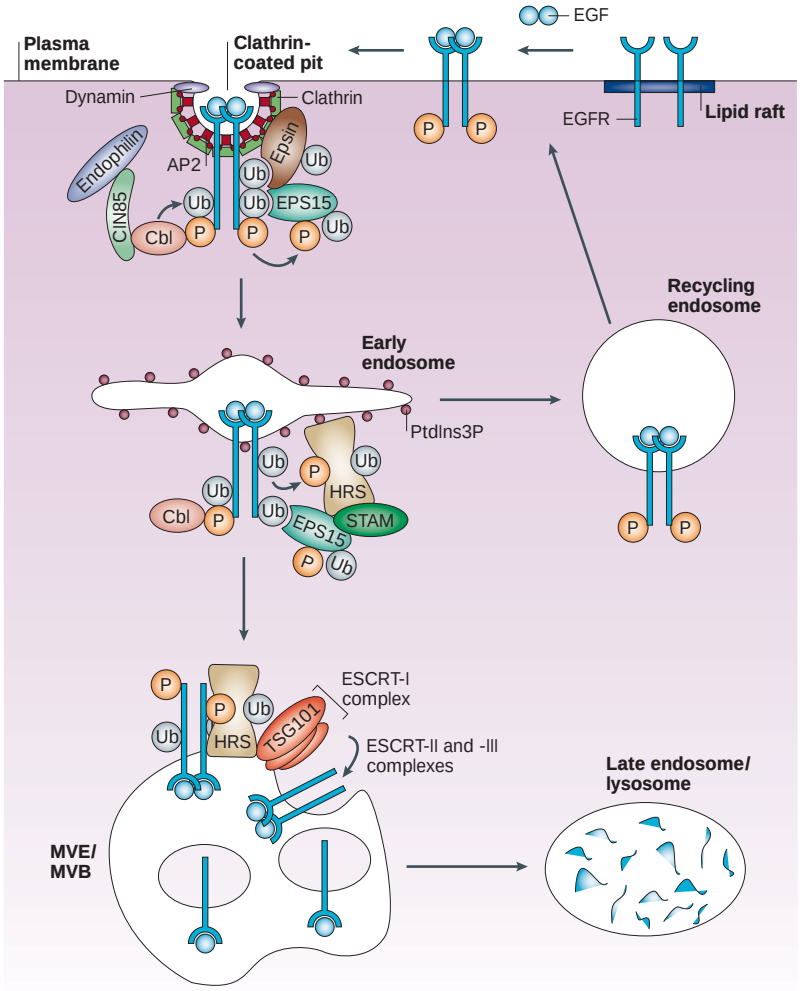


Figure 1 | **Epidermal-growth-factor-receptor trafficking.** From the cell surface, epidermal growth factor (EGF)-bound EGF receptors (EGFRs) might move out of lipid rafts. They are then internalized into clathrin-coated pits. Phosphorylated EGFRs recruit the E3 ubiquitin ligase Cbl, which multiubiquitylates EGFR and associates with Cbl-interacting protein of 85 kDa (CIN85) and endophilins. EGFR phosphorylates and induces the monoubiquitylation of EGFR-pathway substrate-15 (EPS15) and epsin, the latter of which interacts with adaptor protein-2 (AP2), clathrin and phosphatidylinositol-4,5-bisphosphate at the plasma membrane (not shown). Epsin and EPS15 might bind to monoubiquitylated EGFRs through their ubiquitin-interacting motif. In early endosomes, hepatocyte-growth-factor-regulated tyrosine-kinase substrate (HRS) binds to phosphatidylinositol-3-phosphate (PtdIns3P) through its 'Fab1, YOTB, Vac1, EEA1' (FYVE) domain, and forms a ternary complex with signal transduction adaptor molecule (STAM) and EPS15 that interacts with EGFRs. Ubiquitylated HRS is also phosphorylated after receptor-tyrosine-kinase activation. From the early endosomes, EGFRs are recycled back to the cell surface or are sorted towards the multivesicular endosome/multivesicular body (MVE/MVB). At the MVE/MVB, HRS interacts with tumour susceptibility gene-101 (TSG101), a component of endosomal sorting complex required for transport-I (ESCRT-I). This interaction leads EGFRs to ESCRT-II and -III and to the intralumenal vesicles of MVEs/MVBs, and subsequently to late endosomes/lysosomes where they are degraded. P, phosphate; Ub, ubiquitin.

EGFR signalling during trafficking. The complexity of the signalling networks and biological responses that are linked to RTKs might indicate that trafficking could have a significant role in controlling the nature of pathway activation and therefore the biological response that extends beyond the simple downregulation of the receptor. However, although plenty of studies have clearly shown that endocytic pathways have a crucial function in downregulating EGFR, it has been surprisingly difficult to pin down whether trafficking *per se* controls the nature of the molecular response to RTK, and in particular EGFR, activation[62,63]. Early studies with an EGFR that is defective for internalization clearly showed that cell-surface EGFR could mediate signals that induce mitotic responses and induce the transformation of cells at low doses of EGF[64]. Such studies also showed that the inhibition of endocytosis does not result in the attenuation of biological effects such as cell proliferation[65,66]. Nevertheless, EGFR analysis in the presence of active endocytosis has shown that endosomally localized EGFR associates with many, if not all, of its downstream effectors, such as SHC (SH2-domain-containing transforming protein) and GRB2[18,67]. Furthermore, this leads to the recruitment of mamalian son-of-sevenless (mSOS) and to the endosomally localized activation of Ras, Raf, MEK1 and the entire mitogen-activated protein kinase (MAPK) cascade[67–69] (MEK1 stands for 'MAPK and extracellular signal-regulated kinase (ERK) kinase-1'). In addition, by blocking cell-surface EGFR activation and EGFR recycling back to the cell surface, internalized activated receptors have been shown to signal from endosomes and to promote cell survival[70]. Of note, the nerve growth factor (NGF) RTK receptor TRKA can also activate the Ras–MAPK pathway from endosomes in neuronal cells[71]. So, RTKs can clearly signal from endosomes.

However, these studies raise an important point. Is endosomal signalling a passive event that is initiated at the plasma membrane and maintained during trafficking, or is it biologically relevant? The analysis of EGFR signalling when endocytosis is blocked has shown that MAPK activation[66,69] is dependent on endocytosis, whereas the activation of phospholipase Cγ (PLCγ) and SHC is not[66]. Furthermore, p14, a protein that is localized to endosomal compartments, might be key, because it recruits the MP1 (MEK1 partner) MAPK scaffolding protein to endosomes and is important for the robust

EGF-dependent activation of ERK, but not of another kinase known as p38 (REF. 72). Consistent with the idea that unique signalling activities arise from the endosomal compartment, active Rap1, a Ras-related GTPase, has been reported to be preferentially enriched on endosomes, possibly through an endosomally localized Ras guanine nucleotide-exchange factor[73]. Together, these data indicate that the trafficking of RTKs can function to control both the nature and magnitude of molecular signalling events and the biological outcome of cell stimulation.

However, simple receptor internalization might not be sufficient for endosome-associated signalling. Indeed, wild-type Rab5, which stimulates both endocytosis and endocytic recycling, potentiates Raf1 activation by H-Ras (the latter is activated by EGFR activation), whereas a Rab5 mutant that allows only endocytosis and blocks recycling relocalizes H-Ras from the plasma membrane into endosomes, but inhibits H-Ras-dependent Raf1 activation[74]. This is contrasted by K-Ras, the action of which is not dependent on trafficking. Why is cycling between the cell surface and the endocytic compartments a key requirement for signalling through certain downstream effector pathways but not others? As noted above, plasma-membrane lipid-raft microdomains are enriched for various downstream effectors of EGFR — in particular, H-Ras.This contrasts with the localization of K-Ras, which is not found in lipid rafts[7,75]. Interestingly, the H-Ras-dependent activation of Raf is sensitive to the perturbation of lipid rafts[66,67], whereas Raf activation by K-Ras is not[8]. Therefore, it is tempting to speculate that cycles of internalization and recycling might promote H-Ras-dependent Raf signalling by allowing activated EGFR repeated and transient access to plasma-membrane lipid rafts. This further indicates an important interplay between endocytosis, recycling and the partitioning of the plasma and endosomal membranes into microdomains that are enriched in specific signalling constituents.

The exact role that is fulfilled by trafficking in controlling the nature of RTK signalling pathways has been difficult to establish convincingly, although there is little doubt that the other outcome of endocytosis — the downregulation of receptors — is crucially important in biological systems. Indeed, treating cells with EGF leads to the rapid downregulation of the cell-surface EGFR pool and is part of the mechanism that underlies the interesting 'ON/OFF' switch-like response of MAPK activation. The analysis of neuronal differentiation in a neuroendocrine cell line further indicates that the timing of MAPK activation is important for controlling the biological response, with brief activation of MAPK leading to proliferation and sustained activation promoting neuronal differentiation[78]. It is unlikely that biological systems use single-hit stimulations to control developmental or homeostatic pathways, so it is interesting that pulsatory activation of EGF signalling (two pulses separated by eight hours) induces a proliferative response in epithelial cells, whereas a single pulse does not[79]. Consequently, the rate of RTK trafficking can have an important role in controlling the biological response. Indeed, ERBB-family members — which form homodimers and heterodimers with each other — traffic with variable kinetics, and whereas homodimers of EGFR (which is also known as ERBB1) are rapidly degraded, heterodimers of EGFR with either ERBB2 (also known as the oncoprotein HER2) or ERBB3 tend to be recycled[18]. This is dictated, in part, by the stability of the ligand–receptor complex in the endocytic pathway and by the capacity of the receptor to bind Cbl[18]. So, in addition to regulating the preferential activation of distinct signalling pathways, sustained endosomal signalling by receptor complexes is also crucial for sustaining multiubiquitylation of EGFR and for directing it to the degradative pathway.

These studies begin to reveal an important feature of RTK trafficking — that robust MAPK activation occurs in the same endocytic pathway that also regulates receptor degradation. As the magnitude and kinetics of signal activation in RTK pathways are important in disease — in particular, in cancer — integrating trafficking with signalling provides an elegant mechanism for tuning signalling responses. For example, by ensuring the transient nature of the MAPK response. Indeed, interfering with this integration by disrupting RTK turnover is a key molecular pathology that underlies many cancers. This is highlighted by Cbl, which was first identified as v-Cbl. v-Cbl is the protein product of the transforming gene of the Cas NS-1 murine leukaemia virus, and it is a dominant-negative mutant that consists of only the EGFR-binding domain of Cbl. It therefore blocks receptor downregulation, promotes recycling[80] and mediates EGF-induced transformation[80]. The amplification of ERBB2 (HER2), which is downregulated slowly compared to EGFR (ERBB1), is also important in a subset of breast cancers. The crucial role that trafficking has in this oncogenic activity is highlighted by the fact that the L26 antibody can function to decrease cell-surface ERBB2 levels by inducing ERBB2 internalization, ubiquitylation, and degradation in a Cbl-dependent manner[81]. A humanized version of the L26 antibody (Herceptin) is used for the treatment of metastatic breast carcinoma because of the overexpression of ERBB2 in this disease[81,82]. Receptor trafficking is therefore an important target for the design of new therapeutics. These studies highlight the importance of linking signalling to receptor trafficking in the RTK system, and this is a link that might be far more intimate than was first imagined, especially as a growing number of reports are showing direct regulatory interactions between signalling modules and components of the endocytic machinery[83,84].

TGFβRs

The TGFβ superfamily — which includes activins and the bone morphogenetic proteins (BMPs) — stands in stark contrast to RTK pathways and functions through a unique signalling pathway that involves transcriptional regulators called SMADs. The SMAD pathway transmits signals directly from TGFβ-family receptors to the nucleus, where these signals regulate the expression of hundreds of genes in a cell-type- and cell-context-dependent manner[85]. In all animals, TGFβ-family

members function as key morphogens — that is, diffusible factors that control cell fate on the basis of their concentration. They are used repeatedly during development and, in addition to controlling cell fate, they regulate cell proliferation, epithelial-cell plasticity and cell movement. The TGFβ–SMAD signalling pathway has a crucial role in these biological processes, because it accurately interprets extracellular concentrations and translates them into the appropriate expression of target genes at the right time and in the right place. The role of membrane trafficking in TGFβ-superfamily signalling has been studied almost exclusively for the prototypical TGFβ system, so TGFβ is focused on in this review. TGFβ, similar to activins and BMPs, signals through heteromeric complexes of type-I and type-II transmembrane serine/threonine kinases. Activation of signalling occurs when TGFβ binds to constitutively active type-II receptor dimers (known as TβRII), which leads to the recruitment of type-I dimers (TβRI) to form a heterotetrameric receptor. TβRII then *trans*-phosphorylates and activates TβRI (REF. 86), which, in turn, binds and phosphorylates R-SMADS (receptor-regulated SMADs; SMAD2 and SMAD3; FIG. 2). R-SMAD binding to the receptors is facilitated by a protein known as SARA (SMAD anchor for receptor activation)[87], which has a FYVE domain and is predominantly localized to PtdIns3P-enriched early endosomes. After phosphorylation, SMAD2 dissociates from both the receptors and SARA. It then interacts with SMAD4 (the common SMAD) and translocates to the nucleus, where the SMAD complex regulates the transcription of target genes[85]. The inhibitory SMAD, SMAD7, negatively regulates TGFβ signalling by recruiting the HECT-domain-containing E3 ubiquitin ligases SMURF1 or SMURF2, which direct the ubiquitylation and subsequent degradation of the TGFβR–SMAD7 complex[88,89] (FIG. 2).

TGFβRs and clathrin-mediated endocytosis. From the plasma membrane, TGFβRs are internalized[90,91] through clathrin-mediated endocytosis[92]. TβRII, which contains a di-leucine motif[93], is present in clathrin-coated pits[94] and it binds to the β2 subunit of AP2 *in vitro*[95]. TβRII has also been proposed to internalize through clathrin-coated pits, in part, through its association with TβRIII (type-III TGFβR), which can directly bind to β-ARRESTIN-2 (REF. 96). After internalization into clathrin-coated vesicles, TGFβRs are found for extended periods in early-endosome antigen-1 (EEA1)-positive early endosomes[94,97], and have been found in Rab11-positive recycling endosomes[94,98], but not in p62-positive late endosomes[94]. Taken together, these data show that TGFβRs follow the clathrin-mediated route for internalization. Interfering with clathrin-dependent trafficking — using K^+ depletion, or dominant-negative mutants of DYNAMIN[94,97] or EPS15 (REF. 94), all of which prevent the clathrin-dependent trafficking of TβRII — also blocks TGFβ-induced SMAD2 activation and signalling[94,97]. As PtdIns3P-containing EEA1-positive early endosomes are enriched for the SMAD2-anchor SARA[97,99,100], which contains a FYVE domain[87], this indicates that the EEA1-positive endosome is a key compartment for SMAD activation (FIG. 2). Indeed, wortmannin, which inhibits PI3K and depletes the endosomal pool of PtdIns3P, or the overexpression of a SARA mutant that lacks the FYVE domain both inhibit TGFβ signalling[99,100]. Therefore, the clathrin-mediated endocytic pathway might promote the colocalization of receptors with downstream components such as SARA and SMAD2 in early endosomes. Recently, the protein cPML, a cytoplasmic form of promyelocytic leukaemia protein, was shown to promote SMAD binding to SARA and to have a key role in TGFβ signalling by stimulating the recruitment of both the TGFβR complex and SARA to early endosomes[101] (FIG. 2). Interestingly, HRS, which is important for mediating the downregulation of EGFR (FIG. 1), might promote activin signalling, which uses a TGFβ-like pathway in cooperation with SARA[102] (FIG. 2). Furthermore, SARA interacts with cell-surface TGFβRs and can protect the receptors from SMAD7–SMURF2-mediated degradation[94] (see below).

TGFβRs and lipid rafts. Clathrin-dependent trafficking is not the only pathway for internalizing TGFβRs[90,94] and, in MvLu1 cells, about half of the endogenous TGFβRs are in lipid rafts and are internalized through clathrin-independent pathways, which leads to their entry into caveolin-1-positive vesicles. These vesicles are also positive for the SMAD7–SMURF2-ubiquitin-ligase complex[94], which was found to associate preferentially with raft-bound receptors[94]. Indeed, TGFβR turnover is inhibited by disrupting lipid rafts, and a catalytically inactive SMURF2 mutant specifically protects TGFβRs that reside in lipid rafts[94]. By contrast, the expression of caveolin-1 in caveolin-1-deficient cells enhances SMAD7–SMURF2-induced TGFβR turnover (FIG. 2). So, lipid rafts and caveolin-1 — the latter of which has been shown to interact with TβRI (REF. 103) — are important for the regulation of receptor degradation by a non-classic endocytic pathway. Of note, the presence in lipid rafts of NEDD4 — another HECT-domain-containing E3 ubiquitin ligase — indicates that lipid rafts might represent a general membrane location for HECT-domain-containing ubiquitin ligases. It is unclear what signals control TGFβ partitioning between these two pathways. However, chimeric TGFβRs that contain a granulocyte/macrophage colony-stimulating factor-1 extracellular domain are predominantly localized in the EEA1 compartment, which indicates that the determinants might reside in the extracellular domain[98].

TGFβR endocytosis, partitioning and regulation. TGFβRs are therefore internalized through two distinct endocytic routes — the clathrin-mediated pathway and the caveolar/lipid-raft-mediated pathway — that fulfil separate functions in TGFβ signal transduction. The former is important for promoting signalling, whereas the latter mediates receptor degradation (FIG. 2). Interestingly, the presence of these two internalization pathways creates a dynamic balance between TGFβR trafficking and its membrane compartmentalization. Interfering with either pathway therefore shifts the

R-SMADS
(receptor-regulated-SMADs). R-SMADs are transcription factors, and they contain two domains (MAD homologue-1 and -2 (MH1 and MH2)) that are separated by a proline-rich linker. The MH1 domain mediates interactions with proteins and DNA, whereas the MH2 domain mediates protein–protein interactions.

β-ARRESTIN-2
Arrestins are an important family of proteins that are known to be negative regulators of G-protein-coupled receptor (GPCR) signalling. Arrestins bind to phosphorylated GPCRs and recruit clathrin and AP2, which results in receptor internalization and desensitization. These proteins have also been shown to regulate Wnt and TGFβR internalization.

DYNAMIN
An important component of the endocytic machinery that might function both as a regulatory GTPase (by recruiting various components into coated pits and inducing vesicular budding) and as a mechanochemical enzyme (that forms a collar-like structure at the necks of invaginated membranes and promotes fission of the buds).

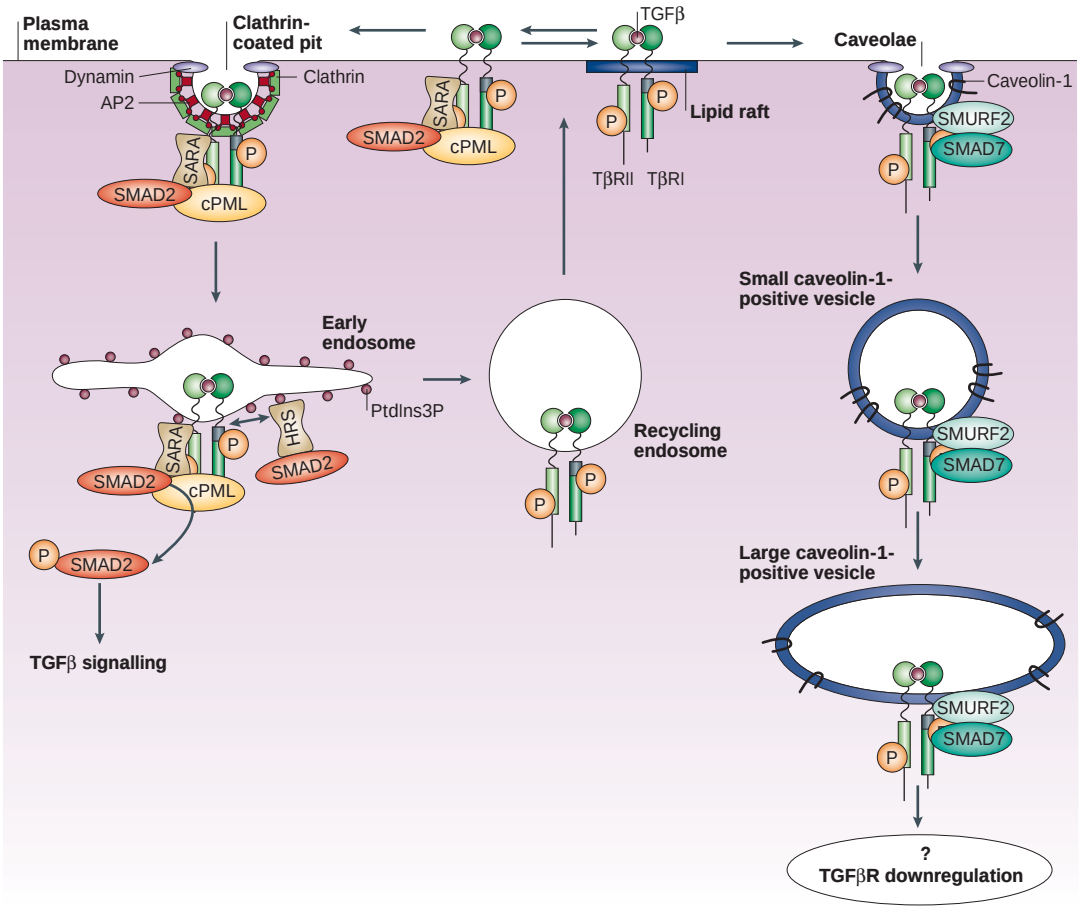


Figure 2 | **Transforming-growth-factor-β-receptor internalization by clathrin- and lipid-raft-mediated endocytosis.** At the plasma membrane, the tetrameric transforming-growth-factor-β-receptor (TGFβR) complex is internalized by two distinct endocytic pathways. The TGFβR complex is composed of two type-I TGFβRs (TβRIs) and two type-II TGFβRs (TβRIIs), and in this complex, constitutively active TβRII transphosphorylates TβRI. For simplicity, this tetrameric complex is represented by a dimeric TβRII–TβRI complex in this figure. In the clathrin-mediated endocytic route, receptors are directed towards the early endosomes, which are enriched in phosphatidylinositol-3-phosphate (PtdIns3P). Here, the receptors interact with two 'Fab1, YOTB, Vac1, EEA1' (FYVE)-domain-containing proteins — SMAD anchor for receptor activation (SARA) and hepatocyte-growth-factor-regulated tyrosine-kinase substrate (HRS) — that are associated with SMAD2. The cytoplasmic promyelocytic leukaemia protein (cPML) that interacts with TGFβRs, SARA and SMADs, is required for the early endosomal localization of these TGFβ signalling components. Of note, SARA, SMAD2 and cPML probably also interact with TGFβ–TGFβR complexes at the plasma membrane. From the early endosomes, TGFβRs are able to signal (through SMAD2 phosphorylation) and are recycled back to the cell surface. In the lipid-raft/caveolae-mediated endocytic pathway, TGFβRs associate with SMAD7–SMURF2 and are internalized into caveolin-1-positive vesicles. This leads to the ubiquitin-dependent degradation of the receptors (SMURF2 is a HECT-domain-containing E3 ubiquitin ligase). The exact compartment in which the receptors are degraded has not been characterized. AP2, adaptor protein-2; P, phosphate.

receptors into the alternative pathway, and overexpression of SARA — like the disruption of lipid rafts — blocks SMAD7–SMURF2-dependent TGFβR turnover in a FYVE-domain-dependent manner[94]. Indeed, sequestering receptors from the caveolar/lipid-raft pathway might be a key function of clathrin endocytosis. Consistent with this hypothesis, concomitant interference with lipid rafts and clathrin is sufficient to remove the block in TGFβ signalling that is caused by interfering with the clathrin pathway[94]. In addition, in some cell types that are deficient in caveolin, clathrin-dependent endocytosis is not essential for TGFβ signalling[104]. These latter studies emphasize the importance that cell-type differences might have in understanding how trafficking regulates cell signalling and receptor turnover. In this respect, the downregulation of caveolin is associated with tumour progression[105], and its loss might be speculated to promote constitutive TGFβ signalling, which has been associated with progression to a metastatic phenotype in late-stage tumours. Altogether, these results point to a key role for the dynamic balance between the clathrin and caveolin-1/lipid-raft compartments in regulating TGFβ signalling.

TβRII trafficking also differs significantly from EGFR trafficking in that ligand binding does not seem to alter the trafficking rate or pathway of internalization. Indeed, in one present model, EGFR is sequestered in lipid rafts and the EGF ligand induces the migration of EGFR out of these lipid rafts, and this is followed by ligand-dependent internalization and degradation[49,53]. By contrast, TGFβR

trafficking through the two endocytic pathways is not affected by the TGFβ ligand[93,94], and this is similar to observations of IL2-receptor trafficking[45]. This indicates that the ligand might not regulate trafficking *per se*, but that it probably stabilizes the TβRII–TβRI complex during trafficking to facilitate the activation of SARA-bound SMAD2 in the clathrin pathway or SMAD7–SMURF2-dependent degradation in the lipid-raft pathway.

It is therefore interesting to compare ligand-independent TGFβR trafficking, which uses distinct endocytic pathways for signalling versus degradation, to that of the EGFR, which uses a ligand-dependent event and intimately links signalling to receptor degradation in the same pathway (FIGS 1,2). The morphogen function of TGFβ-family members probably lies at the core of these unique trafficking mechanisms. Cells must be able to measure the concentration of extracellular factors accurately and continuously to allow them to respond appropriately to morphogens within a developmental field. Work from Gurdon and colleagues has shed light on the biology of this process, and has revealed key features of the activin pathway — a TGFβ-family ligand that uses the same SMAD pathways as TGFβ — that support this biological behaviour[106]. They showed that cells respond continuously to activin and display a ratchet-like behaviour (that is, cells respond to the highest dose of ligand they were exposed to), and that they respond to the absolute number of occupied receptors. Ligand-independent trafficking and uncoupling the signalling compartment from the degradative compartment are elegantly suited to this behaviour. For example, it allows continuous monitoring of ligand concentration and permits ratcheting, because prior exposure to ligand does not downregulate receptors, as would be the case in the EGF system. Receptors in the EEA1 compartment are therefore sequestered from the SMAD7–SMURF2-dependent degradative pathway, which prevents the signal transience that is inherent when signalling and degradative trafficking are tightly linked. The distinct global features of TGFβ-family versus RTK-family trafficking have therefore probably evolved to accommodate the very different biological functions that these signalling systems have.

Other receptors

Clathrin-independent endocytosis is an internalization route that is followed not only by TGFβRs, but also by other receptors and GPI-anchored proteins. IL2Rβ is an example of another transmembrane receptor that is internalized by a non-clathrin pathway[45]. It is one subunit of the high-affinity receptor for IL2, a cytokine that is induced in response to the activation of T-lymphocytes by mitogens or specific antigens. At the lymphocyte cell surface, IL2Rβ is associated with GM1-positive/caveolin-1-negative detergent-resistant membranes[45], and although IL2Rβ internalization is inhibited by a dominant-negative dynamin mutant, it is not affected by a dominant-negative mutant of EPS15 that blocks clathrin-mediated endocytosis. This indicates that IL2Rβ is endocytosed through a non-clathrin, dynamin-dependent, lipid-raft-dependent endocytic pathway[45] (FIG. 3). Because IL2Rβ has been found in transferrin-positive vesicles and the late endosome/lysosome, this pathway might lead the receptor to classic endosomal compartments[107]. However, as clathrin-dependent pathways have also been implicated in the IL2Rβ endocytosis[107], this remains unclear.

Autocrine motility factor (AMF; which is also known as glucose 6-phosphate isomerase) is a cell-motility-stimulating factor that binds to the cell-surface glycoprotein gp78/AMF receptor (AMFR). AMFR is present in caveolae at the cell surface and in smooth-endoplasmic-reticulum tubules, and interfering with clathrin-mediated endocytosis does not disrupt the internalization of biotinylated AMF to AMFR-positive tubules[108]. Furthermore, cholesterol-extracting reagents and dominant-negative dynamin inhibit AMFR internalization to the endoplasmic reticulum[109], which indicates that AMFR — similar to TGFβR, IL2Rβ, SV40 (REF. 110), CHOLERA-TOXIN B SUBUNIT and GPI-anchored proteins[34,111,112] — also uses a lipid-raft-dependent internalization route (FIG. 3).

Emerging areas

Our limited understanding of how Hh and Wnt receptors transduce signals is a handicap in elucidating the role of endocytosis in regulating their signalling. However, recent work has begun to identify a key link between signalling and endocytosis for these two morphogen systems.

The Hh signalling pathway. The Hh signalling pathway has a crucial role in many aspects of embryonic patterning and in cancer. In this system, the present model, which was first characterized in *Drosophila melanogaster*, is that Hh binds to its receptor Patched (Ptc), which relieves the Ptc-dependent inhibition of the seven-transmembrane-protein Smoothened (Smo). Smo then regulates gene expression through Gli transcription factors. Little is known about the internalization routes that are used by Ptc and Smo, although it is known that their trafficking changes significantly in response to Hh. Hh induces Ptc accumulation[113,114] in perinuclear regions[115], with the concomitant redistribution of Smo from intracellular structures to the cell surface[113,115]. Recently, a report showed that the addition of the vertebrate orthologue of Hh, Sonic Hedgehog (Shh), induces the appearance of Ptc–Shh and Smo in a common vesicle and proposed that in late endosomes, the Ptc–Shh complex is segregated from Smo, which recycles back to the cell surface. Ptc–Shh then enters the lysosomal compartment[116]. This model gives the late endosomal compartment a new role, in which it helps to release Smo from the inhibitory Ptc–Shh complex. Interestingly, overexpressed Ptc interacts with caveolin and is present in caveolae, and caveolin-1-enriched vesicles might redistribute Smo to lipid rafts[117]. As the cholesterol-tethered form of Hh has also been found in lipid rafts[118], it is possible that the Ptc–Hh complex is formed in lipid rafts and might regulate signalling by dissociating the Ptc–Smo complex in this compartment. The dynamic trafficking of ligands and receptors in this system is therefore probably crucial for controlling the activity of the pathway.

CHOLERA-TOXIN B SUBUNIT
A type of bacterial toxin that contains an enzymatic A subunit and a binding B subunit. The uptake of cholera toxin is mediated by a glycolipid, the GM1 ganglioside.

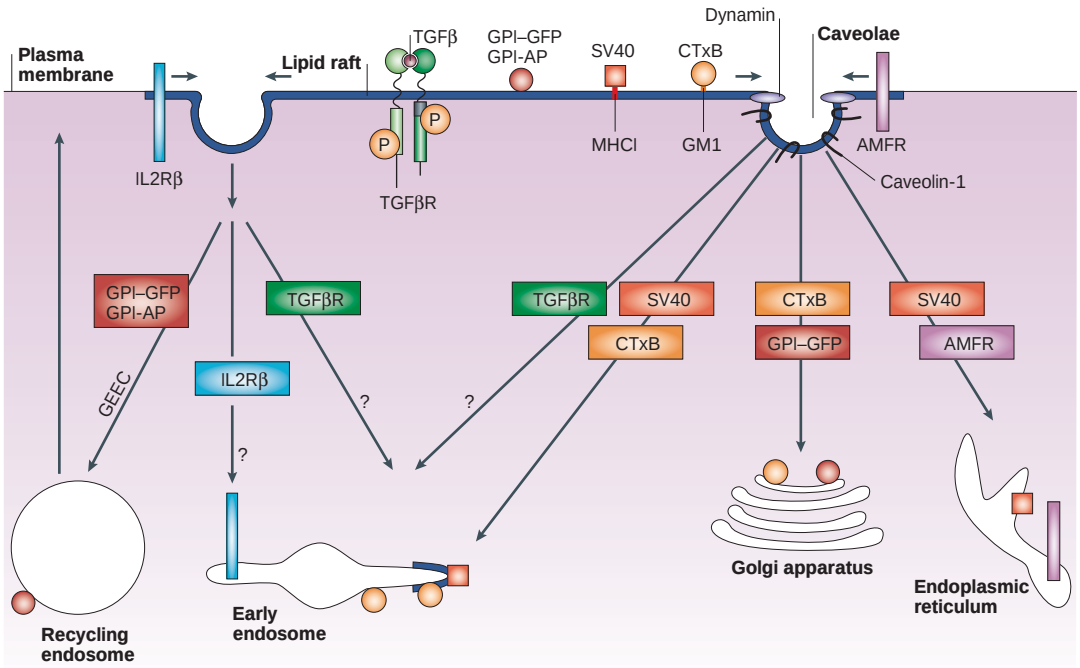


Figure 3 | **Internalization through lipid-raft-dependent endocytosis.** Cell-surface molecules — Simian virus 40 (SV40), cholera-toxin B subunit (CTxB), glycosylphosphatidylinositol–green-fluorescent-protein (GPI–GFP) fusion proteins, GPI-anchored proteins (GPI-APs), transforming-growth-factor-β receptor (TGFβR), interleukin-2 receptor-β (IL2Rβ) and autocrine-motility-factor receptor (AMFR) — that are internalized through lipid-raft and/or caveolin-positive endocytic pathways are shown in a schematic representation. Specific inhibitors can be used to perturb these two lipid-raft endocytic pathways, such as β-methyl cyclodextrin, nystatin, filipin and dominant-negative dynamin. The intracellular targets of these molecules are indicated. Question marks are used for cases in which the intracellular target compartment has not been characterized or has been implicated on the basis of published data, but has not been directly shown. GEEC, GPI-anchored-protein-enriched early endosomal compartment; MHCI, class-I major histocompatibility complex; P, phosphate.

The Wnt signalling pathway. The Wnt signalling pathway is also important in development and carcinogenesis. In the canonical Wnt pathway, Wnt ligands signal through low-density-lipoprotein-receptor-related proteins-5/6 (Lrp5/6; the *D. melanogaster* orthologue is known as Arrow) and Frizzled (Fz). This induces the stabilization of intracellular β-catenin, which, in turn, initiates the transcription of Wnt-responsive genes. In *Drosophila melanogaster*, the Wnt ligand Wingless (Wg) is endocytosed through clathrin-mediated endocytosis into MVBs and lysosomes, which leads to the downregulation of Wg levels and signalling[119]. Little is known about the internalization of Lrp5/6, but, in response to Wnt5, Fz4 is internalized through clathrin-dependent endocytosis by β-arrestin-2, which binds the PKC-phosphorylated, Fz4-associated-protein Dishevelled[120]. However, what role the endocytosis of Fz4 has in Wnt5 signalling — which uses the poorly defined non-canonical Wnt pathway — is unclear. Interestingly, in human cells, Dickkopf1 — which is known to bind to and inhibit the Wnt co-receptor LRP6 — forms a ternary complex with the membrane protein Kremen-2, blocks Wnt/β-catenin signalling, and induces the internalization and clearance of cell-surface LRP6 (REF. 121). Although the analysis of Wnt-receptor trafficking is in its infancy, it is nonetheless interesting that Wnt ligands are highly insoluble, probably due to modification by palmitoylation[122], and the presence of this modification might indicate that they associate with lipid rafts[123].

Taken together, these reports represent the first important steps in establishing connections between the Hh and Wnt signalling pathways and trafficking. In the near future, analysis of the endocytic routes that are used by these two factors and their receptors should yield important insights into how they function as morphogens.

Conclusions

It is becoming increasingly evident that clathrin-mediated endocytosis is not the only internalization pathway. Lipid rafts are membrane microdomains that: are distinct from clathrin-coated pits; are rich in cholesterol, glycosphingolipids, GPI-anchored proteins and signalling proteins; and can function as signalling platforms. Cholesterol and lipid rafts have been shown to be crucial for the assembly and activity of various signalling networks. Moreover, lipid-raft trafficking is also required to turn off signal transduction and has been found to mediate TGFβR degradation by inducing the colocalization of these receptors with the SMAD7–SMURF2-ubiquitin-ligase complex.

One important area that remains poorly defined is the nature of the molecular composition of lipid rafts and how many varieties of them exist in the cell. Indeed,

work at present indicates that caveolae are probably not the only specialized type of lipid raft. Furthermore, the molecular mechanism that underlies lipid-raft-mediated endocytosis has not been fully elucidated. Nevertheless, the existence of lipid-raft endocytic pathways gives cells other ways to regulate signalling, and the interest in this subject ensures that there are many exciting findings to come in the future.

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The authors declare no competing financial interests.

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