Forty Years of Clathrin-coated Vesicles

Margaret S. Robinson

University of Cambridge, Cambridge Institute for Medical Research, Cambridge, UK

e-mail msr12@cam.ac.uk

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Abstract

The purification of coated vesicles and the discovery of clathrin by Barbara Pearse in 1975 was a landmark in cell biology. Over the past 40 years, work from many labs has uncovered the molecular details of clathrin and its associated proteins, including how they assemble into a coated vesicle and how they select cargo. Unexpected connections have been found with signalling, development, neuronal transmission, infection, immunity, and genetic disorders. But there are still a number of unanswered questions, including how clathrin-mediated trafficking is regulated and how the machinery evolved.

Introduction

In 1975, a young postdoc at the MRC Laboratory of Molecular Biology published a paper in the Journal of Molecular Biology, entitled “Coated vesicles from pig brain: purification and biochemical characterization” (1). For the postdoc, Barbara Pearse, this was the culmination of two years’ work, which had started off as an attempt to purify tubulin (2). The classical method for purifying tubulin is to allow brain extracts to go through two cycles of microtubule assembly followed by disassembly, but this only works on very fresh tissue. What Barbara didn’t realise was that the brains she got from the local slaughterhouse were already a day old. So when she looked at her prep in the electron microscope, she didn’t see any microtubules, but she did see intriguing structures that looked to her like sliced tomatoes (Figure 1a). When she showed the images to colleagues, they recognised the structures as the “vesicles in a basket” that had been described by Toku Kanaseki and Ken Kadota some five years earlier (3). So in the end, Barbara decided to purify coated vesicles instead of tubulin, captivated not only by their tomato-like appearance but also by several reports in the literature about their possible role in endocytosis, although that word was not yet in common use. When she analysed her purest fraction by SDS PAGE, she found that it was dominated by a single protein with a molecular weight of ~180K (Figure 1b). In the final paragraph of her paper, reproduced below, she made a number of points that would set the scene for thousands of papers in the years to come.

“Coated vesicles of brain are believed to be derived by micropinocytosis of synaptic membrane (Heuser & Reese, 1973). Whether the same protein is associated with the coated vesicles described in other tissues is unknown. In any event, the 180,000 molecular weight protein is likely to be an important example of a class of proteins involved in membrane movement, and I propose that it be called ‘clathrin’. ‘Clathrin’ itself may be able

to pinch off a vesicle through interactions between its subunits and the membrane phospholipids. The coat may also be able to interact with other molecules, to ensure formation of vesicles at particular sites on the membrane and for their transport to specific cellular locations. Traces of other proteins are visible on the SDS-gel patterns of the final preparation. These may represent slight impurities, or minor proteins associated with the vesicles and involved in such specific functions." (1)

The phrases "coated pit" and "coated vesicle" had actually been coined more than ten years earlier, in another classic paper by Tom Roth and Keith Porter, to describe the structures they saw in electron micrographs of mosquito oocytes taking up yolk proteins (4). Like Pearse, Roth and Porter were extraordinarily prescient when they came to speculate about the significance of their findings. They proposed that the coat might have a mechanical function, and/or that it might have something to do with "the specificity of materials adsorbed" - i.e., a selective role in the uptake of yolk. Another key study (referred to in the final paragraph of the Pearse paper) was the work by John Heuser and Tom Reese implicating coated vesicles in the recycling of synaptic vesicle membrane (5), although the exact contribution of coated vesicles to this pathway remains hotly debated even up until the present day (6-9).

While Barbara Pearse was working out how to purify coated vesicles, Mike Brown and Joe Goldstein were investigating the binding and uptake of low density lipoproteins (LDL) by fibroblasts from normal individuals and from patients with familial hypercholesterolemia (FH). This is a devastating disorder, where people who are homozygous for the mutant gene can have heart attacks as early as age 5. Brown and Goldstein found that normal cells incubated with radioiodinated LDL bound the LDL with high affinity, took it up very rapidly, and eventually degraded it in lysosomes, indicating that the cells had receptors for

LDL, which were efficiently endocytosed. However, this didn't happen in cells from homozygous FH patients, suggesting that the causative mutation was in the LDL receptor gene (10). In most cases, the FH cells were unable to bind any LDL at all, but there was one patient, J.D., whose cells could bind LDL but not internalise it (11). Together with Dick Anderson, Brown and Goldstein investigated how the LDL got inside the cell, by incubating fibroblasts with LDL coupled to ferritin, an electron-dense tag. Their electron micrographs showed that in normal cells, membrane-bound LDL was highly concentrated in coated pits and vesicles, while in J.D.'s cells, the membrane-bound LDL was excluded from coated pits and vesicles (12).

Thus, by the end of the 1970s, both of the functions of the coat proposed by Roth and Porter had been shown to be correct. Pearse's discovery of clathrin, followed by her high-resolution analyses of purified clathrin-coated vesicles in collaboration with Tony Crowther and John Finch, demonstrated that the coat is a cage-like structure consisting of 12 pentagons and a variable number of hexagons, "the most economical covering of a sphere or closed surface" (13). Together with the earlier work of Kanaseki and Kadota, these findings immediately suggested how the coat could play a mechanical role, by forming a cage while attached to a membrane and thereby converting a flat sheet into a vesicle. At the same time, the discovery by Brown, Goldstein, and Anderson that coated pits and vesicles are sites of receptor-mediated endocytosis (their terminology), where some membrane proteins are concentrated and others excluded, confirmed the idea that the coat plays a selective role as well. The clathrin field was now well on its way.

Clathrin structure

Probably the most remarkable thing about clathrin is the way it can assemble into a lattice of hexagons and pentagons. The aesthetic appeal of clathrin has enticed countless

researchers into the field (including myself), and in fact, people have been fascinated by this type of structure for over 2,000 years, beginning with Archimedes and including Leonardo da Vinci (Figure 2). But clathrin is not unique in its ability to assemble into structures made out of hexagons and pentagons; similar types of structures can be found elsewhere in nature, ranging from buckminsterfullerene, which is >100 times smaller than a clathrin coat, to pollen grains and the skeletons of certain protozoans, which are ~1,000 times larger (Figure 2).

However, when Roth and Porter first observed the vesicle coat, they weren't able to discern its geometry, partly because of how they fixed the cells (with osmium tetroxide only), and partly because it is difficult to appreciate that the coat is actually a lattice when looking at a two-dimensional thin section. Thus, Roth and Porter described the coat as a layer of bristles, and when they proposed that it might have a mechanical role in "giving ... the spherical form to the base of the pit and the pit-vesicles", they suggested that this could be due to "a natural repulsion of the outer ends of the bristles" (4). It was Kanaseki and Kadota who first realised that the coat is in fact a "basketwork" composed of hexagons and pentagons. This was because they looked at isolated coated vesicles, so the structure of the coat was not obscured by other proteins in the cytoplasm, and also because they used both negative staining (where the stain fills the crevices of the coat, as in Figure 1a) and thin sections of specimens that had been fixed with glutaraldehyde as well as osmium. Kanaseki and Kadota proposed that the coat starts off as a network of hexagons, some of which are then transformed into pentagons, leading to "the formation of a beautiful sphere containing the vesicle", which they illustrated using a soccer ball as a model (3). This "beautiful sphere" and the hexagonal networks that precede it are probably best appreciated in the spectacular images of John Heuser, first unveiled in 1980 (14), in which

he used his method of “unroofing” cells in order to look down on the inside of the plasma membrane (Figure 3a).

Because Pearse’s 1975 paper had shown clathrin to be by far the most abundant component of the coat, it was generally assumed that the hexagons and pentagons must be made out of clathrin. This was formally demonstrated in 1979 by Jim Keen, who extracted the vesicle coats with high concentrations of Tris buffer and then fractionated the extracts by gel filtration. Clathrin eluted first from the column, behaving as a very high molecular weight oligomer, followed by a second fraction that contained a 100-110 kD protein or proteins (15). Keen found that it was possible to assemble baskets using the clathrin-containing fraction alone, but that assembly occurred much more readily when he added the second fraction, which he subsequently dubbed “assembly polypeptides” or “APs” (16). Negative staining of the first fraction, containing non-assembled clathrin, showed “linear protein aggregates” with no well-defined structure (15). However, rotary shadowing studies carried out two years later by Ernst Ungewickell and Dan Branton revealed that native non-assembled clathrin exists as a three-legged structure called a triskelion (Figure 3b). Ungewickell and Branton proposed that each triskelion is made out of three copies of clathrin together with three copies of a much smaller protein (17). The smaller protein is now known as clathrin light chain, while the orginal 180 kD protein discovered by Pearse is now known as clathrin heavy chain (Figure 3c).

During this time, repeated attempts were made to solve the structure of clathrin by X-ray crystallography. The problem was not getting enough protein (12 bovine brains routinely yield >100 mg of purified clathrin), but getting the protein to crystallise. Finally Tommy Kirchhausen’s lab tried using recombinant clathrin heavy chain, which they had cloned and sequenced in 1987 (18), and succeeded in crystallising the first 494 residues, which

comprise the foot and part of the ankle of a single triskelion leg (19). Unexpectedly, because this was not predicted from the sequence, residues 1-330 were found to form a seven-bladed β-propeller, followed by ten short α helices stacked against each other to form a zigzag (Figure 3d). The following year, Joel Ybe and Frances Brodsky solved the structure of residues 1,210-1,516, comprising most of the thigh, and discovered that it consisted entirely of stacked hairpins of α-helices (Figure 3e). By looking at the sequence, they were able to predict that the entire leg adopts a stacked hairpin structure, with a ~145-residue motif consisting of five hairpins repeated seven times. This clathrin heavy chain repeat (CHR) motif can be found in several other proteins as well, including machinery for trafficking to lysosomes (20).

In addition to X-ray crystallography, structural biologists have investigated clathrin-coated vesicles (CCVs) and reassembled clathrin coats using cryo-electron microscopy, starting with a study in 1986 by Guy Vigers, Tony Crowther, and Barbara Pearse (21). Over the years, Corinne Smith, Tommy Kirchhausen, and Steve Harrison have managed to improve the resolution by an order of magnitude (22-26), so it is now possible to obtain cryo-EM structures at ~8 Å resolution, where the individual triskelions can be seen to intertwine (Figure 3f). The crystal structures and homology models can be fitted into the electron density maps, and by comparing structures that differ by the presence or absence of a binding partner, such as clathrin light chain, the location of the interaction and the shape of the partner can be determined (Figure 3g). Thus, at long last we have a detailed molecular understanding of how clathrin is able to assemble into a vesicle coat.

One of the suggestions Pearse made in her 1975 paper was that clathrin might be able to interact with membrane phospholipids and pinch off vesicles (1). We now know that clathrin cannot interact directly with phospholipids, and that the pinching-off process is mediated by

a different protein, dynamin. Nevertheless, Philip Dannhauser and Ernst Ungewickell showed in 2012 that if purified clathrin is anchored to an artificial liposome by an intrinsically disordered polypeptide containing clathrin binding sites, it is capable of generating spherical buds that progress all the way to the narrow neck stage (Figure 3h). Adding dynamin enables the necks to be severed and free CCVs to be generated (27). This experiment suggests that clathrin assembly alone may be sufficient to drive membrane curvature, at least *in vitro*.

Clathrin function

When Anderson, Goldstein, and Brown published their 1977 paper demonstrating that LDL receptors in J.D.'s cells are unable to enter clathrin-coated pits, they proposed that the cytoplasmic domain of the wild-type LDL receptor contains an amino acid sequence, the "internalisation site", which binds to a component of the coat, possibly clathrin (Figure 4a) (12). This generated a lot of excitement at the time, because the "sorting problem" - how proteins get to the right part of the cell - was just starting to be recognised as one of the major paradoxes in cell biology (28). The idea that the cell could pick and choose what it put into a vesicle, instead of just packaging a random assortment of proteins, was very attractive, because it got around the problem of membrane compartments getting all mixed up by indiscriminate trafficking from one to another. Moreover, structures near the Golgi apparatus that looked very similar to the structures that bud from the plasma membrane had been described in 1967 by Holtzman, Novikoff, and Villaverde (29), and by Friend and Farquhar (30), suggesting that CCVs might sort cargo not only at the plasma membrane, but also at other locations (Figure 4b).

This idea gained strong support in 1980, when Jim Rothman and Dick Fine published two papers reporting that CCVs transport newly synthesised membrane proteins from the ER to

the Golgi, and from the Golgi to the plasma membrane (28, 31). Their strategy was to infect cells with vesicular stomatitis virus (VSV), and then to pulse-label the cells and isolate CCVs after chasing for various lengths of time. The CCV fractions were found to be enriched in radiolabelled VSV G protein, the viral membrane glycoprotein. Depending on how long they chased the cells, the VSV-G was either in its high-mannose form, indicating that it had recently left the ER, or in its trimmed form, indicating that it had moved at least partway through the Golgi apparatus. Largely as a consequence of this work, the first edition of Molecular Biology of the Cell (Alberts et al., 1983) described coated vesicles as “miniature sorting machines”, and proposed that they “could be the principal means by which cells execute their sorting decisions” (32).

It was also in 1980 that Peter Novick and Randy Schekman first published their identification of 23 different yeast genes encoding components of the secretory pathway (33). It would take over a decade for all of these genes to be cloned by complementation, but gratifyingly, some of them turned out to be homologues of machinery identified in mammalian cells by the Rothman lab (34). As an alternative approach (and perhaps a shortcut), Greg Payne and Randy Schekman decided to test the involvement of a candidate protein, clathrin heavy chain. They assumed - as did the rest of the world - that cells would be dead without clathrin, so they disrupted only one of the two alleles in a diploid strain. Astonishingly, when the cells were allowed to sporulate, all four of the haploid spores were viable (35). The two clathrin-null spores gave rise to much smaller colonies than the two wild-type spores, and in a similar study carried out independently by Sandy Lemmon and Beth Jones, the clathrin-null cells rarely grew at all (36). Nevertheless, both labs found that under certain conditions, yeast cells could survive without clathrin. Not surprisingly, there were effects on endocytosis (37). There was also mislocalisation of proteins normally resident in the late Golgi (38). In addition, a temperature-sensitive clathrin heavy chain

mutant generated a few years later had a block in receptor-mediated trafficking of hydrolases to the vacuole, the yeast equivalent of the mammalian lysosome, although with time the cells were able to adjust (39). However, in both the null mutant and the temperature-sensitive mutant, the kinetics of invertase secretion were nearly normal, arguing against an essential role for clathrin in the secretory pathway.

Although yeast was the first organism in which clathrin function was tested using a genetic approach, there are now many more examples. In animals, clathrin appears to be necessary for survival (40), but some cultured animal cells are able to get by without clathrin (41). Similarly, clathrin-null Dictyostelium cells are viable (42). However, RNAi-mediated knockdown of clathrin is lethal in trypanosomes, the unicellular parasites that cause sleeping sickness (43); and clathrin is also essential in the plant Arabidopsis (44). The loss-of-clathrin phenotype is always severe, whether or not it leads to death, with profound effects on both endocytosis and intracellular trafficking, in particular to lysosomes. So far, every eukaryote whose genome has been sequenced (>100 organisms) has been found to have a clathrin heavy chain gene, with one exception: microsporidians, which are parasitic fungi that have lost many of the genes found in all other eukaryotes (45). These observations indicate that clathrin is an ancient protein, which must have been present in the last eukaryotic common ancestor (LECA) ~1.5 billion years ago, and that there is normally a strong selective pressure against losing the clathrin heavy chain gene. But in spite of clathrin's importance, it does not appear to be required for constitutive secretion in any of the organisms where its function has been tested.

So how to reconcile Rothman's data with all the subsequent studies arguing against a role for clathrin in the secretory pathway? In retrospect, it seems likely that the VSV-G protein-containing transport intermediates isolated by Rothman were not clathrin-coated, but had

similar fractionation properties. In fact, a year after the yeast clathrin knockout paper came out, the Rothman lab published a paper on “a new type of coated vesicular carrier that appears not to contain clathrin” (46), which were subsequently purified, leading to the discovery of the COPI coat (47) (Figure 5a). Then in 1994, the Schekman lab discovered another type of non-clathrin coat, the COPII coat (48) (Figure 5b). Hundreds of studies have now been carried out on COPs I and II, demonstrating that COPII is the coat used for ER-to-Golgi traffic, while COPI facilitates Golgi-to-ER traffic and probably other pathways as well (49). Both COPI and COPII contain proteins with a similar architecture to clathrin heavy chain, consisting of one or two seven-bladed β-propellers followed by stacked α-helical hairpins, although whether these proteins are true homologues or the result of convergent evolution is unclear (50). In addition, part of the COPII coat assembles into a polyhedral cage, although the geometry is different from that of the clathrin cage (51). Thus, although we now know that clathrin is not the only type of coat used in membrane traffic, it remains the best understood, and in many ways it has been a paradigm for shaping our understanding of other types of coats.

Cargo recognition

But clathrin never acts alone. Even in 1975, Pearse pointed out that “traces of other proteins are visible on the SDS-gel patterns of the final preparation” (1). These proteins include the 100-110 kD bands that Keen found in his clathrin assembly-promoting fraction (15), and in 1981 Unanue, Ungewickell, and Branton provided evidence that the same proteins might also attach clathrin to the membrane (52). This meant that there was a good chance that the proteins could be involved in what John Heuser called “the critical exciting issue of how the coat ‘recognizes’ receptor-rich regions of the donor membrane compartment and selects them from the rest” (53). So in 1982, I took up a postdoctoral position with Barbara Pearse to try to purify and characterize the 100-110 kD proteins

(intending to stay in Cambridge for two years although I never left). It turned out that there were two distinct protein complexes in the clathrin assembly-promoting fraction, both of which contained 100-110 kD proteins. We were able to separate these two complexes by hydroxylapatite chromatography, so we called them HA-I and HA-II (54). Using a different column chromatography-based method, Jim Keen purified the same two complexes, which he called AP-1 and AP-2 for assembly polypeptides 1 and 2 (55), and this is the name that has stuck, because conveniently it can also stand for adaptor proteins 1 and 2.

Both AP-1 and AP-2 are heterotetramers, consisting of two large subunits corresponding to the 100-110 kD gel bands, which are sometimes called adaptins (β-adaptin and γ- or α-adaptin), together with a medium-sized (μ) subunit of ~50 kD and a small (σ) subunit of ~20 kD (Figure 6a). After several years of trying to raise antisera against the large subunits, eventually we succeeded in making polyclonal and then monoclonal antibodies that were specific for the subunits of AP-2 (56, 57). One year later, Ernst Ungewickell's lab made a monoclonal antibody against AP-1 (58). Together, our immunofluorescence images showed that AP-2 is associated with the plasma membrane, and AP-1 with intracellular membranes (Figure 6b). These were exciting findings, because if it was true that the APs were involved in cargo recognition, then the differential localization of AP-1 and AP-2 would "help to explain how different membrane proteins can be sorted into coated vesicles in different parts of the cell" (57).

But it was still many years before the APs were shown definitively to select cargo. In the meantime, a number of sorting signals for clathrin-mediated endocytosis were identified (Figure 6c). The first of these was the "internalization site" of the LDL receptor, discovered in 1986 by the Brown and Goldstein lab when they compared the sequences of the wild-type receptor and the internalization-deficient receptor from J.D. They found that the J.D.

receptor had a single point mutation in its cytoplasmic tail, converting a tyrosine to a cysteine (59). Then in 1988, Mike Roth's lab took a membrane protein that is normally not taken up by clathrin-mediated endocytosis, influenza hemagglutinin, and mutated three different residues in its cytoplasmic tail to tyrosines. One of these changes converted the hemagglutinin into a CCV cargo protein (60). Two other CCV cargo proteins were investigated in 1989-1990, the cation-independent mannose 6-phosphate receptor (CIMPR) (61) and the transferrin receptor (TfR) (62), and in both cases mutating a key tyrosine residue strongly inhibited the receptor's ability to be endocytosed.

All of these studies pointed a finger at tyrosine residues, and yet most transmembrane proteins have tyrosines in their cytoplasmic tails but are not picked up as coated vesicle cargo. This suggested that the tyrosine needed to be in a particular context to work, but it was difficult to find any common features in all of the cargo proteins, other than the presence of a tyrosine residue. However, when some of the neighbouring residues were mutated, patterns began to emerge. For instance, Mike Roth's lab showed that endocytosis of hemagglutin was four times more efficient if, in addition to mutating the first cysteine to a tyrosine, they also mutated the second cysteine to a phenylalanine or leucine (63). Conversely, when the valine or phenylalanine downstream from the tyrosine in the CIMPR or the TfR was mutated to another bulky hydrophobic residue, the proteins were still internalised, but any other residue caused them to remain on the cell surface (64, 65). These studies and others led to the proposal that the consensus sequence for clathrin-mediated endocytosis is YXXΦ, where Φ is a bulky hydrophobic residue (65). The odd one out was the LDL receptor: not only did it not fit the consensus sequence, but mutagenesis studies indicated that it was the residues upstream of the tyrosine, not downstream, that were important (66).

But how were the sorting signals recognized? Although there was a lot of enthusiasm for the idea that they bind to AP complexes, there was very little evidence for this, not for want of trying. So in 1995, Hiroshi Ohno and Juan Bonifacino decided to use an unbiased approach to find cargo recognition machinery, whatever it might be. They made a “bait” construct containing three copies of a YXXΦ motif, and they used it to screen a yeast two-hybrid library. Out of 2.5 x 10^6 independent recombinants, they found exactly two that specifically recognised the sorting signal. When they sequenced the two clones, they found that both encoded the C-terminal portion of the medium (μ) subunit of AP-2. They went on to show that the μ subunit of AP-1 could also bind YXXΦ motifs, but with slightly different preferences (67). By this time, another clathrin-dependent sorting signal had been identified: the dileucine motif, discovered in 1992 by François Letourneur and Rick Klausner (68). Again, the Bonifacino lab was able to demonstrate an interaction with AP complexes, although in this case they needed to co-express the small (σ) subunit and one of the large subunits, using the yeast three-hybrid system, before they could detect binding (69).

However, neither YXXΦ motifs nor dileucine motifs were able to bind robustly to the entire AP heterotetramer, only to pieces of it. Yet the four subunits of an AP complex are tightly attached to each other and do not normally exist as single proteins or partial complexes. This paradox was finally explained when David Owen and colleagues solved the structures of various AP components by X-ray crystallography. Their first structure was the C-terminal domain of the AP-2 μ subunit with a bound YXXΦ peptide, which showed how the two key amino acids, the tyrosine and the bulky hydrophobic residue, slot into compatible pockets (70). They went on to solve the structure of the entire AP-2 core, which demonstrated that AP-2 is normally in a “closed” conformation with its cargo binding sites obscured (71) (Figure 6d). Eventually, after seven years, the Owen lab managed to solve the structure of

the complete AP-2 core containing two bound peptides, a YXXΦ and a dileucine. The inclusion of the dileucine was unintentional; one of the subunits carried a myc tag for ease of purification, and the LI sequence of the myc tag conveniently slotted into the dileucine binding pockets of the σ subunit. As a consequence, the complex was propped open (Figure 6d), allowing the first glimpse of what an AP complex might look like when it is actively engaged in cargo recognition (72). Presumably, the complex remains closed in the cytosol to prevent promiscuous binding to all of the other proteins in the cell that have YXXΦ or dileucine sequences. But it is not just the cargo binding sites that are inaccessible when AP-2 is in the cytosol; the Owen lab recently showed that the "clathrin box" (the clathrin binding site on the long flexible linker of the large β subunit) is buried in the core of the complex when it is in its closed conformation (73). Thus, when free in the cytoplasm, AP-2 and AP-1 keep their binding sites well shielded. However, when they are recruited onto the appropriate membrane, the initial binding event is thought to trigger a conformational change, which enables the complex to interact with both cargo proteins and clathrin, and so kick-starts the process of CCV formation.

Additional adaptors

In the early 1990s, subunits of the COPI coat began to be cloned and sequenced. The Rothman lab had already noted the similarities in size between the components of the COPI and clathrin coats (47, 74), and four of the seven COPI subunits turned out to be distantly related to the subunits of AP-1 and AP-2 (75, 76). This made us wonder whether there might be other heterotetrameric complexes that could be involved in the formation of other types of vesicles. By this time, the human genome project was well underway, so we took advantage of all the sequences that were available in the public database and began to carry out BLAST searches of expressed sequence tags (short stretches of random

cDNAs) to try to find new homologues of the COPI and AP subunits. Juan Bonifacino and Esteban Dell'Angelica had exactly the same idea at exactly the same time, so for several years there was intense competition between our two labs, as we identified the subunits of a third and then a fourth AP complex. Fortunately, we managed to publish more or less simultaneously (77-82), and to remain on good terms.

The reason AP-3 and AP-4 hadn't been found earlier was that they were not detectable in purified CCVs, suggesting that they didn't act together with clathrin. The Kirchhausen lab had recently mapped the clathrin binding sites on AP-1 and AP-2 to the flexible linker regions of their large β subunits (83), so Dell'Angelica and Bonifacino used the comparable region of the AP-3 β subunit in a pulldown assay, to find out whether it might bind to some other type of scaffolding protein that could be taking the place of clathrin. They isolated a prominent 180 kD protein, which they sequenced. Surprisingly, this protein turned out to be clathrin heavy chain itself. Narrowing down the binding site for clathrin led to the discovery of the clathrin box, which has the sequence LLDLD in the β subunit of AP-3, and LLNLD in the β subunits of APs 1 and 2 (84).

So does AP-3 interact with clathrin after all? In mammalian cells, the two show some colocalisation, although it is never as complete as the colocalisation between clathrin and AP-1 or AP-2 (85, 86). In yeast, however, where the AP-3 β subunit lacks a clathrin box, clathrin and AP-3 are thought to act completely independently, based on their non-overlapping mutant phenotypes (87). The availability of a mouse mutant lacking the AP-3 β subunit enabled us to test whether the clathrin interaction was essential for function, by transfecting fibroblasts with either wild-type AP-3 β or a mutant lacking the clathrin box. Both constructs were able to rescue the mutant phenotype, but the rescue was never quite

as good with the clathrin box mutant, and we also couldn't rule out the possibility that the AP-3 complex might have additional clathrin binding sites (88). Thus, the final verdict seems to be that AP-3 can use clathrin in some organisms, but it doesn't absolutely have to.

However, AP-4 doesn't appear to bind clathrin at all, even though all four AP complexes are able to recognise YXXΦ motifs on cargo proteins (89). The completion of the human genome project, and the sequencing of other genomes from a wide range of eukaryotes, revealed that all four AP complexes are widespread and ancient, dating back to pre-LECA times (90) (Figure 7b). BLAST searches didn't pull out any likely candidates for additional AP subunits, so for over ten years we all thought that the inventory was complete (91, 92). However, through the use of structure-based searching combined with wet work, we recently managed to identify a fifth AP complex (93) and a more distant relative called TSET (94). Although both are even more ancient than APs 1-4 (Figure 7b), they have been lost from many organisms. For instance, animals like ourselves have retained only a small fragment of the original TSET complex. Interestingly, this fragment gave rise the muniscin family of proteins, which contribute to clathrin-mediated endocytosis, although their precise role is in some dispute (95-100). The TSET complex has been most extensively characterised by Daniël Van Damme and colleagues (101-103), who showed that it is essential for clathrin-mediated endocytosis in Arabidopsis, unlike the AP-2 complex, which is dispensable.

In addition to the heterotetrameric AP/AP-like like complexes, which can be either clathrin-dependent or clathrin-independent, there are a number of alternative adaptors, generally monomeric, which contribute to clathrin-mediated trafficking by increasing the repertoire of cargo proteins in a CCV (Figure 8a). The mystery of how the LDL receptor gets into coated

vesicles was finally solved when its FXNPXY sorting signal was shown to bind to proteins with PTB (phosphotyrosine-binding) domains, like Dab2 and ARH (104-106). The “PTB” acronym is actually a misnomer, because both Dab2 and ARH preferentially bind to non-phosphorylated tyrosines. Linton Traub’s lab demonstrated that both of these proteins also have binding sites for clathrin and AP-2, and act as cargo-selective adaptors for the LDL receptor and related proteins (107, 108).

Two other families of monomeric adaptors, the GGAs and the stonins, were originally identified as proteins with domains that are homologous to those of AP subunits (91, 109-112). The GGAs have a C-terminal domain derived from the γ subunit of AP-1, while the stonins have a C-terminal domain derived one of the medium subunits (either μ1 or μ2). In mammals, GGAs bind to cargo proteins with a DXXLL motif, including the mannose 6-phosphate receptors and sortilin, all of which function as hydrolase receptors (113). Stonin-2 (the better characterised of the two stonin isoforms in mammals) has so far been found to have only one cargo, synaptotagmin-1 (112, 114). All of these cargo proteins can also bind to AP complexes, raising the question of why GGAs and stonins are needed as well. In Drosophila, null mutations in stonin (Stoned-B) are embryonic lethal (115), indicating a non-redundant function. However, the single GGA gene in flies is dispensable (116). In yeast, there is some functional redundancy between GGAs and AP-1: knocking out either one on its own causes at most a mild defect in endosomal sorting, but knocking out both severely compromises cell viability (117, 118).

PTB domain-containing adaptors, GGAs, and stonins are relatively recent innovations, found only in metazoans or, in the case of GGAs, metazoans and fungi (i.e., opisthokonts) (119, 120). Two other families of alternative adaptors are found in nearly all eukaryotes: the epsinR/epsin family and the CALM/AP180 family (121), which have ENTH (epsin N-

terminal homology) and ANTH (AP180 N-terminal homology) domains respectively. In animal cells, both epsinR and CALM recognise SNAREs as cargo proteins (122-124). There is a huge body of work, stemming from a classic paper published by Jim Rothman's lab in 1993 (125), showing that SNAREs are required for vesicles to fuse with the appropriate target membrane. This means that SNAREs are essential CCV cargo proteins, because without them the vesicles would be "all dressed up with nowhere to go", i.e., non-fusogenic. Structures of the ENTH and ANTH domains with SNAREs bound to them, solved by David Owen's lab in 2007 (126) and 2011 (127), have revealed a completely different type of cargo recognition from the short linear motifs that can exist on multiple proteins (e.g., humans have 2,154 predicted transmembrane proteins with YXXΦ motifs in their cytoplasmic tails (128)). When the cargo protein is a SNARE, the interaction surface is extensive, involving a folded domain with multiple residues in contact with the adaptor. Because the SNAREs don't have to compete with "ordinary" cargo proteins for binding sites on epsinR and CALM, they can be guaranteed "reserved seating" in the vesicle. In fact, this is exactly the type of situation envisioned by Pearse in 1975, when she suggested that the coat might interact with other molecules "to ensure ... transport [of vesicles] to specific cellular locations" (1).

Other machinery

With recent advances in mass spectrometry, many additional components of the coat have been identified (129-138) (Figure 8b). Some of these were found in preparations of purified CCVs, others were found as binding partners for the appendage or "ear" domains of the large subunits of AP-1 and AP-2, and many (including all of the known alternative adaptors) were found using both approaches. We now know that intracellular CCVs contain at least 100 different proteins, with cargo and coat components split approximately 50:50 (135, 139, 140), and the situation is likely to be similar for endocytic CCVs (135). Some of the

uncharacterised coat components may be other types of adaptors; some may play regulatory roles; and some may have more than one function. For instance, the CALM and epsin family proteins act as cargo adaptors, but they have also been implicated in membrane deformation (141, 142), so they may work together with clathrin to shape the vesicle and control its size.

But although proteomic analyses can provide a list of proteins associated with budded-off CCVs, there are other proteins that contribute to CCV formation that might be missed, because they act too early or too late. One way of finding such proteins is to carry out screens for mutants that are deficient in clathrin-mediated trafficking, similar to the approach adopted by the Schekman lab for the secretory pathway. In the mid-1980s, Howard Riezman's lab began screening yeast cells for defects in endocytosis, and discovered among other things a role for the actin cytoskeleton (143, 144) and a new internalisation signal, ubiquitin (145). Fifteen years later, Barth Grant and colleagues looked for genes in *C. elegans* by monitoring the uptake of yolk proteins in oocytes (the same pathway that Roth and Porter described in mosquitoes some 35 years earlier). The machinery they identified includes the worm yolk protein receptor (146), which is a member of the LDL receptor family, and a nucleotide exchange factor for the small GTPase rab5 (147). With the advent of siRNA libraries, this approach has become feasible in mammalian cells. High throughput screens have revealed that a number of protein kinases contribute to clathrin-mediated endocytosis in mammals (148, 149), as well as other machinery whose function may be indirect, such as the vacuolar ATPase (V-ATPase) (150), which is needed to maintain sufficient levels of cholesterol in the plasma membrane (151, 152).

Another very powerful approach for analysing clathrin-associated machinery is live cell imaging. Jim Keen's lab was the first to visualise the dynamic behaviour of clathrin in 1999

(153), and since then many papers have been published using increasingly sophisticated technology. One particularly elegant system was devised by Christien Merrifield and colleagues, who transfected cells with transferrin receptor coupled to superecliptic pHluorin, a highly pH-sensitive derivative of GFP. By cycling the pH of the medium between 7.4 and 5.5 every second, they were able to determine precisely when a clathrin-coated pit pinched off as a vesicle, because the transferrin receptor suddenly became visible at pH 5.5 (154, 155). Co-transfection with various mCherry-tagged coat components enabled them to monitor the behaviour of each protein relative to the scission event, to obtain its "recruitment signature" (155). For a comprehensive analysis of coated vesicle dynamics, Sandy Schmid's lab, together with Gaudenz Danuser, used automated particle-tracking software to image tens of thousands of clathrin-coated structures. After carrying out mathematical modelling to identify kinetically distinct populations, they then depleted or overexpressed particular proteins and repeated their analyses to look for changes in the relative amounts of the different populations (156). The Kirchhausen lab managed to image single molecules as they were being recruited onto the plasma membrane, and then used their images to reconstruct "the first five seconds in the life of a clathrin-coated pit" (157).

These complementary approaches have led to conclusions that are largely in agreement with each other. Together, they provide strong support for the "coincidence detection" model of coat protein recruitment, where multiple low affinity interactions combine to get the right machinery to the right place at the right time (or as Pearse proposed, "to ensure formation of vesicles at particular sites on the membrane" (1)). For endocytic CCVs, a particularly important interaction is with phosphatidylinositol 4,5-bisphosphate (PIP2) (158-160). Because PIP2 is primarily generated at the plasma membrane, this interaction helps to target AP-2 and associated proteins to the right compartment. Most of the initial AP-2 recruitment events are non-productive, with lots of rapid cycling on and off the plasma

membrane (157) (Figure 9a). However, electrostatic interactions between PIP2 and the μ subunit may open up the complex and expose its multiple binding sites (72) (Figure 9b). The open conformation is further stabilised by binding to cargo and clathrin (156, 157) (Figure 9c-d), committing the coated pit to go on to become a coated vesicle.

Once the formation of the coated pit is underway, other machinery is added on. Recruitment of the GTPase dynamin peaks just 2-4 seconds before scission (155) (Figure 9g), although low levels of dynamin are associated with the coated pit at earlier stages (155, 156). Dynamin is a particularly important component of the machinery, because it severs the neck of the coated pit to give rise to a coated vesicle. In 1995, Jenny Hinshaw and Sandy Schmid showed that dynamin can self-assemble into rings (161), while Pietro De Camilli and colleagues discovered that in isolated nerve terminals, the non-hydrolysable GTP analogue GTPγS caused the plasma membrane to form long tubular invaginations, decorated with closely spaced rings of dynamin and often capped with a clathrin-coated bud (162). These studies immediately suggested a mechanism for constricting the collar of the coated pit, although precisely how constriction leads to fission is less clear (163). Another enigma is how dynamin is recruited at exactly the time when it is needed. An actin-dependent feedback loop has been suggested (164), but studies with inhibitors like latrunculin indicate that unlike yeast, many mammalian cells do not require actin for endocytosis (165). Another possibility, suggested by Volker Haucke and colleagues, is that recruitment of SNX9 (which itself may be facilitated by production of PI(3,4)P2 via the PI 3-kinase PI(3)K C2α) in turn facilitates the recruitment of dynamin (166). However, there is a discrepancy between the Merrifield and Haucke labs about which is recruited first, SNX9 or dynamin, with the Merrifield lab reporting that SNX9 recruitment peaks after scission, not before (155).

In any case, immediately after scission, there is recruitment of uncoating machinery like auxilin/GAK and OCRL1 (155) (Figure 9h). Again, it is not clear how these events are timed so precisely. Auxilin, which is a cofactor for the “uncoating ATPase” Hsc70, preferentially binds to PI4P over PIP2, so one suggestion is that its recruitment coincides with changes in the phosphoinositide composition of the vesicle just it dissociates from the plasma membrane (167). This is consistent with the observation that the PIP2 5-phosphatase synaptojanin has a similar recruitment signature to dynamin, peaking just before scission (155); however, OCRL1 is also a PIP2 5-phosphatase, and yet it has exactly the same recruitment signature as auxilin (155). So there is still a lot that we don’t understand about how the recruitment of so many different molecules is orchestrated. Probably the best way to visualise the order of events in CCV formation is to look at the beautiful computer-generated animation (complete with orchestration) made by Janet Iwasa and Tommy Kirchhausen (www.youtube.com/watch?v=o_EUHu4OJus).

To what extent can we extend the observations on clathrin-mediated endocytosis to clathrin-mediated intracellular trafficking? Endocytosis is much easier to visualise, not only because it can be followed using probes added to the outside of the cell, but also because it is amenable to TIRF (total internal reflection fluorescence) imaging. However, studies from the Kirchhausen lab, making use of a 3D tracking method, have revealed that AP-1-containing clathrin carriers are somewhat shorter-lived than those that contain AP-2 (45 ± 16 sec vs. 53 ± 18 s) and appear to be dynamin-independent (86). Perhaps the major difference is that the intracellular pathway does not make use of the plasma membrane-specific phosphoinositide PIP2. A different phosphoinositide, phosphatidylinositol 4-phosphate (PI4P), has been implicated in AP-1 recruitment (168), but probably the most important interaction is with the small GTPase Arf1. The Bonifacino and Hurley labs showed that adding GTP-bound Arf1 to the AP-1 core enabled cargo binding in vitro, and

the crystal structure of AP-1 in complex with ARF1-GTP revealed that the AP-1 had adopted an open conformation (169). However, Arf1 also facilitates the recruitment of AP-3, AP-4, and COPI, but onto different membranes, so once again recruitment must be by coincidence detection rather than by a single interaction. In the case of AP-1, there are several studies showing that cargo plays an important role (170-173).

Multiple pathways

The archetypal clathrin-mediated pathway is endocytosis. However, clathrin-independent endocytic pathways also exist, although they are much less well understood (174). In some cell types, clathrin-dependent endocytosis appears to be the dominant pathway (175, 176), but other pathways can be upregulated to compensate if the clathrin pathway is switched off. For instance, in 1995 the Schmid lab expressed a temperature-sensitive dynamin mutant in HeLa cells. They found that immediately after shifting to the non-permissive temperature, endocytosis was reduced by ~50%. However, after an hour there was actually an increase in endocytosis, and after 12 hours, endocytosis was exactly the same as in control cells (177). This indicates that cells are able to sense whether there is too much or too little plasma membrane and to respond accordingly, although without clathrin and adaptors, uptake of cargo is likely to be non-selective.

A wide range of cargo proteins can be accommodated in endocytic CCVs, including receptors for growth factors (178). Unlike “housekeeping” proteins such as the receptors for transferrin and LDL, which are endocytosed constitutively, growth factor receptors are only endocytosed when they are activated by ligand binding. Their sorting signals can be much more complex than those in other cargo proteins. In 2010, Sasha Sorkin and colleagues carried out a heroic study on the EGF receptor, in which they mutated 21 lysines to arginines (interestingly, some of these are substrates for acetylation rather than

ubiquitylation), and also got rid of both a YXXΦ motif and a dileucine motif, but found that they still hadn't quite abolished the receptor's ability to be internalised into CCVs (179).

Initially everybody assumed that endocytosis of activated growth factor receptors was a way of preventing too much signalling (180), and there is no question that this is part of the story. However, endocytosis can also be used to bring signalling molecules into contact with new substrates (181). For instance, SARA (SMAD anchor for receptor activation) is recruited to PI3P-containing endosomes via its FYVE domain, where it meets up with TGF-β receptors that have been internalised by clathrin-mediated endocytosis (182). Eventually, through a signalling cascade involving several downstream effectors, this interaction leads to changes in gene expression.

Because of the links between endocytosis and signalling, it is not surprising that there are also links with embryonic development (183). Several components of the endocytic machinery, including the PTB domain-containing adaptors Numb and Disabled, were originally identified in Drosophila screens for developmental mutants (184, 185). Dynamin also made its first appearance in Drosophila, in a 1973 screen by David Suzuki's lab for temperature-sensitive mutations causing paralysis (186), where one of the genes, *shibire*, turned out to encode dynamin (187, 188). A follow-up study showed that brief heat pulses applied at various stages during embryogenesis "caused a broad spectrum of phenotypically distinct defects" (189), and the temperature-sensitive *shibire* fly has been a useful tool for developmental biologists ever since. Two widely researched signalling pathways that make use of endocytosis (although there is still some controversy as to how) are the Notch pathway and the Wingless pathway, both of which were first found in Drosophila screens for odd-looking mutants (190, 191) but are now of clinical interest because they are often dysregulated in cancer (192, 193). There are also examples of cells

using clathrin-mediated endocytosis for specialised functions, such as synaptic vesicle recycling in neurons (5) and MHC class II presentation in cells of the immune system (194-196).

The AP-1 pathway has been harder to dissect than the AP-2 pathway, in part because the “donor” compartment is difficult to define. Do the vesicles bud from the TGN, some type of endosome, or both? And to what extent is the TGN also some type of endosome (197)? Moreover, where do the vesicles go? As Werner Franke pointed out in a 1974 paper, coated vesicles “do not come with small arrows that tell us unequivocally in what direction they are going" (198). Knockdowns and knockouts have confirmed that AP-1 facilitates the trafficking of newly synthesized lysosomal hydrolases bound to mannose 6-phosphate receptors (199, 200), a pathway worked out by Stuart Kornfeld’s lab in the 1980s (201). If AP-1 is depleted or deleted, the hydrolases get secreted instead of delivered to lysosomes. Yet in other respects the knockdown and knockout phenotypes have been surprisingly mild (139). But is this a true reflection of the AP-1 pathway? We know from studies on temperature-sensitive clathrin and dynamin mutants that cells can often adjust to the gradual loss of an important protein (39, 177). To investigate what happens if AP-1 is lost quickly, we developed the “knocksideways” system, which rapidly diminishes the available pool by using a small molecule to reroute it to mitochondria (202). This produced a much stronger phenotype, with nearly 100 coat and cargo proteins depleted two-fold or more from isolated CCVs (139). Interestingly, all of the well-characterised transmembrane cargo proteins turned out to be itinerants: proteins that move about between different post-Golgi compartments, rather than proteins that reside at a fixed address. These data, together with earlier studies (203, 204), support a role for AP-1, and for clathrin-mediated intracellular trafficking in general, in a bidirectional cycling route between the TGN and endosomes. In contrast, knocking the GGAs sideways only depleted lysosomal hydrolases and their

receptors from the CCV fraction, supporting a role for GGAs as cargo-selective adaptors for the TGN-to-endosome leg of the AP-1 pathway (139).

But AP-1 and clathrin can also be used for polarized trafficking of proteins to the plasma membrane. Vertebrates have an epithelial cell-specific isoform of the AP-1 μ subunit, which contributes to basolateral sorting (205-207), and the Bonifacino lab showed that several basolateral membrane proteins preferentially bind to this isoform (208). In addition, AP-1 (without a special subunit) and clathrin contribute to polarity in neurons, in both worms (209) and mice (210), by directing proteins to the somatodendritic plasma membrane. It is difficult to reconcile these findings with everything else we know about the role of AP-1 in trafficking ***away*** from the plasma membrane. How could AP-1 also be involved in trafficking ***to*** the plasma membrane? One possibility is that AP-1 acts at a post-Golgi compartment to traffic proteins away from the ***wrong*** part of the plasma membrane (i.e., apical or axonal), after which some other type of (non-clathrin) machinery can ensure that the proteins get to the ***right*** part of the plasma membrane (i.e., basolateral or somatodendritic) (211).

Two other pathways that make use of clathrin are regulated secretion and multivesicular body (MVB) formation. It has been known since the mid-1980s that immature secretory granules often have partial clathrin coats associated with them (212, 213), and Sharon Tooze's lab showed that these coats are also positive for AP-1 (214). Initially it was assumed that the function of the clathrin was to facilitate the packaging of granule proteins. However, subsequent studies making use of immunogold EM revealed that the clathrin patches contain AP-1 cargo proteins, which normally cycle between the TGN and endosomes (215, 216). These findings indicate that the immature granule should be viewed as more of an outpost of the TGN, and that AP-1 and clathrin facilitate the removal of non-

granule proteins. In other words, the role of clathrin and AP-1 in regulated secretory cells is not all that different from their role in other types of cells.

However, the role of clathrin in MVB formation appears to be very different from its all other roles. Electron-dense plaques were first observed on the limiting membrane of MVBs nearly 50 years ago, when Marilyn Farquhar pointed them out (217), but their composition was unknown until the late 1990s, when Judith Klumperman and colleagues showed that they could be labelled with anti-clathrin antibodies (218, 219). As Klumperman wrote in a 2002 paper, “unlike clathrin coats at other sites in the cell, the endosomal coat consists of two layers, a thin and highly electron-dense layer closely opposed to the limiting membrane of the endosomal vacuole, and a second, more fuzzy and less electron dense layer facing the cytoplasm” (220). In fact, the coats on MVBs look so different from other coats that it is possible that the clathrin doesn’t even form a typical polyhedral lattice. Harald Stenmark’s lab showed that clathrin is recruited to the MVB by Hrs (221, 222), a component of the ESCRT-0 (endosomal sorting complex required for transport-0) complex. ESCRTs package ubiquitylated membrane proteins into intralumenal vesicles, which have the opposite topology to CCVs in that they bud away from the cytoplasm rather than towards it. Thus, the current view is that the function of MVB-associated clathrin is not to shape the membrane, but rather to form a patch that sequesters ubiquitylated proteins, which can then be handed over to other components of the ESCRT machinery (223).

Disease

There are several examples of genetic disorders in humans caused by mutations in clathrin-dependent cargo proteins. Familial hypercholesterolemia, caused by mutations in the LDL receptor, is probably the most famous of these. Others include Menkes disease and Wilson disease, caused by mutations in the copper-transporting ATPases ATP7A and

ATP7B, respectively (139, 224, 225). Both ATP7A and ATP7B have dileucine-containing sorting signals, which bind to AP-1 and help to localise them to the TGN and endosomes, where they provide copper as a cofactor for various enzymes (226, 227). However, high copper concentrations cause the transporters to be translocated to the plasma membrane, where they detoxify the cell by pumping out excess copper. This is concomitant with the transporters no longer being packaged into CCVs, suggesting that their dileucine motifs somehow become masked (139).

Another protein reported to be trafficked by clathrin is amyloid precursor protein (APP), the progenitor of the amyloid plaques found in the brains of Alzheimer's patients (228-230). There is no doubt that alterations in the cell's trafficking machinery can increase the frequency of the proteolytic event that makes the protein amyloidogenic (231), and a genome-wide association study identified the clathrin adaptor CALM as a susceptibility locus for Alzheimer's disease (232). However, the situation is complicated by the involvement of other proteins that are also trafficked in a clathrin-dependent manner, such the APP sorting receptor SORL1 (233, 234), and the β-secretases, which cleave APP (234-236). A further complication is that clathrin-mediated trafficking can also be used to clear away the amyloidogenic peptide, and thus prevent the build-up of plaques (237),

Mutations in coat proteins tend to be less common than mutations in cargo proteins. Experiments on animals indicate that null mutations in single copy genes encoding components of clathrin, AP-1, or AP-2 are likely to be lethal (40, 199, 238, 239). However, in some cases there are two or more genes encoding different isoforms of a particular subunit, and null mutants are viable. Patients have now been identified with mutations in all three of the closely related genes encoding the AP-1 small subunit, *AP1S1*, *AP1S2*, and *AP1S3* (240-242). Mutations in both *AP1S1* and *AP1S2* result in severe intellectual

disability, and mutations in both *AP1S1* and *AP1S3* affect the skin, but for the most part the three phenotypes are different. It has been proposed that these differences are due to differential sorting of particular cargo proteins, such as ATP7A/ATP7B and sortilin, both of which have dileucine motifs (243, 244). However, there is at present no evidence that these dileucines preferentially interact with one isoform over the other, so another possibility is that the different phenotypes are due to the relative expression levels of the three isoforms in various tissues.

Vertebrates have two genes encoding different isoforms of clathrin heavy chain, *CLTC* and *CLTCL1* (245). In humans, these genes are located on chromosomes 17 and 22 respectively, so the proteins are called CHC17 and CHC22. Most human tissues express more *CLTC* than *CLTCL1*, sometimes by as much as two orders of magnitute, but in skeletal muscle the two are expressed at comparable levels (www.proteinatlas.org). CHC22 has been implicated in the trafficking of the insulin-responsive glucose transporter GLUT4 (246); however, patients have recently been identified with a probable loss-of-function mutation in *CLTCL1* who have normal glucose tolerance but severe intellectual disability and an inability to feel pain (247). The molecular basis for this phenotype is still unknown, nor is it clear why some animals, including mice and pigs, have lost the *CLTCL1* gene entirely, with no apparent phenotype.

Unlike AP-1 and AP-2, APs 3, 4, and 5 are not essential for viability in animals. There are naturally occurring mutations in AP-3 in Drosophila (80, 248-250) and mice (251, 252), as well as in humans with Hermansky Pudlak syndrome (253), a disorder mainly affecting lysosome-related organelles. There are also patients with mutations in AP-4 or AP-5, both of which cause hereditary spastic paraplegia. It is not clear precisely why, because we still don't know for sure what AP-4 and AP-5 actually do, although AP-4 has been implicated in

polarised sorting and AP-5 in lysosome maintenance (211, 254-258) (Figure 7a). So far, there is only one example of a genetic disorder involving an alternative adaptor. Autosomal Recessive Hypercholesterolemia is caused by mutations in the cargo-selective adaptor ARH, which recognises the LDL receptor (105, 108), resulting in a very similar clinical phenotype to that of FH, caused by mutations in the receptor itself.

In addition to genetic disorders, there are links between clathrin-mediated trafficking and infectious diseases. The first of these was described in 1980, when Ari Helenius and colleagues showed that Semliki Forest virus hijacks clathrin-coated pits to gain entry into the host cell (259). Since then, many other viruses and even bacteria have been shown to exploit clathrin for infectivity (260, 261). But there are other links as well. For instance, two proteins encoded by all strains of human and simian immunodeficiency virus (HIV and SIV), Nef and Vpu, contribute to the pathogenesis of the virus by modifying the surface of infected cells. Proteins that are downregulated include MHC class I (262), the viral co-receptor CD4 (263), and tetherin, a protein that prevents the release of viral particles by keeping them attached to the plasma membrane (264). In all three cases, clathrin and AP complexes have been shown to be involved (265-268).

The next forty years

We've come a long way since Barbara Pearse first identified clathin 40 years ago, but there are still many open questions. For instance, we know very little about how all the clathrin-mediated events are regulated and coordinated. How does the cell "know" how much endocytosis or intracellular trafficking it needs, so as to maintain each compartment at a steady state? How are the fundamental pathways adapted in different cell types, and how are they coupled to all the other events taking place in the cell and organism?

Even at the nuts-and-bolts level, where the most progress has been made, there is still a lot we don't know. For instance, how does the clathrin coat assemble? Does it first makes a flat lattice of hexagons, which then rearranges itself into a curved one as hexagons convert into pentagons (as proposed by Kanaseki and Kadota in 1969 (3)), or is curvature built into the lattice from the start? There is no question that flat lattices exist (see Figure 3a), but are they precursors of coated vesicles, or are they artefacts, produced when a cell is tightly stuck to a glass cover slip (269)? Because of the extensive interactions between the clathrin legs, with four triskelia contributing to each edge (Figure 3f), converting hexagons into pentagons is not trivial; in fact, the entire lattice would have to be taken apart and put back together again (270). However, a very recent correlative light and electron microscopy study from the labs of Marko Kaksonen and John Briggs suggests that this is in fact what happens. Using fluorescence recovery after photobleaching, Briggs and Kaksonen showed that there is rapid exchange of clathrin throughout the lifetime of a coated pit, right up until the burst of dynamin recruitment (Figure 9g) (271).

Another ongoing debate is whether clathrin is really what curves the membrane *in vivo*. Is it strong enough to be the driving force (272-274), or does it mould itself around a membrane that is being reshaped by some other mechanism? Clathrin's ability to form a cage while attached to a lipid bilayer may be sufficient to bend the membrane of an artificial liposome (Figure 3h (27)). However, even in this minimal *in vitro* system, the clathrin-binding linker proteins attached to the liposome probably contribute as well, by crowding together and creating steric pressure (275, 276). (This is somewhat akin to the "natural repulsion of the outer ends of the bristles" proposed by Roth and Porter over 50 years ago (4), except that the proteins aren't bristles but disordered polypeptides with large hydrodynamic radii (276).) And in a living cell, the other side of the membrane is normally densely packed with cargo, driving the curvature in the opposite direction (276, 277). Increasing the rigidity of the

scaffold may help, and this is one of the roles that has been attributed to the clathrin light chains (277-279). The right lipid environment is also important: depleting cholesterol from the plasma membrane prevents coated pits from curving more than 180° (150-152). Other membrane bending mechanisms have been invoked, including driving a “wedge” into the membrane (e.g., the amphipathic helix of epsin (141)) and attaching an inherently curved protein (e.g., BAR domain-containing proteins like the muniscins (96)). And of course, there is no reason why all of these mechanisms couldn’t contribute to vesicle formation *in vivo* (280).

There are also questions about how certain types of cargo are sorted. For instance, intracellular CCVs contain a lot of SNAREs that don’t seem to be able to bind to epsinR or CALM (139), so are there other SNARE-adaptor interactions we don’t know about? And how many of the pieces of the core machinery have additional functions (281)? Clathrin heavy chain can “moonlight” as a component of the mitotic spindle (282), while some of the other coat components, like epsin and CALM, can get inside the nucleus (283). What are they doing there?

An even more fundamental question is, where did all this machinery come from? Phylogenetic analyses indicate that TSET predates the five AP complexes, and that the last AP complexes to emerge were AP-1 and AP-2, the two that are clathrin-dependent (94) (Figure 7b). TSET can also act together with clathrin, at least in plants (103), but in some respects it is more similar to COPI than to an AP complex, and its core heterotetramer is tightly associated with two additional proteins, which have the β-propeller plus stacked α-helical hairpin architecture, and are most closely related to the two largest subunits of COPI (94). So if TSET has its own associated scaffold, does it really need clathrin as well? One possibility is that TSET started off as a very early clathrin-independent coat, but then

clathrin arrived on the scene as a “Johnny-come-lately”. Clathrin may have been so good at its job that TSET was rendered redundant in a number of lineages, including the one that gave rise to humans, while in other organisms TSET may have evolved ways of cooperating with clathrin. Of course, these ideas are very speculative because they are based on attempts to reconstruct events that occurred some two billion years ago. However, the recent exciting discovery of a phylum of archaea “that bridge the gap between prokaryotes and eukaryotes”, and the tantalising suggestion that that there may be “even closer relatives of eukaryotes”, raise the possibility that in a few years we may be able to find out a lot more about the evolution of coat components (284). This in turn may tell us about how intracellular membrane compartments were first generated, during the prokaryote-to-eukaryote transition (50).

Thus, one short paper in 1975 spawned an entire field. As of August 2015, over 8,000 papers on clathrin have been published, with the number per year going up and up (Figure 10). Authors of these papers include Nobel laureates like Brown, Goldstein, Rothman, and Schekman; and the field has taken off into all sorts of unexpected directions, including medical ones. But Barbara Pearse didn’t start working on coated vesicles because she thought they would lead to benefits in health and wealth. She was driven entirely by curiosity. She wanted to know what coated vesicles were made out of, what they did, and how they worked. Yet like much of basic science, her work and the work that followed has turned out to have clinical relevance, impacting on diseases as diverse as atherosclerosis, cancer, dementia, influenza, and AIDS. There are likely to be more medical implications to come. For instance, there is a lot of interest in the use of nanoparticles to deliver therapeutic agents to the right target (285). However, in order to get these agents to work, we will need to know as much as we can about the cell’s gatekeepers, and this can only

come about through an even deeper understanding of clathrin and its associated machinery.

Happy anniversary, clathrin, and many happy returns!

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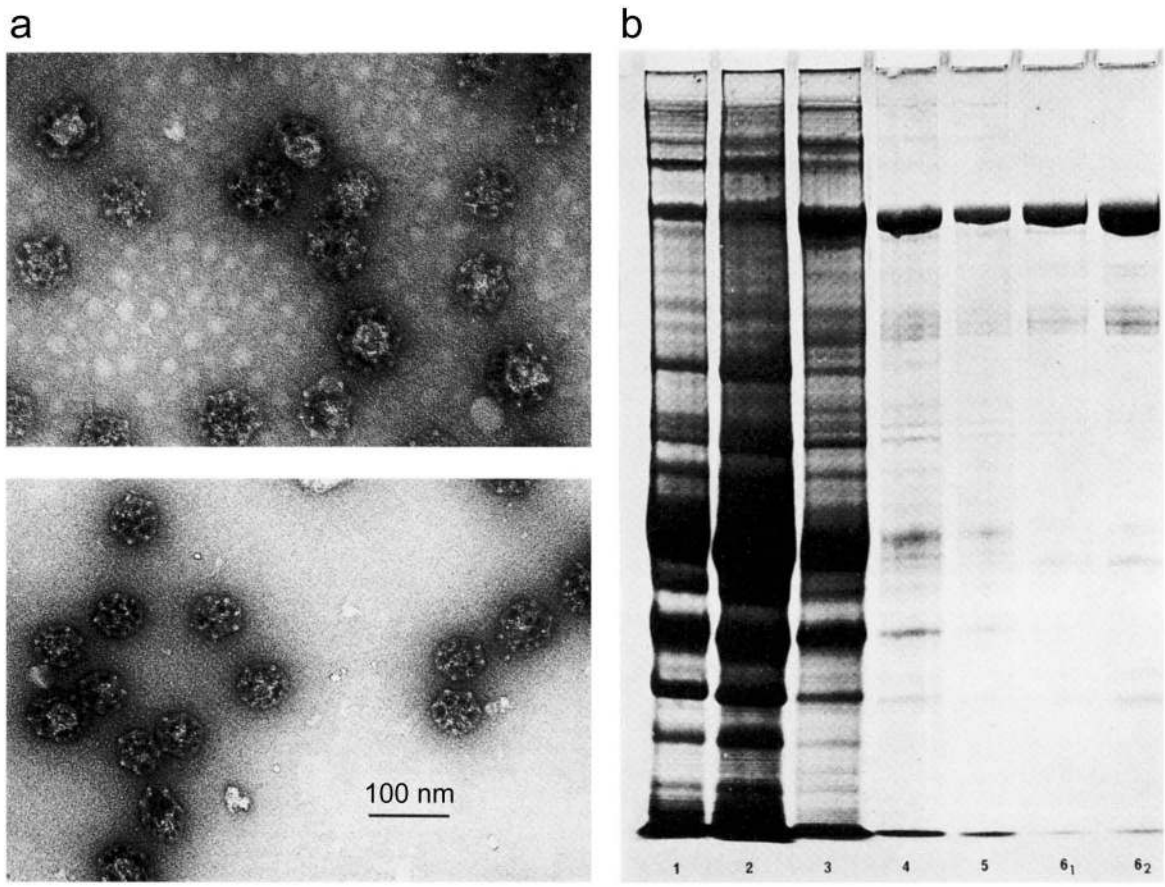

Figure 1. Figures from the original 1975 paper by Barbara Pearse (1). a, Negatively stained purified CCVs on a coated EM grid (top) and suspended in stain (bottom). b, SDS polyacrylamide gel electrophoresis of successive stages in the purification of CCVs from pig brain. As the CCVs become more enriched, the high molecular weight clathrin band becomes more predominant.

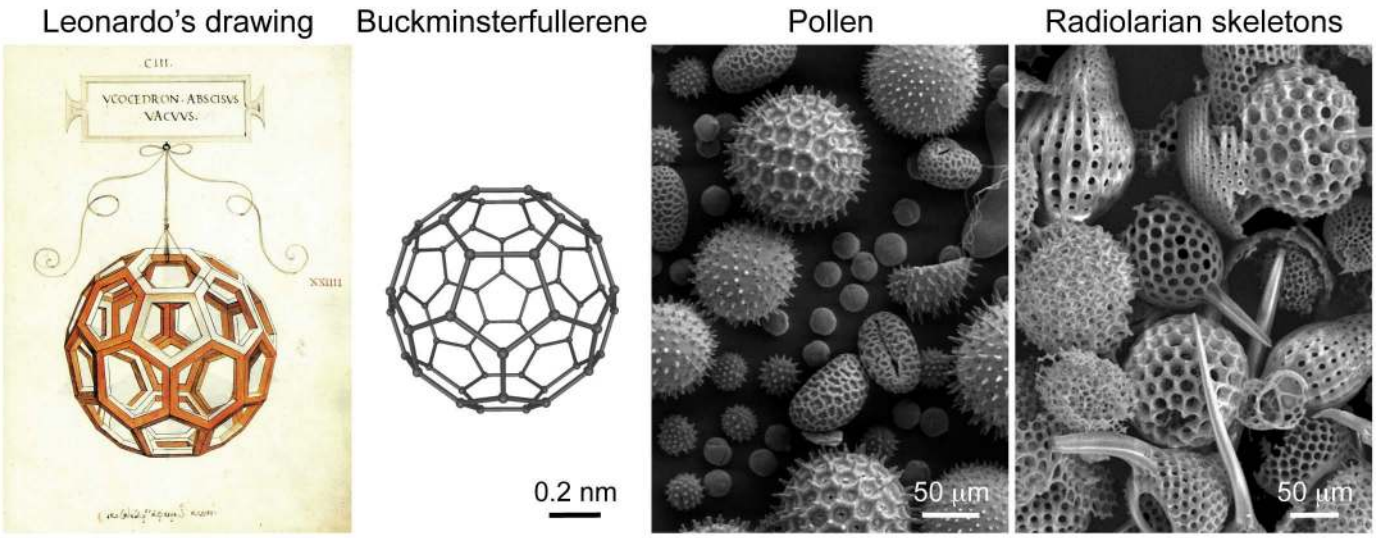

Figure 2. Other structures resembling a clathrin coat. From left to right, Leonardo da Vinci's drawing of a truncated icosahedron (http://www.georgehart.com/sculpture/leonardo-project.html), model of Buckminsterfullerene (http://en.wikipedia.org/wiki/Fullerene), and scanning electron micrographs of pollen grains (http://en.wikipedia.org/wiki/File:Misc_pollen.jpg) and the silicon-containing skeletons of radiolarians (image courtesy of the University of Dayton Nano-scale Engineering Science and Technology Laboratory http://www.nestlaboratory.com/content.php?id=11).

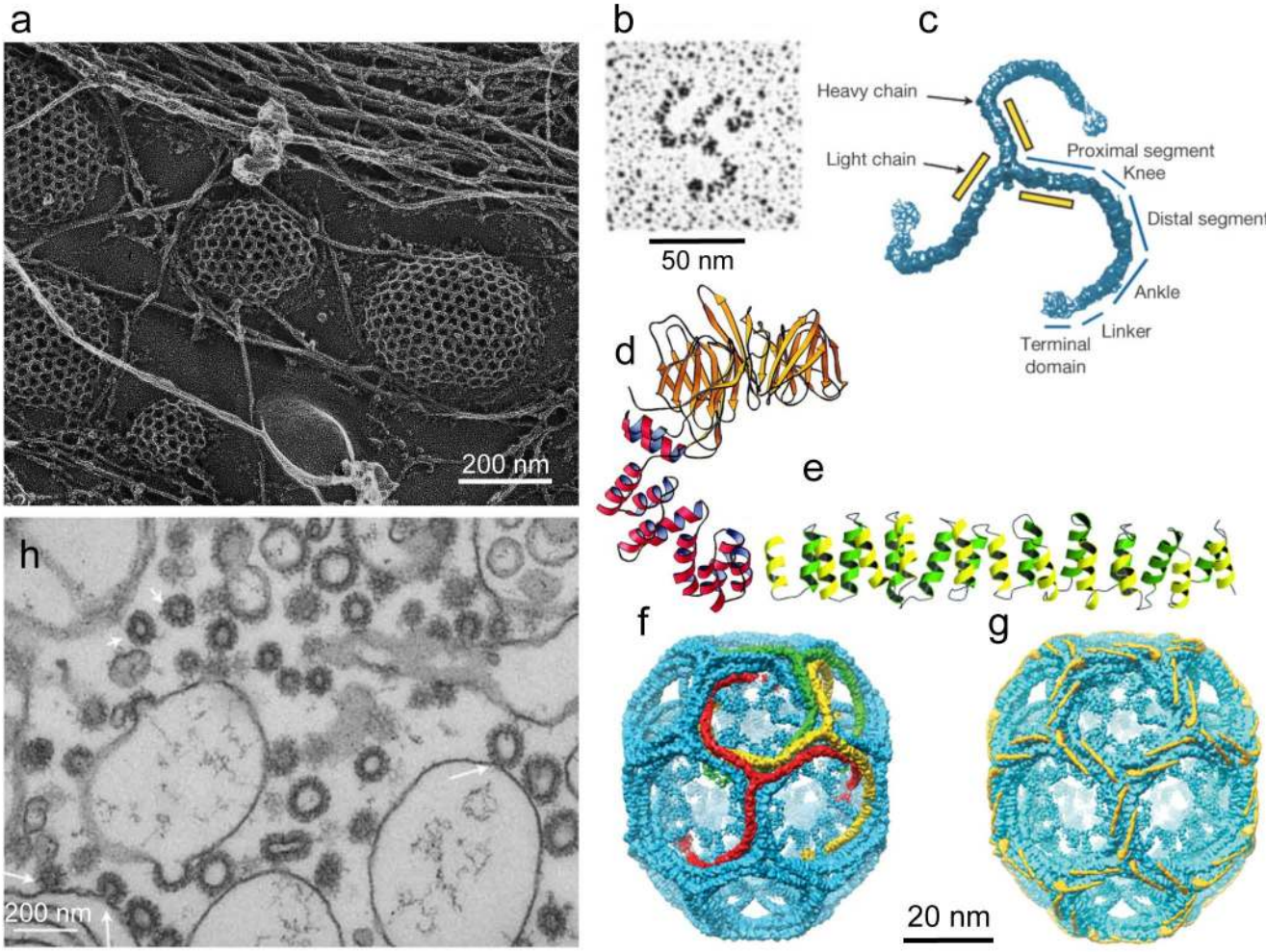

Figure 3. Clathrin structure. a, One of John Heuser's electron micrographs of an "unroofed" cell, showing clathrin-coated pits budding from the plasma membrane (http://www.heuserlab.wustl.edu/images/galleries/classics/pages/17.shtml). b, Electron micrograph of a clathrin triskelion from Ungewickell and Branton's 1981 paper (17). c, Clathrin triskelion, showing the various domains (25). d, Structure of the clathrin N-terminal "foot" and "ankle" (19). e, Structure of a portion of the clathrin "leg" (20). f, Image reconstruction of a clathrin hexagonal barrel, showing the heavy chains only (25). g, Hexagonal barrel showing the positions of the clathrin light chains in yellow (25). A subsequent X-ray crystallography study showed that the light chains are in fact longer than in the reconstructed image, and cover each heavy chain "thigh" more completely (286). h, Electron micrograph of purified clathrin forming spherical buds on a liposome (27).

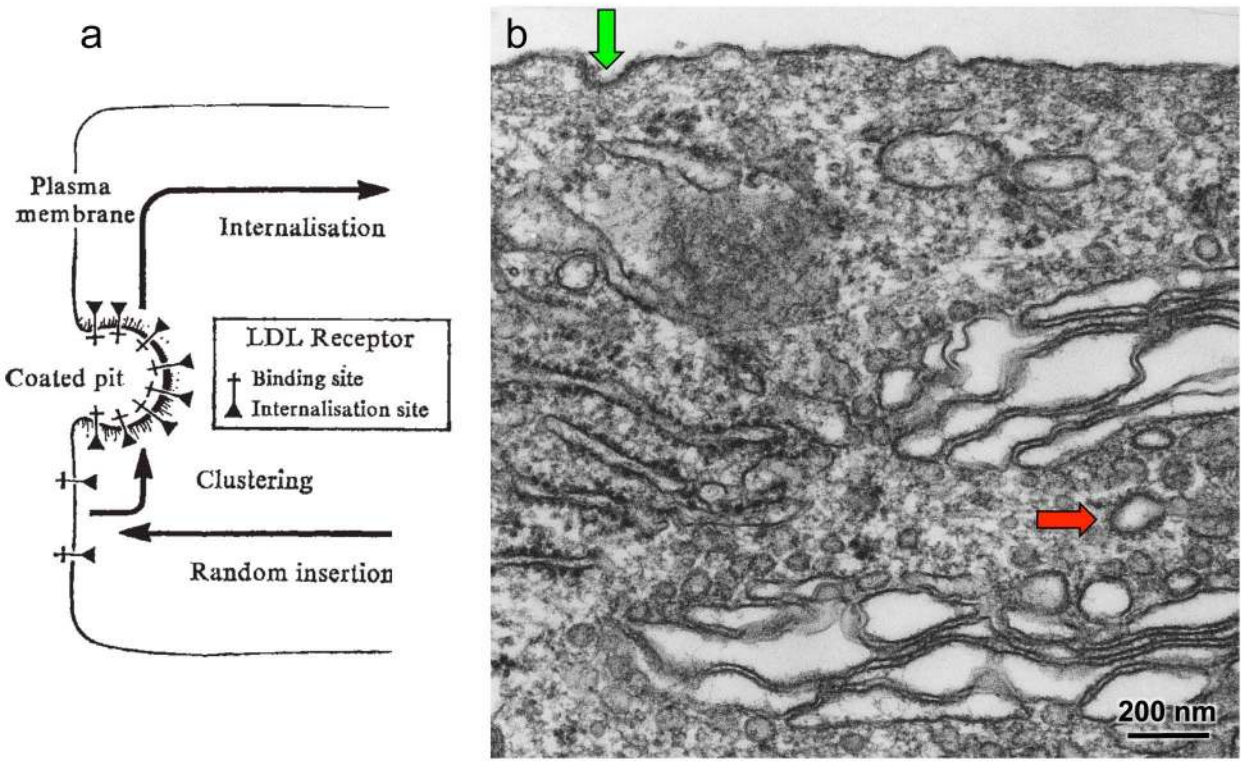

Figure 4. Clathrin function. a, Schematic diagram from a 1977 paper by Anderson, Goldstein, and Brown, showing how the LDL receptor might use an “internalisation site” to become incorporated into a coated pit (12). b, Electron micrograph of a granulosa cell showing two clathrin-coated budding profiles, one at the plasma membrane (green arrow) and one at an intracellular membrane near the Golgi apparatus (red arrow).

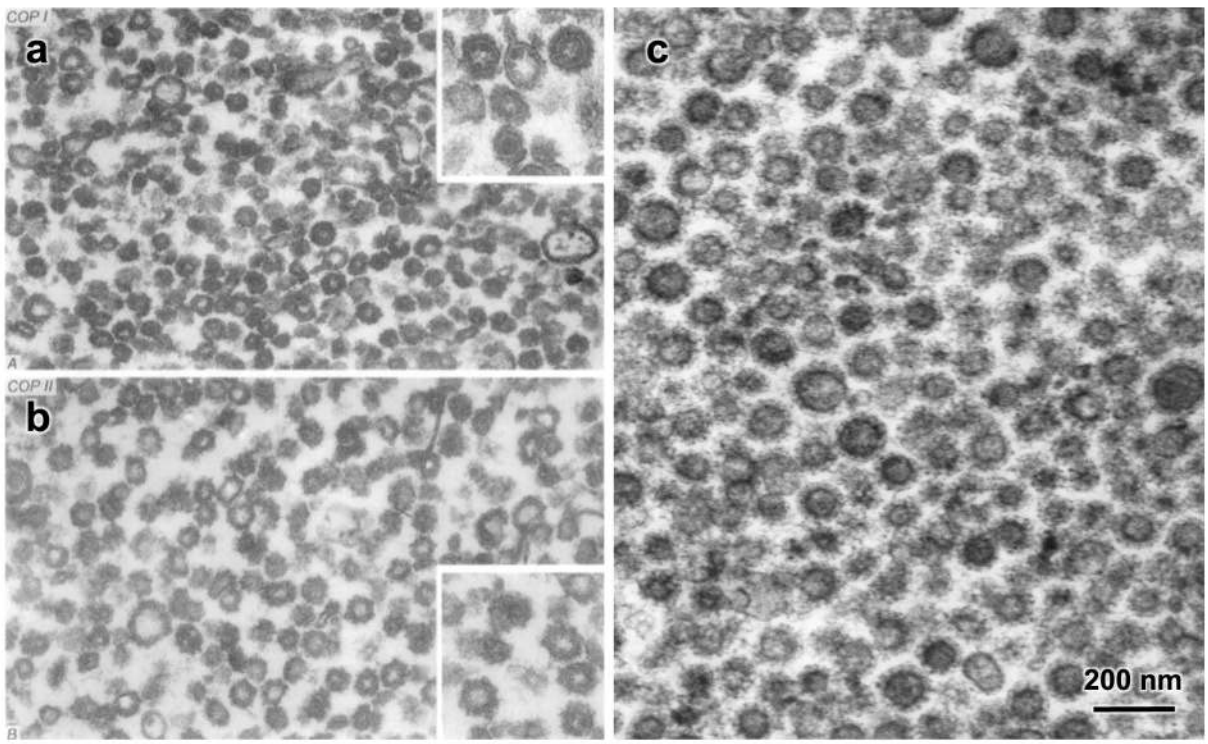

Figure 5. Different kinds of coated vesicles. Electron micrographs of thin sections of purified COPI-coated vesicles (a) (287), COPII-coated vesicles (b) (287), and clathrin-coated vesicles (c).

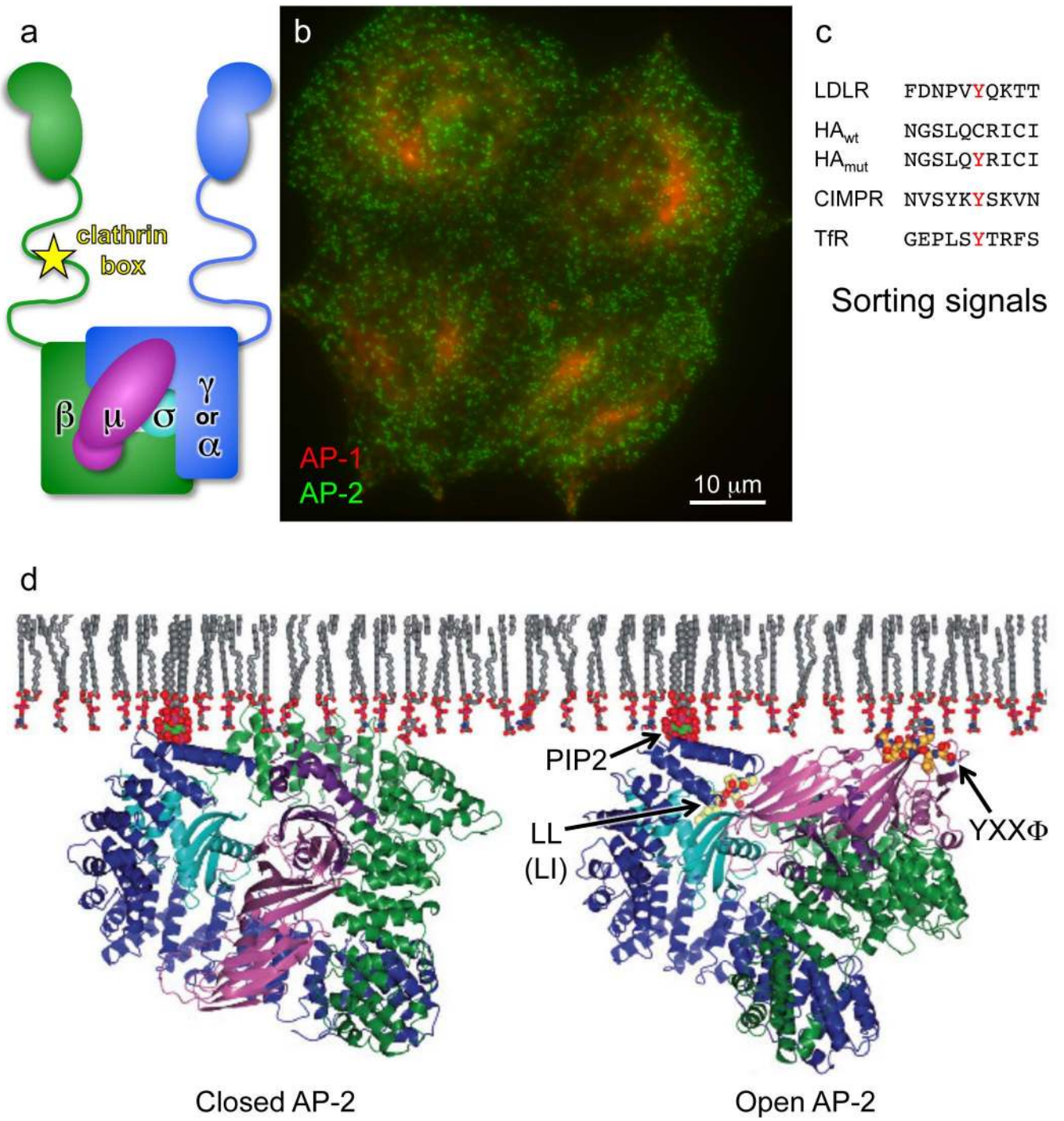

Figure 6. AP complexes and sorting signals. a, Schematic diagram of an AP complex, showing the four subunits and the clathrin box. b, Immunofluorescence micrograph of HeLa cells double labelled for AP-1 and AP-2. c, Sorting signals on several clathrin-dependent cargo proteins, with the key tyrosine residues indicated in red. d, Structure of an AP-2 core in its closed and open forms, with the same colour scheme for the four subunits as in (a). In the open conformation, the binding sites for both the YXXΦ motif and the dileucine motif are accessible (72).

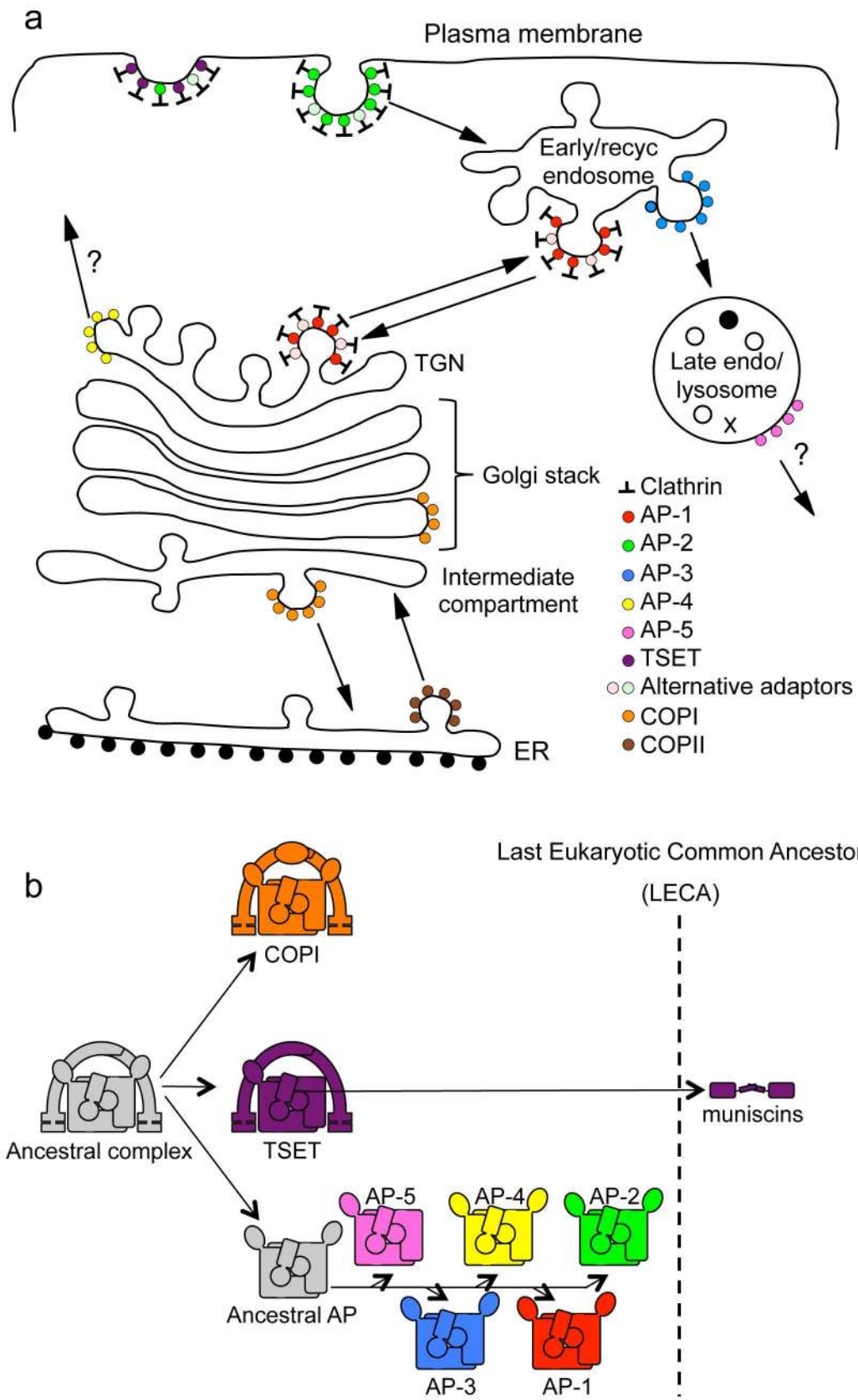

Figure 7. Clathrin-associated machinery and how it evolved. a, Diagram of a cell showing clathrin, APs and other complexes including the COPI and COPII coats, and alternative adaptors. The TSET-mediated endocytic pathway does not occur in animals. b, Proposed evolution of the seven related heterotetramers: the five AP complexes, COPI, and TSET. Modified from (94).

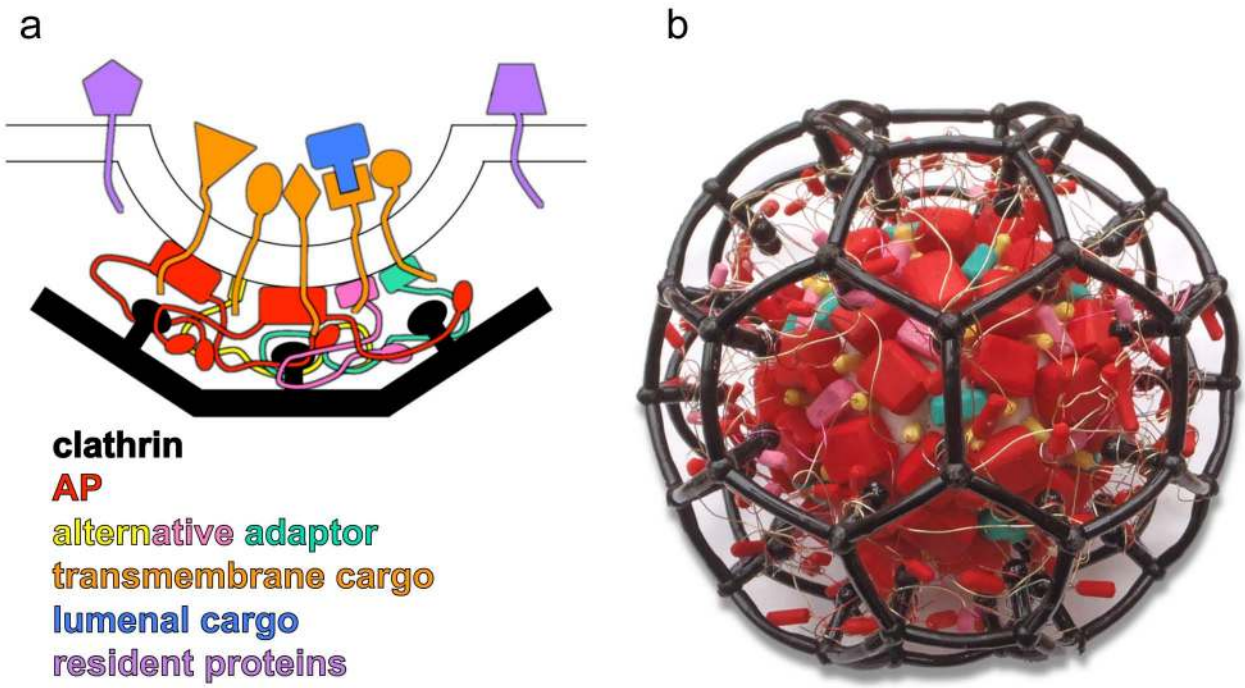

Figure 8. Models of a clathrin-coated pit and coated vesicle. a, A coated pit showing machinery, cargo, and excluded resident proteins. b, A CCV, with copy number based on proteomic analyses of the AP-1 dependent population (140).

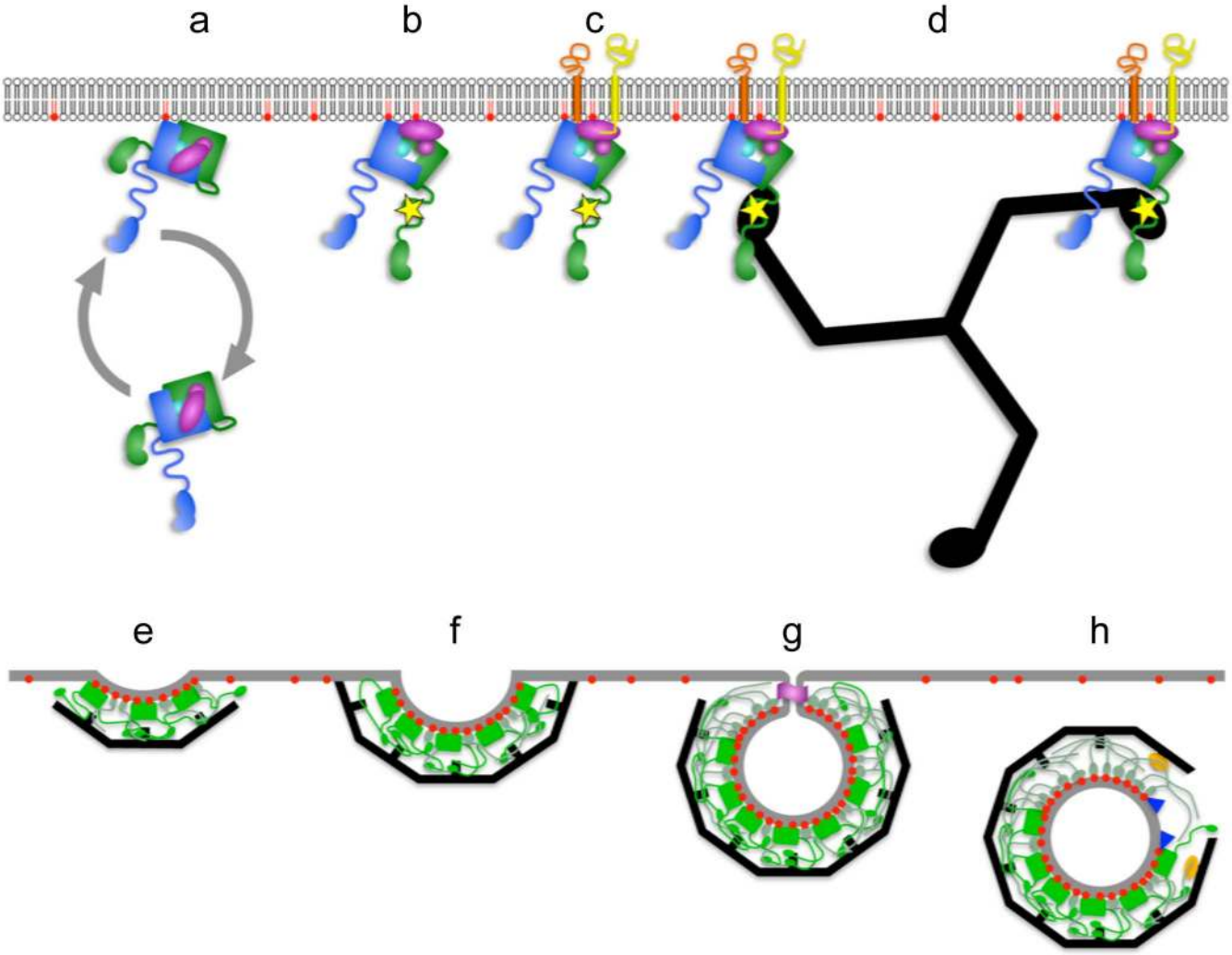

Figure 9. Diagram showing proposed steps in the formation of an endocytic CCV. a, AP-2 complexes (same colour scheme as in Figure 6) are recruited to the plasma membrane by the α subunit binding to PIP2 (red), but rapidly cycle on and off. b, Electrostatic interactions between PIP2 and the μ subunit may open up the complex and expose additional binding sites. c, Interactions with cargo (orange and yellow) further stabilise the open conformation of AP-2 and enable the coated pits to mature more efficiently (156). d, Clathrin also stabilises the association of AP-2 with the plasma membrane: one triskelion recruited to two AP-2 complexes increases the residence time of the AP-2 at the plasma membrane and results in the recruitment of more clathrin and AP-2 (157). e and f, The coated patch (now shown at lower magnification) keeps growing, incorporating various early-arriving alternative adaptors, such as CALM and the muniscins FCHo1/FCHo2 (grey-green) (155). g, Dynamin (purple) is recruited to the neck of the deeply invaginated coated pit to facilitate scission from the plasma membrane. h, Immediately after scission, uncoating machinery is recruited, including auxilin (orange) and OCRL1 (blue). The entire sequence of events, from a to h, takes about two minutes.

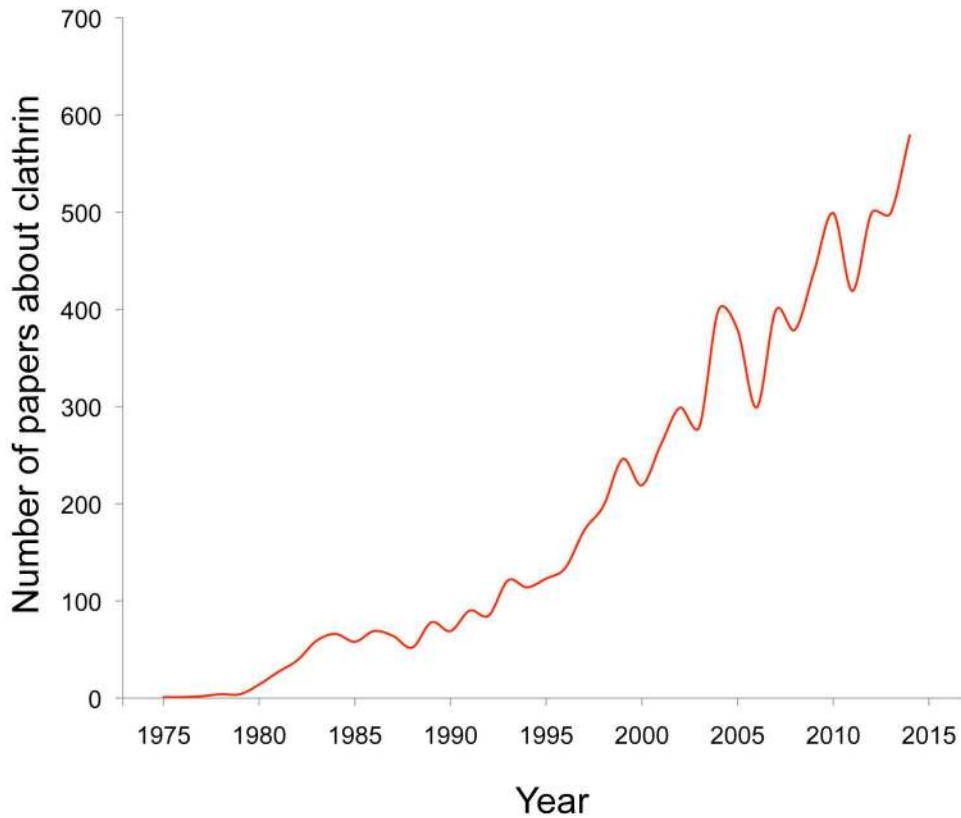

Figure 10. Papers about clathrin over the last 40 years. PubMed was used to search for papers containing the key word “clathrin” from 1975 through 2014. The number of papers per year has increased from one in 1975 to 579 in 2014.