Yosef Yarden · Gabi Tarcic *Editors*

Vesicle Trafficking in Cancer

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Editors
Yosef Yarden
Department of Biological Regulation
Weizmann Institute of Science
Rehovot, Israel

Gabi Tarcic
Department of Biological Regulation
Weizmann Institute of Science
Rehovot, Israel

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Preface

The engulfment of small portions of the plasma membrane, along with an assortment of specific surface proteins, and their packaging in vesicles, which subsequently travel to various organellar destinations, is a vital process tuned by multiple lipids, nucleotides, and proteins, which undergo ubiquitination, phosphorylation, and other reversible covalent modifications. The highly complex endocytic process is both ubiquitous and robust. The reason why endocytosis and intracellular trafficking are essential for life is dictated by the nature of their diverse surface-bound cargo; nutrient receptors and transporters, intercellular and matrix adhesion molecules, as well as a plethora of receptors for growth factors, chemokines, and cytokines are all transported to and from the plasma membrane by means of vesicular trafficking. Hence, the endocytic process critically regulates metabolism, signal transduction, and cell polarity/migration. The remarkable robustness of intracellular trafficking is the outcome of a unique hub-centric design: distinct modular hubs (e.g., AP2-EPS15 and HRS-LST2) comprising a phosphoinositol-binding, membrane-anchoring component, an ubiquitin-binding module, and a machinery enabling homo-assembly are distributed along the pathway. Their bistable regulation entails a set of small GTP binders of the RAB family. Scheduled hub transitions define points of commitment to vesicle docking, fusion, scission, and and subtly manipulates conferring the characteristic unidirectional nature of intracellular trafficking.

While viruses and other cellular invaders utilize the endocytic machinery as their port of entry, according to observations reported over the last decade, cancer multiplies and subtly manipulates vesicular trafficking to imbalance energy and metabolism, signal transduction, and cellular invasion. This volume reviews the plethora of molecular mechanisms that manipulate vesicular trafficking in tumors. The notion of derailed endocytosis in cancer first emerged from studies of receptors for growth factors, such as the epidermal growth factor receptor (EGFR) and c-MET, the receptor for the hepatocyte growth factor (HGF). However, later studies extended the notion to additional families of surface molecules, such as integrins and cadherins, and G-protein-coupled receptors. At this rather initial phase of research, it is still

difficult generalizing the strategies enabling deliberate manipulation of the endocytic process by malignant transformation. Nevertheless, it seems safe arguing that malignancies target the fundamental attributes ensuring unidirectional cargo progress, such as phosphoinositol metabolism, specific RAB proteins, certain E3 ubiquitin ligases or deubiquitinating enzymes (DUBs), as well as the actin/tubulin meshwork.

Importantly, mammalian cells maintain several endocytic pathways and portals, such as the caveolae-mediated pathway and macropinocytosis, but the best understood and apparently most relevant to cancer is the clathrin-mediated pathway. In the opening chapter of this book, **Alexander Sorkin** and **Manojkumar Puthenveedu** review the sequence of events taking place within clathrin-coated areas of the plasma membrane when cargo is actively recruited to the coated area. They also describe how such receptors influence nucleation, stabilization, and size of the clathrin-coated pits, which are considered the bottleneck of receptor endocytosis. Accordingly, overexpression of c-MET and other growth factor receptors, a frequent aberration in carcinomas, appears to saturate the pit and thereby slows down the rate of receptor inactivation. In another chapter, **Tal Hirschhorn** and **Marcelo Ehrlich** describe one of the major regulators of endocytosis, namely, actin dynamics, which control not only invagination and scission but also vesicle movement along actin cables. One critical regulator, the huntingtin-interacting protein-1 related (Hip1R), binds both clathrin and actin, hence acting as a coordinator of actin remodeling and vesicle dynamics. Remarkably, overexpression of HIP1R has been observed in brain and prostate tumors. In a subsequent chapter, **Eli Zamir**, **Nachiket Vartak**, and **Philippe I. H. Bastiaens** highlight the importance of the concentration parameter of membrane proteins. This is determined by the spatial distribution of proteins, their translocation to membrane surfaces, and the interactions between mutant and wild-type versions.

Giorgio Scita and colleagues explain how endocytosis and recycling ensure the asymmetric distribution of membrane proteins, which is crucial for proper polarized cellular functions, including directed cell migration. Viewing the topic from a different perspective, **Shreya Mitra** and **Gordon Mills** propose that abnormal vesicular trafficking disturbs cell polarity by eliminating tight junctions and diminishing apical-basal polarity, which regulate a myriad of cellular functions, including metabolism and asymmetric division of stem cells. Concentrating on cadherins and integrins, especially on their roles in epithelial to mesenchymal transition, a process thought to precede epithelial cell migration and metastasis, **Crislyn D'Souza-Schorey** and **Guangpu Li** argue that sustained signaling from endosomes leads to the formation of invasive structures reminiscent of tumorigenic phenotypes. In line with this view, **Iwona Pilecka** and **Marta Miaczynska** consider endosomes as platforms that can sustain signals generated by internalized G-protein-coupled receptors and receptor tyrosine kinases, thereby enhancing downstream biological outcomes, such as cell migration. They also highlight the presence of endosomal proteins in the nucleus, where they might regulate transcription or chromatin remodeling. **Ying-Nai Wang, Jennifer Hsu, and Mien-Chie Hung** extend this to evidence favoring shuttling of internalized receptors, for example, EGFR and HER2, a

cancer-promoting kin of EGFR, to the nucleus and to other subcellular compartments, where they act as transcriptional regulators. Furthermore, they review evidence linking the translocation of EGFR into the nucleus with poor clinical prognosis, as well as with the outcome of anticancer treatments (e.g., specific kinase inhibitors and monoclonal antibodies).

Cargo ubiquitination, along with ubiquitination of the endocytic machinery, has emerged in the last decade as drivers or by-products of malignant transformation. This is the reason why four chapters of this volume are devoted to ubiquitin-mediated regulation of the endocytic process. **Daniela Hoeller** and **Ivan Dikic** provide an introduction to the versatility of the ubiquitin system and the crosstalk to other posttranslational modification. In another chapter, **Elena Maspero**, **Hans-Peter Wollscheid,** and **Simona Polo** describe ubiquitination of a vast array of mammalian signaling receptors, such as growth factor receptors, G-protein-coupled receptors, NOTCH, various channels, and interferon receptors. They highlight putative roles of monoubiquitination of a set of endocytic adaptors, which bind ubiquitin. In addition, they review implications for cancer, such as the ability of HER2, an internalization-defective receptor, to shunt internalized EGFRs to the recycling route. Several E3 ubiquitin ligases play critical roles in the regulation of endocytosis and malignancy. The list includes Hakai, Nedd4, Deltex, and the CBL family. **Stanley Lipkowitz** and colleagues devote a chapter to the three CBL proteins, emphasizing, on the one hand, their regulation by phosphorylation and more than 50 interacting proteins, and, on the other hand, the variety of oncogenic mutations that inactivate CBL. Remarkably, over the past five years, CBL mutations have been found in ~5% of a wide variety of myeloid neoplasms, including the myelodysplastic syndrome, myelofibrosis, refractory anemia, acute myeloid leukemia, atypical chronic myelogenous leukemia (aCML), and juvenile myelomonocytic leukemia (JMML; up to 15%).The action of CBL and other E3 ubiquitin ligases is reversed by a large set of deubiquitinating enzymes (DUBs). **Han Liu**, **Sylvie Urbé**, and **Michael Clague** describe in depth the DUBs engaged in the regulation of vesicular trafficking. For example, two endosome localized DUBs, AMSH and USP8, accelerate recycling of receptors by reducing active sorting to lysosomal degradation. Predictably, such DUBs might act as tumor suppressors, but their actions appear more complicated than expected, as clarified by Clague and colleagues.

To deepen the description of aberrant endocytosis, several chapters of the book concentrate on specific cargos: c-MET, EGFR, and integrins. **Stéphanie Kermorgant** and colleagues underscore the importance of c-MET's signaling from endosomes, as well as the ability of certain oncogenic mutants of the receptor to enhance downstream signals, by means of defying normal endocytosis. Similarly, **Sergio Anastasi**, **Stefano Alemà**, and **Oreste Segatto** present endocytosis as an element of spatial receptor regulation. They focus on the diverse mechanisms through which receptors escape from downregulation in cancer cells. Specifically, they highlight the intrinsic refractoriness of brain and lung mutants of EGFR to endocytosis-mediated downregulation. In contrast to the well-understood behavior of ligand-activated receptors, ligand-independent activation mechanisms of EGFR remain poorly understood. **Tzipora Goldkorn**, **Simone Filosto**, and **Samuel Chung** highlight stress-dependent

activation, internalization, and trafficking of EGFR. Accordingly, under cellular oxidative stress, p38-MAPK, c-SRC, caveolin-1, and ceramides, membrane sphingolipids generated during oxidative stress undertake driver roles in vesicular sorting. In a subsequent chapter, **Elena Rainero**, **Peter V.E. van den Berghe**, **and Jim Norman** argue that endocytosis and recycling of integrins is important during tumor progression and clarify how mutations of p53 drive invasion and metastasis by altering integrin and EGFR recycling.

Finally, because of the pivotal roles played by derailed endocytosis in cancer, future studies will likely translate this new understanding to improved cancer therapy, as well as to efforts that link aberrant trafficking to mechanisms promoting patient resistance to specific drugs. An exemplification is provided in Jim Norman's chapter: the overall lack of efficacy of anti-integrin drugs (i.e., $\alpha v\beta 3$ inhibitors) in tumor angiogenesis is attributable to enhanced recycling of both $\alpha 5\beta 1$ integrin and receptor tyrosine kinases. Along this vein, the closing chapter, written by **Gabi Tarcic** and **Yosef Yarden**, discusses the possibility that antireceptor monoclonal antibodies commonly used to treat various types of cancer (e.g., trastuzumab, an anti-HER2 antibody) actually force endocytosis of their oncogenic antigens and subsequently target them to degradation in lysosomes. Thus, future studies will not only shed new, endocytosis-relevant light on molecular modes of oncogenic processes; they also might open new avenues in cancer therapy.

Contents

Contributors

Stefano Alemà Institute of Cell Biology and Neurobiology, CNR, Monterotondo, Italy

Sergio Anastasi Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy

Department of Physiology and Pharmacology, University of Rome La Sapienza, Rome, Italy

Rachel Barrow Spatial Signalling Team, Centre for Tumour Biology, Barts Cancer Institute – a Cancer Research UK Centre of Excellence, Queen Mary University of London, John Vane Science Centre, London UK

Philippe I.H. Bastiaens Department of Systemic Cell Biology, Max Planck Institute of Molecular Physiology, Dortmund, Germany

Technische Universität Dortmund, Dortmund, Germany

Peter V.E. van den Berghe Beatson Institute for Cancer Research, Glasgow, UK

Samuel Chung Center for Comparative Respiratory Biology and Medicine (CCRBM), University of California School of Medicine, Davis, CA, USA

Michael J. Clague Cellular and Molecular Physiology, Institute of Translational Medicine, The University of Liverpool, Liverpool, UK

Crislyn D'Souza-Schorey Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA

Ivan Dikic Buchmann Institute for Molecular Life Sciences and Institute of Biochemistry II, Goethe University School of Medicine, Frankfurt, Germany

Andrea Disanza IFOM Foundation, Institute FIRC of Molecular Oncology, Milan, Italy

Marcelo Ehrlich Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel

Simone Filosto Center for Comparative Respiratory Biology and Medicine (CCRBM), University of California School of Medicine, Davis, CA, USA

Emanuela Frittoli IFOM Foundation, Institute FIRC of Molecular Oncology, Milan, Italy

Tzipora Goldkorn Center for Comparative Respiratory Biology and Medicine (CCRBM), University of California School of Medicine, Davis, CA, USA

Signal Transduction Internal Medicine, University of California School of Medicine, Davis, CA, USA

Tal Hirschhorn Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel

Daniela Hoeller Buchmann Institute for Molecular Life Sciences and Institute of Biochemistry II, Goethe University School of Medicine, Frankfurt, Germany

Jennifer L. Hsu Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan, R.O.C

Mien-Chie Hung Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan, R.O.C

Carine Joffre Spatial Signalling Team, Centre for Tumour Biology, Barts Cancer Institute – a Cancer Research UK Centre of Excellence, Queen Mary University of London, John Vane Science Centre, London, UK

Institut Curie, INSERM U-830, Paris, France

Stephen C. Kales Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Stéphanie Kermorgant Spatial Signalling Team, Centre for Tumour Biology, Barts Cancer Institute – a Cancer Research UK Centre of Excellence, Queen Mary University of London, John Vane Science Centre, London, UK

Guangpu Li Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Stanley Lipkowitz Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Han Liu Cellular and Molecular Physiology, Institute of Translational Medicine, The University of Liverpool, Liverpool, UK

Ke Ma Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Elena Maspero IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy

Ludovic Ménard Spatial Signalling Team, Centre for Tumour Biology, Barts Cancer Institute – a Cancer Research UK Centre of Excellence, Queen Mary University of London, John Vane Science Centre, London, UK

Marta Miaczynska International Institute of Molecular and Cell Biology, Laboratory of Cell Biology, Warsaw, Poland

Gordon B. Mills The Department of Systems Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Shreya Mitra The Department of Systems Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Marion M. Nau Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Jim C. Norman Beatson Institute for Cancer Research, Glasgow, UK

Andrea Palamidessi IFOM Foundation, Institute FIRC of Molecular Oncology, Milan, Italy

Iwona Pilecka International Institute of Molecular and Cell Biology, Laboratory of Cell Biology, Warsaw, Poland

Simona Polo IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy

Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' degli Studi di Milano, Milan, Italy

Manojkumar A. Puthenveedu Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA

Elena Rainero Beatson Institute for Cancer Research, Glasgow, UK

Giorgio Scita IFOM Foundation, Institute FIRC of Molecular Oncology, Milan, Italy

Dipartimento di Medicina, Chirurgia ed Odontoiatria, Università degli Studi di Milano, Milan, Italy

Oreste Segatto, MD Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy

Sara Sigismund IFOM Foundation, Institute FIRC of Molecular Oncology, Milan, Italy

Alexander Sorkin Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Gabi Tarcic Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel

Sylvie Urbé Cellular and Molecular Physiology, Institute of Translational Medicine, The University of Liverpool, Liverpool, UK

Nachiket Vartak Department of Systemic Cell Biology, Max Planck Institute of Molecular Physiology, Dortmund, Germany

Ying-Nai Wang Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan, R.O.C

Hans-Peter Wollscheid Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' degli Studi di Milano, 20122 Milan, Italy

Yosef Yarden Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel

Eli Zamir Department of Systemic Cell Biology, Max Planck Institute of Molecular Physiology, Dortmund, Germany

Chapter 1
Clathrin-Mediated Endocytosis

Alexander Sorkin and Manojkumar A. Puthenveedu

Abstract Clathrin-mediated endocytosis is the main portal of entry into the cell for many soluble and membrane molecules. Clathrin-coated vesicles are formed from the plasma membrane in a sequence of coordinated protein-lipid and protein-protein interactions, starting with adaptor-mediated recruitment of clathrin to the membrane, proceeding to clathrin polymerization and assembly into deeply curved coated buds, and ending with the dynamin-dependent scission of a coated vesicle. Clathrin coats trap and concentrate endocytic cargo by using a multitude of adaptor proteins that recognize specific sequence motifs in the cytosolic domains of receptors and other transmembrane cargo molecules. Endocytic cargo that is concentrated in this manner, such as signaling receptors, may regulate the stability, size, and dynamics of individual clathrin coats and thereby influence endocytosis.

1.1 Introduction

Endocytosis is a process by which cells internalize extracellular and cell-surface materials. These materials include membrane proteins, which comprise of a third of the genome. Many of these proteins have critical functions at the plasma membrane or need to pass through the cell surface on the way to their intracellular sites of function. Therefore, the localization and function of all these proteins (also referred as endocytic "cargo") are regulated by endocytosis. Endocytic cargo is captured in small areas of the plasma membrane that invaginate to form buds, which eventually

A. Sorkin (✉)
Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
e-mail: sorkin@pitt.edu

M.A. Puthenveedu (✉)
Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA
e-mail: map3@andrew.cmu.edu

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pinch off to form vesicles that move into the cytoplasm. Newly formed endocytic vesicles typically fuse with early endosomes, and the endocytosed cargo is then sorted into recycling, lysosomal, or other trafficking pathways.

Endocytic vesicles are formed by several mechanisms. Clathrin-mediated endocytosis (CME) is an evolutionary conserved pathway that is the main and best characterized pathway of endocytosis, although several clathrin-independent endocytosis pathways have also been described [1, 2]. CME is essential for many fundamental cellular processes, such as synaptic transmission, signal transduction, nutrient uptake into the cell, and membrane homeostasis. It is the fastest and highly regulated pathway of endocytosis. The specific internalization rate constants measured for the CME cargo, such as transferrin and epidermal growth factor (EGF) receptors, can be as high as 0.3–0.4 min^{-1} (30–40% of surface receptors are endocytosed in 1 min) [3].

The process of formation of an endocytic clathrin-coated vesicle (CCV) has been studied intensively for about 50 years using various methodologies. CCV is formed by the assembly of clathrin-coated pits (CCPs)—small areas of the cytosolic surface of the plasma membrane that are covered by a protein coat consisting mainly of clathrin—followed by their scission from the membrane generating free vesicles. In recent years, remarkable progress has been made in understanding the molecular mechanisms of this process through the development of two main experimental approaches: One, resolving the atomic structures of clathrin and other components of clathrin assembly has ultimately led to the high-resolution structure of an entire CCV formed in vitro. Two, total interference reflection fluorescence (TIRF) and other live-cell optical microscopy methods have allowed detailed real-time imaging of the CME process and have unveiled a sequence of events and protein recruitment to the clathrin coat during endocytosis with very high temporal resolution. Many comprehensive review articles have been dedicated to the mechanisms of CME (for example [2, 4, 5]). In this chapter, we will discuss recent advances in understanding the molecular machineries involved in CME in mammalian cells. The endocytosis of "signaling" receptors, e.g., receptors that are capable of triggering cellular signal transduction processes, will be specifically described, and examples of the regulatory mechanisms by which these receptors can modulate CME will also be illustrated.

1.2 Structure of Clathrin and Clathrin Vesicles In Vitro

Clathrin is the main component of coated pits and vesicles. It is a soluble cytosolic protein that cannot bind lipids directly and is therefore recruited to the membrane by a specialized set of proteins called adaptors (discussed below) to assemble into coated pits and buds. The ability of clathrin to polymerize into a lattice and assemble into vesicles in vitro under mildly acidic pH allowed comprehensive biochemical analysis of the assembled state of clathrin. Elucidation of the high-resolution structure of the clathrin molecule and the clathrin lattice has led to a remarkable level of understanding of the mechanisms of lattice assembly and clathrin interactions

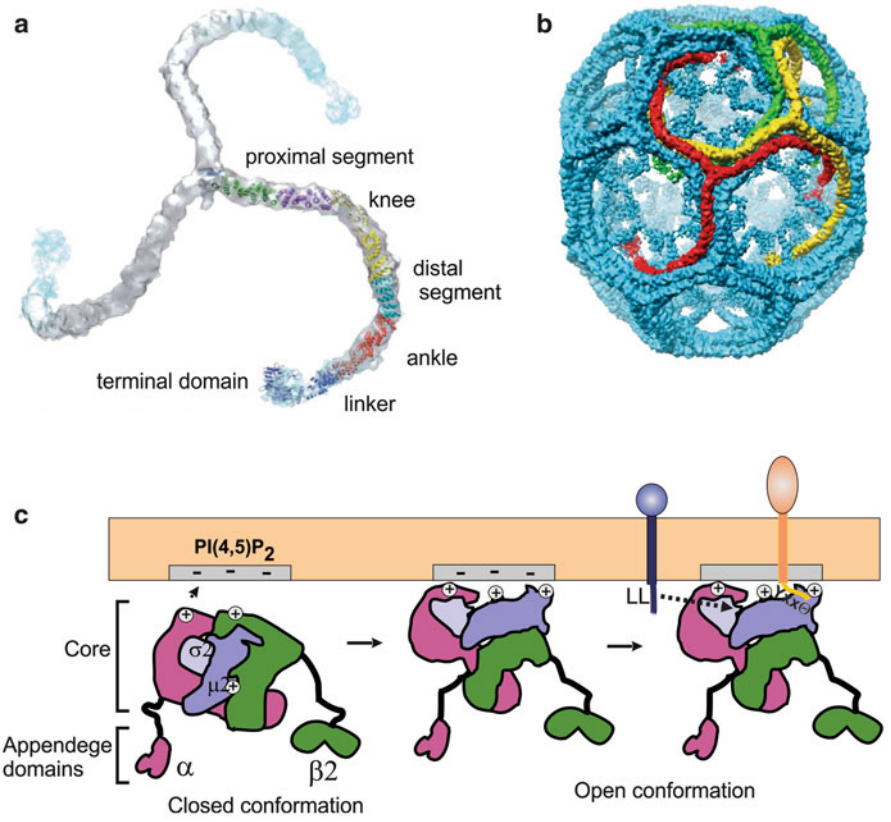

Fig. 1.1 Schematic structures of clathrin triskelion, polymerized clathrin, and AP-2. (**a**) The clathrin triskelion consists of three CHC and three CLC (not shown) molecules. Names of segments of one heavy chain are indicated with the terminal domain at the amino-terminus and the vertex at the carboxyl-terminus (adapted from [6, 11] with permission from *Nature*). (**b**) Image reconstruction at 7.9 Å resolution of a clathrin coat (in a shape of "hexagonal barrel") assembled in vitro from the bovine-brain clathrin (heavy chains only) and AP-2 using cryo-electron microscopy images. There are 36 clathrin triskelions in the structure. The colored triskelions show three independent triskelions. Noisy central density, from spatially disordered and substoichiometric AP-2 complexes, has been flattened (adapted from [6, 11] with permission from *Nature*). (**c**) Clathrin adaptor complex AP-2 consists of the core and appendage domains connected to the core by flexible hinge domains. AP2 binds initially to negatively charged PI (4,5)P2 headgroups in the membrane mainly through the positively charged interfaces in the α subunit and, additionally, in the β2 subunit. The large conformational change in AP-2 is then triggered by the electrostatic attraction of C-terminal lipid-binding patches of μ2 to the negatively charged membrane, which results in an "open" conformation form of AP-2. AP-2 in open conformation can bind tyrosine- and leucine-based internalization motifs present in the cytosolic domains of the membrane cargo

involved in this process [6]. Clathrin functions as a heterohexamer, adopting a three-legged triskelion structure of three heavy chains and three light chains [7, 8] (Fig. 1.1a). Approximately 100 clathrin triskelion units are present in the assembled coated vesicle.

Clathrin heavy chains (CHC) serve as structural element of the clathrin lattice, whereas clathrin light chains have mainly regulatory functions.

The CHC consists of eight CHC repeat (CHCR) motifs (CHCR0-7) forming a right-handed super-helix coil of α-helices [6, 9], arranged into proximal, distal, and terminal domains [10]. The proximal domains (CHCR6 and 7) of three CHC molecules interact to form the vertex of the triskelion. CHCR5 makes the "knee," CHCR3 and 4 constitute the distal domain, and CHCR1 and 2 with the carboxyl (C)-terminal part of CHCR0 make the ankle domain (Fig. 1.1a). The clathrin vertex and three proximal domains form the clathrin hub. The hub provides stability to the triskelion and allows assembly into the characteristic polyhedral lattice. High-resolution cryo-electron microscopy structure analysis revealed a helical tripod, composed of the C-terminal domains of CHCs and positioned beneath the vertex towards the center of the lattice. This tripod contacts the ankles from neighboring triskelia, thus stabilizing the lattice (Fig. 1.1b) [6, 11].

The clathrin knee is flexible, and this allows the lattice to adapt to varying vesicle sizes in vitro and varying membrane curvatures in vivo. Each triskelion vertex is centered at a lattice vertex, and the heavy chain legs form two adjusted edges of a planar (consisting mostly of hexagons) or polyhedral lattice (consisting of pentagons and hexagons). The legs appear to interact via proximal and distal domains, each edge consisting of two antiparallel proximal domains with two antiparallel distal domains situated beneath proximal domains. Because the knee is flexible, these interactions can generate either hexagons or pentagons. An increase in the number of pentagons leads to increased curvature of the polyhedral lattice.

The amino (N)-terminal domain is attached to the distal domain by an α-zigzag linker that positions the terminal domain inside the lattice. The N-terminal domain is structured as a seven-bladed β-propeller [12], in which the β-sheets are organized regularly around the central axis. This β-propeller has at least three distinct binding sites for many proteins, including adaptors that recruit clathrin to the membrane. Proteins containing a "clathrin box" motif Lϕxϕ[D/E], where ϕ is a bulky hydrophobic amino acid and x is any residue, have been shown to bind the site between blades 1 and 2 [12]. Peptides with W-based motif (PWxxW) are thought to fit into a pocket in the center of the domain, formed by the upper regions of blades 1, 4, 6, and 7 [13]. A third binding site, between blades 4 and 5, binds specific sequences via hydrophobic interactions [12].

Clathrin light chains (CLCa and CLCb) bind residues 1438-51 in the proximal domains of CHC through their carboxyl-terminal domains [14–16]. CLCs have a helical rodlike shape and face the outside of the lattice when they contact the proximal domain [6]. Interestingly, in contrast to RNA interference (RNAi) knockdown of CHC, which results in complete disappearance of coated pits at the plasma membrane and strong inhibition of the endocytosis of many types of cargo, knockdown of CLC does not lead to inhibition of endocytosis of classical CME cargo such as the transferrin receptor [17]. Thus, although CLC has been proposed to stabilize the lattice [18], this function of CLCs is apparently not essential for general endocytosis. Recently, CLCs have been implicated in clathrin-mediated endocytic processes that

require actin [19, 20]. In this situation, CLCs bind huntingtin-interacting protein related protein (HipR1) that in turn binds cortactin and F-actin, thus linking the clathrin coat to actin filaments [21, 22]. Further, knockdown of CLC or overexpression of its non-phosphorylatable version has been shown to impair the endocytosis of G protein-coupled receptors (GPCRs) [23]. Thus, CLCs might play a role in specialized endocytic scenarios.

1.3 Steps of Coated Vesicle Formation

1.3.1 Coat Nucleation/Initiation

Formation of an endocytic coated pit at the membrane is initiated by concentrating several clathrin triskelions in a small area of the inner leaflet of the plasma membrane leading to triskelion interactions, clathrin polymerization, and lattice assembly. Clathrin triskelions are recruited from the cytosol to the membrane by adaptor proteins that are capable of simultaneous interaction with triskelions and lipids containing negatively charged head groups. Clathrin lattices are also formed on membranes of endosomes and the Golgi apparatus. The specificity of the formation of CCPs at the plasma membrane is achieved by adaptors with preferential binding to phosphatidylinositol-4,5-biphosphate [PI (4,5)P2], a lipid that is enriched in the plasma membrane (Fig. 1.2).

Historically, adaptor protein complex-2 (AP-2) has been considered to be the main adaptor that recruits clathrin to the plasma membrane (Fig. 1.1c). AP-2, present with the highest stoichiometry to clathrin among all other CCV components, is a heterotetramer consisting of tightly associated α, β2, μ2, and σ2 subunits [24, 25]. The 200-kDa core domain of AP-2, that contacts the membrane, consists of the trunk domains of large α and β2 subunits, assembled together with the μ2 and σ2 subunits [26, 27]. α and β2 subunits have 30 kDa bilobal C-terminal appendages connected to the trunks with long flexible linkers [28]. Appendage domains are capable of binding to different sequence motifs found on many accessory/regulatory proteins (reviewed in [29]). The β2 hinge domain contains two clathrin-binding motifs and strongly interacts with the terminal domain of CHC. Structures of the AP-2 core bound to a phosphoinositol phosphate headgroup analog (inositol hexakisphosphate) were solved [26]. Four positively charged interfaces, that can bind PI (4,5)P2, on α, β2, and μ2 subunits were identified [26, 30–33]. The α subunit site appears to play a key role in the initial docking of AP-2 onto PI (4,5)P2 [30].

Several other proteins, such as epsin, clathrin assembly lymphoid myeloid leukemia (CALM), and its neuronal homolog AP180, satisfy the criteria that define clathrin adaptors: an ability to bind lipids and the terminal domain of CHC [34–37] (Fig. 1.2). The formation of the clathrin lattice in vitro on liposomes and the entire process of coated vesicle formation was reconstituted using a membrane-bound fusion protein of epsin-1 and, with lesser efficiency, AP180 [38]. In these

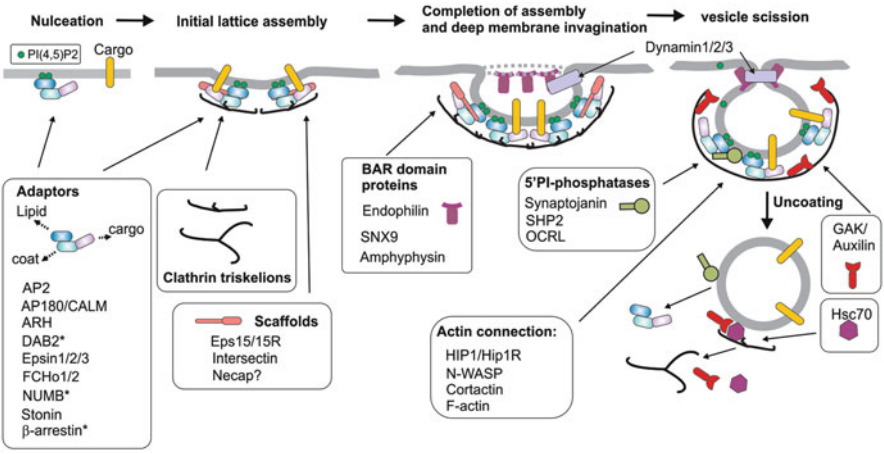

Fig. 1.2 Formation of clathrin-coated vesicle at the plasma membrane. Schematic representation of consecutive stages of the cycle of CCV assembly and disassembly in mammalian cells, based on the heroic systematic analysis by Merrifield and coworkers [61] and numerous other studies. Various adaptors, scaffolds, and other accessory proteins are recruited to the site of forming or assembled clathrin structures at different stages of the process (indicated by *arrows*). Adaptor proteins are shown in generic modular shape consisting of cargo, clathrin coat, and lipid-binding interfaces. *Asterisk* points out on adaptors that require AP-2 binding for linking their cargo to a CCP. Scaffolds do not bind lipids directly but interact with multiple membrane-associated adaptors and may interact with cargo (Eps15/Eps15R)

experiments, the truncated mutant of epsin-1, containing clathrin-binding motifs and lacking ENTH (lipid-binding) domain, but fused to an artificial membrane attachment moiety, was incorporated into liposomes. Recruitment of soluble clathrin to the membrane-bound epsin mutant resulted in the assembly of a slightly invaginated lattice adopting the concave shape of individual clathrin triskelions and ultimately formation and scission of CCVs. These experiments supported the previously proposed hypothesis that the membrane-bending ability of polymerized clathrin is sufficient for generating an initial curvature of coated pits [6, 39]. The question still remains—what is the relative contribution of individual adaptors in the coated pit formation in an intact cell? Is AP-2 sufficient for the initiation of the lattice assembly, or does an assembly process initiated by monomeric adaptors take place in parallel to the AP-2-mediated assembly? Single-molecule imaging analysis demonstrated that two consecutive events of sequential recruitment of two molecules of AP-2 and one clathrin triskelion to a future endocytic site on the plasma membrane typically precede the assembly of a clathrin-coated structure [40]. This recent study, therefore, suggested that membrane AP-2 recruitment is sufficient and essential for coat initiation.

How initial clathrin assembly at the membrane and the initial membrane invagination are coordinated and the mechanisms of this invagination are currently under debate. Recent studies by McMahon and coworkers implicated proteins containing

the membrane shaping F-BAR domain (see below), FCH domain only (FCHo1 and 2) in the nucleation step [41]. FCHo proteins bind Eps15, highly homologous Eps15-related protein, Eps15R, and intersectin, all three proteins with scaffold properties, capable of interacting with multiple clathrin coat accessory/regulatory proteins. It was proposed that binding of the FCHo1/2 "module" to the future endocytic site on the membrane takes place prior to AP-2 recruitment and that F-BAR domain recognizes low membrane curvatures and generates initial curvature of the coated pit. siRNA knockdown of FCHo1/2 blocked formation of coated pits and buds [41]. Subsequent studies confirmed the role of FCHo proteins in coat nucleation [42]. However, translational silencing of FCHo1/2 did not eliminate clathrin- and AP-2 positive structures in the plasma membrane or inhibit endocytosis of the transferrin receptor [43], and FCHo siRNA had only partial effects on endocytosis [44]. Likewise, RNAi experiments demonstrated that depletion of Eps15 and Eps15R causes no significant inhibition of CME [17] or at best a partial inhibition [45]. Furthermore, at least in some cells, all cellular Eps15 could be co-immunoprecipitated with AP-2, suggesting that an AP-2 independent function of Eps15 is unlikely [46]. Therefore, further research is necessary to clarify the precise role of FCHo1/2 and associated proteins and reconcile contrasting models of coordinated coat nucleation and membrane bending.

A number of other adaptor proteins found in CCPs, such as Disabled-2, NUMB, β-arrestins, and autosomal recessive hypercholesterolaemia (ARH), are capable of binding PI (4,5)P2, the terminal domain of clathrin (directly or through AP-2), and transmembrane cargo (Fig. 1.1) but are not directly demonstrated to be sufficient to promote coat assembly [47]. For instance, the clathrin box on β-arrestins is exposed only after they interact with their transmembrane cargo, GPCRs [48, 49]. The common theme is that all clathrin adaptors including AP-2 and monomeric adaptors are capable of binding to the transmembrane endocytic cargo, suggesting that cargo recruitment into a forming coat may have a regulatory function during the CCV formation [47]. In fact, FCHo1 has been proposed to act also as the cargo adaptor, as the μ-homologous domain in FCHo can interact with the BMP receptor [43]. The possibility that cargo alone is sufficient to initiate coat assembly is difficult to envision, because coated pits are not formed on other intracellular membranes where the same cargo molecules are present. It is more plausible to propose that cargo binding to the adaptor proteins occurs after the nucleation step and plays a regulatory role in the kinetics of the CCV cycle. Indeed, the hypothesis was proposed whereby cargo recruitment into forming clathrin coat is necessary for the completion of the CCV formation process and that coats not bearing cargo undergo disassembly before full assembly of the coated bud (see below) [50].

1.3.2 Formation of the Clathrin-Coated Bud

Following initiation of clathrin lattice assembly on the membrane that is slightly bent by assembled clathrin and/or F-BAR proteins, the coat expands simultaneously

with the increase in the membrane curvature, thus forming the coated "bud" connected to the membrane by the "neck" (Fig. 1.2). The intrinsic curvature of assembled clathrin due to pentagonal faces of the polyhedral lattice makes it capable of deforming membranes into stable highly curved buds. During the growth of the coat and formation of the bud, proteins such as Eps15 that are involved in the initial nucleation step and, possibly, cargo recruitment are "pushed" to the edge of the coat [46] (Fig. 1.2). In the same time, epsin, which interacts with lipids and is directly bound to clathrin, remains distributed throughout the clathrin coat [51].

The mechanisms of coat assembly on highly curved membranes are not fully understood. According to one model, the shape of assembled clathrin provides sufficient energy to promote high curvature of the membrane ([38] and references therein). Another model suggests that the energy provided by assembled clathrin coat would not be sufficient to support highly curved membranes, because clathrin triskelions bind to flexible regions of adaptors. This model proposes that additional proteins must provide energy to bend the membrane and form coated buds [2]. Several proteins might bind to slightly curved areas of the membrane and further increase the membrane curvature, either by inserting an amphipathic helix into the inner layer of the membrane (in the case of the ENTH domain of epsin) or through membrane shaping by BAR (Bin/amphiphysin/Rvs) and N-BAR domain scaffolds [52, 53] (Fig. 1.2). Dimerization of BAR domains produces a crescent-shaped structure with a positively charged concave interface, thus enabling generation of the membrane curvature by electrostatic interactions of this surface with negatively charged head groups of lipids [53]. Proteins containing N-BAR and BAR domains also have capacity to bind accessory proteins located in coated pits (Fig. 1.2). This second model proposes that clathrin plays a role in stabilizing the curved membrane and the entire coat structure. The ability to reconstitute the process of clathrin-coated bud formation and vesicle scission from liposomes in the absence of BAR or other lipid-binding domains supports the first model [38]. Furthermore, lack of effects of siRNA knockdown of epsins and BAR domain proteins on endocytosis rates of the conventional CME cargo like the transferrin receptor [17, 51, 54] argues that clathrin is sufficient to carry out all stages of membrane remodeling during CME until vesicle scission. However, it is possible that membrane-bending proteins are required for more efficient membrane bending in the situation when cargo molecules with larger extracellular domains are being packaged into the CCV. Furthermore, there is significant redundancy among membrane-bending proteins, and the absence of the effects of single and double RNAi knockdowns on endocytosis should be interpreted with caution.

1.3.3 Vesicle Scission

As the CCP nears completion, the large GTPase dynamin concentrates at the narrow neck that attaches the forming vesicle to the membrane and promotes vesicle scission (reviewed by Ferguson and De Camilli [55] and Schmid and Frolov [56]).

There are three mammalian dynamin genes, which encode proteins that are 80% homologous. Dynamin 1 is expressed at high levels exclusively in neurons, although low levels of dynamin 1 are detected in many non-neuronal cultured cell lines. Dynamin 2 is expressed ubiquitously. Dynamin 3 is found predominantly in the testis and, at low levels, in brain and other tissues. All three have the same domain organization: an amino-terminal G domain, a "middle" or "stalk" region, a pleckstrin homology (PH) domain, a GTPase effector domain (GED), and a Pro-rich carboxy-terminal region (Pro-rich domain, PRD). Although dynamin can bind to the membrane through its PH domain, its concentration at the neck appears to require the interaction of the PRD with the SH3 domains of amphiphysin, endophilin, and/or SNX9, proteins that contain BAR and N-BAR domains. At the neck, dynamin assembles into dimers and higher oligomers, and this assembly stimulates GTP hydrolysis and results in a conformational change in the dynamin oligomeric complex. This conformational change provides energy necessary for constricting the neck of the nascent vesicle, fission of the membrane at the constricted neck, and pinching off a CCV [55, 56]. The mechanisms of dynamin-dependent fission are not fully understood. When the activity of dynamin is inhibited by addition of non-hydrolyzable analogs of GTP or mutations, long membrane necks covered by the dynamin collar are observed, indicative of the function of long dynamin oligomers at the late stage of membrane fission [57]. However, long necks are not observed under normal conditions, and it is likely that fission is mediated by short dynamin scaffolds. Analysis of dynamin's interaction with membrane nanotubes suggested that dynamin catalyzes membrane remodeling by generating regulated curvature constraints and bringing membranes to the point of spontaneous fission [58].

In living cells, the fission process is very rapid, and it is likely that the dynamin activity is coordinated with the activity of other accessory proteins. Recently, the ENTH domain of epsin was proposed to be involved in membrane fission by inserting the amphipathic helix into the membrane [59]. Under certain experimental conditions, epsin was shown to rescue the inhibition of fission in the dynamin-depleted cells. On the other hand, accumulation of N-BAR domain containing proteins at the forming neck of coated buds was found to inhibit fission, suggesting that release of these proteins from the nascent vesicle is an important checkpoint during the vesicle scission process. Furthermore, in another recent study, a distinct mechanism of coated pit closure was proposed [60]. In this study, coated pits located on the top surface of substrate-adherent cultured cells were often seen in the close proximity to microvilli. Actin-based structures in the microvilli were proposed to participate in the closure of the clathrin-coated buds, presumably by projecting over an open neck from one side of the narrow and fusion of the end of a microvilli with the opposite side of the neck [60]. In mammalian cells, F-actin and regulators of actin branch assembly such as Arp2/3 (actin-related protein 2/3), N-WASP (Wiscott-Aldrich syndrome-like), and cortactin are transiently recruited to clathrin structures at the time of or immediately before vesicle scission [61, 62]. Actin cytoskeleton has been implicated in CME, although, in mammalian cultured cells, actin polymerization is not essential for coated pit assembly and endocytosis of various cargos [19]. The role of actin in endocytosis is discussed in detail in the Chap. 2.

1.3.4 Uncoating

After pinching off from the plasma membrane, CCVs are rapidly uncoated by the ATPase heat shock cognate 70 (Hsc70) and its co-chaperone, the J domain-containing protein auxilin [63, 64]. Auxilin 1 is expressed in neurons, whereas auxilin 2/GAK (cyclin G-associated kinase) is ubiquitously expressed. The LLGLE motif of auxilin binds to the terminal domain of CHC [65]. High-resolution structure of the clathrin vesicle demonstrated that auxilin also interacts with an ankle region of CHC [11]. Binding of auxilin to the clathrin lattice formed in vitro causes change in CHC interactions and moves the clathrin terminal domains slightly outwards. This repositioning causes substantial structural alterations in the lattice, increasing the diameter of a barrel-shaped CCV. The J domain of clathrin-bound auxilin recruits Hsc70, thus positioning Hsc70 in the proximity to several critical interactions of assembled clathrin. In vitro, one auxilin and three or less Hsc70 molecules per triskelia are necessary for uncoating. Hsc70 is a chaperone protein involved in many folding, degradation, and translation processes, and therefore it is difficult to conduct functional experiments to analyze the uncoating function of Hsc70 in intact cells. Hsc70 binding causes a global distortion in the lattice, presumably by interfering with interactions of proximal and distal domains of CHC. In cells, it is likely that budded vesicles have a coat that is interrupted at the site of membrane fission. Therefore, it is possible that the process of uncoating starts from the exposed edge of the coat [2].

The lipid phosphatase, synaptojanin, is recruited to nascent vesicle at the time of scission with kinetics similar to that of dynamin and several BAR domain proteins (Fig. 1.2) [61, 66]. Binding of synaptojanin to the SH3 domain of endophilin is proposed to mediate the association of synaptojanin with forming vesicles [67]. Synaptojanin dephosphorylates the head group of PI (4,5)P2 at position 5′ [68]. Decrease in PI (4,5)P2 concentrations releases AP-2, AP180, CALM, and other PI (4,5)P2-binding adaptors to the cytosol. Phosphoinositol-3-kinases that use PI (4,5) P2 as substrate and that are capable of binding to clathrin may also contribute to the reduction of PI (4,5)P2 concentration [30]. Another lipid phosphatase, SHIP2, is shown to be recruited to CCPs earlier than synaptojanin and may also participate in PI (4,5)P2 dephosphorylation [66]. Additionally, oculocerebrorenal syndrome of Lowe (OCRL) lipid phosphatase is proposed to participate in lipid remodeling at the late stages of endocytosis, e.g., immediately after vesicle scission [61, 66, 69]. Finally, released clathrin triskelions, adaptors, and accessory proteins recycle back to the plasma membrane to form new clathrin-coated structures.

1.4 Cargo Recruitment and Endocytosis

A key feature of endocytosis of membrane proteins is that it is highly selective. That is, some proteins are concentrated in CCPs compared to the surrounding membrane, while many others are not. Endocytic proteins are concentrated by a

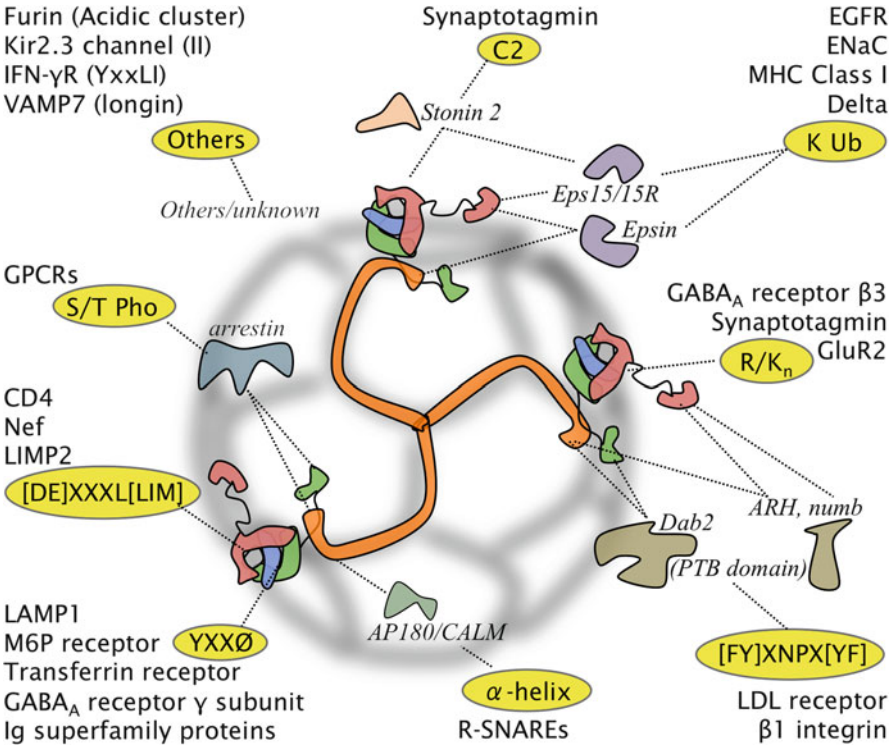

Fig. 1.3 Endocytic sorting signals and adaptors. Schematic of the main endocytic sorting motifs, example cargo molecules that contain these motifs, and the adaptors that mediate their interactions with clathrin. *Dotted lines* indicate the proposed interactions. Single letter amino acid notations are used for the sequence motifs, and *square brackets* indicate alternate residues at the same position. X indicates any residue, and ϕ indicates a bulky hydrophobic residue. Pho denotes phosphorylation, and Ub denotes ubiquitination

simple affinity principle, whereby they are physically linked to the clathrin coat machinery by "adaptor" proteins that recognize specific sorting sequences on cargo (Fig. 1.3). The sorting sequences of some proteins bind adaptors irrespective of whether they are bound to extracellular ligands, leading to "constitutive" internalization of these receptors [47]. For many other proteins, such as signaling receptors, adaptor-cargo binding requires posttranslational modifications and conformational changes of the cargo protein, often induced by ligand binding. Therefore, the internalization of these cargo molecules is regulated [70]. The molecular mechanisms involved in cargo sorting into CCPs in both these modes of endocytosis are discussed below.

1.4.1 Constitutive Endocytosis

Many surface proteins, such as nutrient receptors like the transferrin receptor (TfR) and the low-density lipoprotein (LDL) receptor, are continuously internalized from the plasma membrane and recycled back to the cell surface from the endosome. Most of these receptors use specific sorting sequences on their cytoplasmic surfaces to either directly or indirectly bind adaptors. Careful analysis of many examples over the years has yielded several common sequence motifs that mediate adaptor binding and endocytosis [47, 71].

The best-known example of such endocytosis signals is the Yxxϕ motif, in which x is any amino acid and ϕ a bulky hydrophobic amino acid. Originally identified on TfR, this motif directly binds AP-2, the main endocytic adaptor [72]. Structural studies have since pinpointed this binding interface. The extended Yxxϕ motif forms a transient antiparallel β-strand with the C-terminal β-16 strand of μ2, stabilized by interactions of the Y and the ϕ residues with compatible pockets on either side of the β-16 strand [73]. In some cargo, like the γ subunit of the $GABA_A$ receptor (YGYECL), the affinity of Yxxϕ-μ2 binding is substantially increased by residues upstream of Yxxϕ, which interact with additional hydrophobic pockets on μ2 [74]. A highly related YDYCRV sequence has been identified in BST-2/tetherin [75], a clinically relevant protein due to its antiviral properties. However, this sequence appears to bind the α-appendage domain outside the AP-2 core [76].

Interestingly, while the binding of Yxxϕ to AP-2 is typically independent of the presence of ligand, the accessibility of the binding domain on μ2 is regulated to prevent uncontrolled internalization of cargo. The μ2 subunit is normally in a closed conformation, with the Yxxϕ-binding region masked by the β2 subunit [26, 31]. During CCP initiation and assembly, binding of AP-2—in particular, positively charged patches in the C-lobe of μ2—to PI (4,5)P2 frees the μ2 from the β2 subunit and switches it into an open conformation that allows Yxxϕ binding [31, 77] (Fig. 1.1c). It has also been proposed that phosphorylation of a specific threonine 156 residue on μ2 by adaptor-associated kinase-1 (AAK-1), stimulated by clathrin assembly, plays an additional stabilizing role in this switch in conformation [31, 78–83]. Further, the affinity of Yxxϕ with μ2 may be inhibited by phosphorylation of this motif, such as with the $GABA_A$ receptor and CTLA-4 [74, 84]. In the case of the $GABA_A$ receptor, phosphorylation of either tyrosine in the YGYECL sequence (where the last four residues form the Yxxϕ motif) by Src family kinases inhibits AP-2 binding [74, 85].

The acidic di-leucine ([DE]xxxL[LIM]) motif is another well-studied internalization motif. These motifs bind AP-2 on the σ2 subunit adjoining the PI (4,5) P2-binding region of the α subunit in the AP-2 core [32]. Much like with the tyrosine motif, diLeu binding to AP-2 is also regulated at multiple levels. The binding pocket on σ2 is masked by the N-terminal domain of the β2 subunit and must be made accessible before cargo binding. Lipid binding of multiple subunits of AP-2 [31] might contribute to the large-scale movements required for moving the β2 segment away (Fig. 1.1c). Evidence also suggests that phosphorylation of a specific

tyrosine residue on β2 that packs against the binding domain [86–88] may stabilize a conformation of AP-2 that is accessible to diLeu motifs [32]. Interestingly, structural studies show that the μ2 subunit can remain in the closed conformation even under conditions where diLeu can bind σ2 [32], suggesting that AP-2 might differentially bind these two motifs. However, evidence also suggests that different sorting motifs can influence the binding of each other to adaptors [89, 90]. A more straightforward way of regulating diLeu binding to AP-2 is seen in the regulation of E-cadherin turnover by p120 catenin [91]. p120 contains a diLeu-binding motif that competes with AP-2 for E-cadherin binding and prevents its internalization. However, p120 itself has an internalization motif that binds Numb, an alternate adapter discussed below, and evidence suggests that the complex itself may be internalized in a Numb-dependent manner [92–94]. The molecular details of how all these diverse cargo can influence adaptor binding and endocytosis of each other are still not fully understood.

In addition to these well-studied sequence motifs, a cluster of basic residues has been shown to act as internalization signals for a set of cargo proteins, including the GluR2 subunit of AMPA receptors, the β3 subunit of the $GABA_A$ receptor, and the synaptic Ca^{2+} sensor Synaptotagmin 1. While these are thought to bind the μ2 subunit of AP-2, the exact binding interface and regulation are not clear.

A fourth signal, the [FY]xNPx[YF] motif, was in fact the first internalization signal identified on any cargo protein, in the classic experiments by Brown and Goldstein on LDL receptor (LDLR) internalization [95, 96]. This tyrosine-based motif has since been identified on several proteins, including β-integrins and amyloid precursor protein (APP). Surprisingly, despite the similarity to the Yxxϕ motif, this motif does not directly bind AP-2. Further, the internalization of LDLR proceeds efficiently even when most of AP-2 is depleted from cells, even though TfR internalization is inhibited [97, 98]. This led to the idea that alternate adaptors, not AP-2, drive LDLR internalization. Several alternate adaptors that show remarkable specificity to [FY]xNPx[YF], including ARH, Dab2, and Numb, have been now identified [99–102]. Overexpression of exogenous Dab2 selectively increases the internalization of β-integrins without affecting TfR [103]. Further, depletion of both Dab2 and ARH, but not either alone, inhibits the internalization of LDLR, but not TfR [104–106]. This suggests that ARH and Dab2 are largely functionally redundant in LDLR internalization, although ARH might depend on AP-2 for CCP localization [105, 106].

A characteristic of these adaptors is that they contain "phosphotyrosine-binding" (PTB) domains, which are 100–150 amino acid modules that were originally identified as protein folds that bind phosphorylated tyrosines in an NPxY motif [107]. PTB folds are characterized by an orthogonal β-sandwich, on which the NPxY peptide forms a temporary antiparallel β-strand with the NP residues stabilizing a tight β-turn to present the Y into its binding pocket [108]. In the case of these adaptors, however, it seems that the PTB domain is a misnomer, as they mostly prefer non-phosphorylated Y or F residues [101, 109]. A recent crystal structure suggests that ARH recognizes a longer sequence of the LDLR motif and that it uses an atypical hydrophobic pocket to bind the critical tyrosine [110]. This variation might

explain the flexibility of ARH in recognizing pY/Y/F on that position. At the other end, ARH uses a consensus helical motif that is shared by several adaptors such as epsin and arrestin (see below) to bind the β2 appendage of AP-2 and link cargo to the coat [111].

Much like AP-2, these alternate adapters are also regulated. A well-studied example is Numb, which mediates the internalization of APP, Notch receptor, and integrins, although it requires AP-2 for its localization in CCPs [102, 112, 113]. Phosphorylation of Thr102 by AAK1 induces Numb redistribution from the plasma membrane into endosomes, while a T102A mutant is constitutively localized to surface puncta [114]. Further, phosphorylation of multiple serines by calcium/calmodulindependent protein kinase or by atypical protein kinase C also modulate Numb activity by inhibiting AP-2 binding [94, 115, 116]. Similarly, ARH function could be potentially regulated by phosphorylation of Tyr888 on the ARH-binding domain on the β2 appendage of AP-2 [111, 117]. Such selective phosphorylation of different adaptors by spatially restricted kinases provides a mechanism for localized endocytosis of cargo from defined areas of cells, as is required for cell migration [115].

In addition to these general mechanisms, several cargo proteins use relatively distinct alternate signals and adapters. Arginine (R)-soluble NSF attachment protein receptor (SNARE) proteins—key mediators of membrane fusion—are interesting examples [118]. These need to be included in forming CCVs, as they mediate fusion steps including that of endocytic vesicles and endosomes. However, SNAREs do not have the linear internalization motifs discussed above. Instead, small R-SNAREs, such as synaptobrevin and VAMP8, 3, and 2, are internalized by specific and direct interactions between the N-terminal halves of their SNARE motifs and the ANTH (AP180 N-terminal homology) domain of the endocytic clathrin adaptor CALM [119, 120]. Other SNAREs such as the R-SNAREs VAMP7, and Vti1b use a folded N-terminal domain, called the longin domain, to interact with the ArfGAP Hrb and EpsinR, which might serve as the respective clathrin adapters for these SNAREs [121, 122]. Other adaptor proteins implicated for specific cargo include stonin 2, which binds a set of basic residues on the C2A domain of Synaptotagmin 1 [123, 124], and endophilin, which, in addition to its role in membrane bending, might moonlight as a sorting adapter for the transporter VGLUT-1 [125].

1.4.2 Regulated Endocytosis

In contrast to many of the proteins above, various signaling receptors, channels, and transporters are internalized in response to specific triggers. In the case of signaling receptors, the most common trigger is the binding of extracellular ligands. These triggers typically initiate one of two covalent and reversible modifications on the cargo—phosphorylation and ubiquitination.

Phosphorylation on defined serine (Ser) and threonine (Thr) residues form the internalization signal for many signaling receptors. This has been best established

for members of the GPCR family of signaling receptors [126]. Agonist-binding and activation of GPCRs on the cell surface causes hyperphosphorylation of multiple Ser/Thr residues mainly on the third intracellular loop and C-terminal tail of the receptors [127, 128]. This recruits the specific adaptor β-arrestin (or nonvisual arrestin) to the GPCRs [129, 130]. β-arrestins consist of an N- and a C-terminal globular domain linked by a flexible region and an extended C-terminal tail that contain one or two regions with consensus LϕxϕD/E clathrin-binding sequences [131–133] and an IVFxxFxRxR domain that binds the β2 appendage domain of AP-2 [134, 135]. Before GPCR activation, β-arrestins are kept in an inactive or "closed" conformation by intramolecular interactions of the IV residues in the AP-2 binding domain with a hydrophobic pocket on the N-terminal domain [133, 136]. GPCR activation and binding induce a conformational shift that releases the C-terminal tail and exposes the clathrin- and AP-2-binding motifs [137]. Interestingly, the AP-2-binding motif exists as a part of a β-strand in the closed conformation of β-arrestin. Upon activation, the released sequence undergoes a strand-to-helix transition to adopt a structure highly similar to the AP-2 binding motif on ARH [111]. Arrestin activity is also regulated by PI (4,5)P2 binding [138], phosphorylation [139], and ubiquitination [140, 141], but the precise roles of these regulations appear to be complex and not fully understood. Emerging data suggest that arrestins and related proteins might serve as adaptors for non-GPCR cargo molecules such as the transforming growth factor beta receptors [142] and surface transporters [143], while some GPCRs might use alternate/additional adapters such as disheveled 2 [144].

Ubiquitination is widely used as an internalization signal by many endocytic cargo including growth factor receptors [70], GPCRs [145, 146], and various channels and transporters [147]. Ubiquitination is a posttranslational modification, where ubiquitin (Ub), a conserved 76 amino acid peptide, is covalently conjugated onto typically lysine residues by sequential reactions involving Ub-activating (E1), Ub-conjugating (E2), and Ub-ligase (E3) enzymes [148]. These Ub signals are proposed to be recognized primarily by epsins, Eps15 and Eps15R, clathrin- and AP-2-associated proteins (Figs. 1.2 and 1.3) which contain tandem arrays of ubiquitin-interacting motifs (UIMs), and which are capable of binding simultaneously to multiple Ub moieties conjugated to cargo [149]. The distance between individual UIMs in this array might define the specificity of epsins and Eps15 to different Ub-linked cargo [150, 151]. Epsins and Eps15/Eps15R use a helical motif similar to ARH and arrestins to bind the α- and β-appendages of AP-2 [47]. Epsins also bear a clathrin box allowing their direct binding to the clathrin terminal domain [152], and a colocalization study suggested that clathrin binding might negatively regulate epsin's ability to bind Ub [21] (for further reading, please see Chap. 9).

The EGF-receptor (EGFR), a receptor tyrosine kinase (RTK) frequently overactive in cancer [153], has been extensively studied as an example of Ub-dependent endocytic cargo. Upon ligand binding, EGFR is dimerized, which leads to activation of its intrinsic tyrosine kinase and cross-phosphorylation of several tyrosines on the C-terminus [154, 155]. Among the many effectors that these phosphotyrosines recruit is the adapter Grb2, which in turn recruits the E3 Ub-ligase Cbl, which also