Development of an inducible model of clathrin-mediated endocytosis.

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A thesis submitted to the University of Warwick for the degree of Doctor of Philosophy

Warwick Medical School

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<tr>
<td>ANTH</td>
<td>AP180 N-terminal homology</td>
</tr>
<tr>
<td>AP</td>
<td>Assembly polypeptide</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Arf</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>ARH</td>
<td>Autosomal recessive hypercholesterolemia</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
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<td>ATP7A</td>
<td>Copper transporting ATPase 1</td>
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<tr>
<td>β2-AR</td>
<td>β2 adrenergic receptor</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CALM</td>
<td>Clathrin assembly lymphoid myeloid leukaemia</td>
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<tr>
<td>CBM</td>
<td>Clathrin box/binding motif</td>
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<td>CCP</td>
<td>Clathrin-coated pit</td>
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<td>CCV</td>
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<td>Cluster of differentiation 4</td>
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<td>CD8</td>
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<td>CIMPR</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>Abbreviation</td>
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<td>EGFR</td>
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<td>Fab</td>
<td>Fragment antigen-binding</td>
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<td>FKBP and rapamycin binding</td>
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<td>GAP43</td>
<td>Growth associated protein 43</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GGA and Tom1</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LOV</td>
<td>Light-oxygen-voltage</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>mTORC1</td>
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<td>NECAP1</td>
<td>Adaptin ear-binding coat-associated protein 1</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PSD-95/Dlg1/zo-1</td>
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<td>Paraformaldehyde</td>
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<td>PHD</td>
<td>Pleckstrin homology domain</td>
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<tr>
<td>PI(3,4)P₂</td>
<td>Phosphatidylinositol 3,4-bisphosphate</td>
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<td>PI(4,5)P₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PI-4-P</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIPK</td>
<td>Phosphatidylinositol kinase</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PRD</td>
<td>Proline rich domain</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PX-BAR</td>
<td>Bin/amphiphysin/Rvs with phox homology domain</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured illumination microscopy</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SLF</td>
<td>Synthetic ligand of FKBP</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
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<td>SXN9</td>
<td>Sorting nexin 9</td>
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<tr>
<td>TACC3</td>
<td>Transforming acidic coiled-coil protein 3</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-golgi network</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>TULIP</td>
<td>Tunable, light-controlled interacting protein</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-associated membrane protein</td>
</tr>
<tr>
<td>VHS</td>
<td>Vps27/Hrs/Stam</td>
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<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Acknowledgements

Firstly, I would like to thank my supervisor, Steve Royle, for his support and encouragement throughout the PhD. For giving me the freedom to learn from my own mistakes whilst always being there to pick up the pieces. Also, for coming up with the ‘bonkers’ ideas that made this project so much fun.

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Last but not least, a heartfelt thank you to my parents for always being there, no questions asked. I could not have done this without you.

This work was generously funded by the Medical Research Council.
Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented was carried out by the author except correlative light-electron microscopy experiments, which were performed by Nick Clarke, and the isolation of mouse hippocampal neurones, which was done by Gabrielle Larocque.
Abstract

Clathrin-mediated endocytosis is the major route of internalisation of a wide range of cargo at the plasma membrane. Despite the high frequency of endocytic events, it is difficult to predict when and where a clathrin-coated pit will form, complicating any analysis of initiation mechanisms. This thesis describes the development of an inducible model of endocytosis that provides spatiotemporal control over initiation. This is achieved through chemical- or light-induced dimerisation of a clathrin-binding protein (hook) to a plasma membrane cargo (anchor), bypassing any preparation steps prior to clathrin recruitment. It was found that this rapidly induced clathrin-mediated endocytosis that could function independently of AP-2 and without detriment to endogenous CME.

This synthetic endocytosis was found to be a useful measure of functional interaction between clathrin and its adaptors, for example β3 adaptin hinge + appendage was non-functional for CME despite previous reports of in vitro clathrin binding. Also, these clathrin hooks can be easily mutated to pinpoint interaction sites. Mutagenesis of a β2 hinge + appendage hook revealed that a low level of endocytic activity could be maintained when either the hinge or appendage site was disrupted, but mutation of both sites or removal of the entire appendage inhibited synthetic endocytosis. Additionally, the ability of clathrin alone to promote clathrin-coated vesicle formation was shown by the success of a GTSE1 hook, a protein not known to interact with endocytic accessory proteins.

This technique offers great potential for further examination of molecular level clathrin/adaptor interactions as well as CME mechanics on a cell-wide scale. In particular, the localised initiation of endocytosis demonstrated here using optogenetics is ideally suited to manipulate cells displaying polarised endocytosis.
1. Introduction

1.1 Clathrin-mediated endocytosis

Endocytosis describes the process of internalisation from the plasma membrane (PM), through the formation of new membrane-bound compartments. This may occur constitutively, or in response to environmental changes and stimuli. There are multiple mechanisms for this transport depending on the cargo that needs to be internalised, which can be broadly divided into clathrin-dependent and clathrin-independent pathways (Doherty & McMahon, 2009). Examples of the latter include phagocytosis (Flannagan et al., 2012) and macropinocytosis (Lim & Gleeson, 2011), these are large scale mechanisms with a key role in the immune response, trapping pathogens and sampling antigens. The best characterised of the small scale clathrin-independent mechanisms is caveolar endocytosis, which internalises a diverse range of cargos, including insulin receptors and G protein-coupled receptors (GPCRs) in vesicles coated with a caveolin/cavin scaffold (Shvets et al., 2014).

Clathrin-mediated endocytosis (CME) transports a diverse range of cargoes and therefore operates in most cell types. Coated pits and vesicles were first observed in mosquito oocytes where they were responsible for the uptake of yolk proteins (Roth & Porter, 1964). The organisation of this coat into hexagons and pentagons was first observed on vesicles isolated from guinea pig brains (Kanaseki & Kadota, 1969). Further purification of neuronal clathrin-coated vesicles (CCVs) determined that the cages were largely composed of one protein found at 180 kDa on an SDS gel, clathrin heavy chain (Pearse, 1975). The triskelion structure of this clathrin monomer was revealed by electron microscopy (EM) imaging of dissociated cages (Ungewickell & Branton, 1981).

CME is particularly abundant in neurones where it is one mechanism proposed for the fast retrieval of synaptic vesicles, evidenced by defects in retrieval following inactivation or knockdown of clathrin or its adaptor proteins AP-1 and AP-2 (Royle & Lagnado, 2010). CME in non-neuronal tissues can be broadly separated into two
Figure 1.1 The process of clathrin-mediated endocytosis. (A) Recognition of plasma membrane cargo by adaptor protein AP-2, followed by clathrin recruitment. (B) Invagination of clathrin-coated pit through clathrin polymerisation, assisted by accessory proteins such as epsin and CALM (AP180 in neuronal cells). (C) Scission of the vesicle from the membrane by dynamin. (D) ATP-dependent uncoating of the vesicle by GAK (or neuronal homolog auxilin) and Hsc70. (E) Uncoated vesicles fuse to form early endosomes, from here, cargo may be recycled to the cell surface, sorted further in late endosomes, or sent to lysosomes for degradation.
categories: stimulated and constitutive internalisation. Both types follow the same basic procedure (Figure 1.1). Clathrin cannot bind to membranes directly (Unanue et al., 1981), so first, adaptors proteins must bind to cargo in order to recruit clathrin, then accessory proteins and clathrin bend the membrane inwards forming the clathrin-coated pit (CCP). Finally, the neck of this pit is pinched off by the GTPase dynamin, forming the CCV. Once internalised and uncoated, the contents of the vesicle may be recycled back to the plasma membrane or sent to lysosomes for degradation (Robinson, 2015, Elkin et al., 2016).

Two of the most well-known examples of cargo that are constitutively internalised are the transferrin (Tf) and LDL receptors (LDLR), which provide iron and fatty acids to cells. These contain sorting signals in their cytoplasmic tails, a short sequence of peptides (YTRF and FDNPVY respectively) which bind adaptor proteins. The principal adaptor at the plasma membrane is AP-2, which recognises various cargoes including transferrin and GABA_A receptors. LDL receptors however are recognised by proteins with a phosphotyrosine-binding (PTB) domain such as ARH and Dab2 (Traub, 2009). Mutation of the LDL sorting signal and loss of endocytic activity is one cause of familial hypercholesterolaemia, an observation that led to the discovery of the FXNPXY sorting signal (Chen et al., 1990). The variety of adaptor proteins at the plasma membrane allows recognition of many different sorting signals providing targeted uptake and reduced competition for binding partners (Sorkin (2004), Figure 1.2)

Many receptors need to remain on the cell surface until they are activated, so these undergo stimulated internalisation. These receptors control the timing of endocytosis by recruiting adaptors and clathrin only once a ligand has bound. One example of this is the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK) that dimerises in response to ligand binding, causing autophosphorylation and downstream ubiquitination of its cytoplasmic tail. Only now is it recognised by the adaptors epsin and Eps15, which go on to recruit clathrin and begin the CME process, potentially with the assistance of AP-2 (Fortian et al., 2015). The internalisation of EGFR prevents continued activation but signalling continues with further pathways activated from endosomes. Downregulation of EGFR activity through endocytosis is critical to limit EGF activity, loss of this control is implicated in multiple cancers (Tomas et al., 2014).
Figure 1.2 Interaction of cargo with adaptors and clathrin. Representation of different cargo proteins (grey shapes) and their specific sorting signals. The interactions between these cargoes, alternative adaptors (yellow boxes), AP-2, and clathrin are indicated by a dashed line. Figure adapted from Sorkin (2004).
Multiple methods of controlled endocytosis are employed by GPCRs to control surface expression levels and recycle desensitised receptors. The best characterised of these is arrestin-dependent internalisation, exemplified by the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)AR). Following agonist binding and G protein activation, the GPCR is phosphorylated by G protein-coupled receptor kinases (GRKs); this activated state is recognised by non-visual arrestins. Once bound, arrestin undergoes dephosphorylation and a conformational change that releases binding sites for the N-terminal domain of clathrin and the AP-2 subunit, \( \beta_2 \) adaptin (Moore et al., 2007). Additionally, arrestins interact with membrane phosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) and inositol hexaphosphate (IP_6) to up- and down-regulate CME respectively (Nelson et al., 2008). Once internalised there are two possible fates: reactivation and recycling to the plasma membrane, or lysosomal degradation. Arrestin binding also modulates this behaviour, dissociation of receptor and arrestin near the plasma membrane allows for rapid recycling (e.g. \( \beta_2 \)AR), whereas prolonged association into endosomes results in delayed recycling or degradation (Simaan et al., 2005).

1.1.1 Initiation

The core components required for the initiation of CME have been identified as PI(4,5)P_2, cargo, AP-2, and clathrin (Godlee & Kaksonen, 2013). However, the initiation event is not well understood, particularly regarding the regulation of timing and location. Key to understanding these properties is the identification of the initiating protein(s). In vitro, clathrin cages can self-assemble with no other material present, although when supplemented with adaptor proteins the cages form more readily, with a better defined morphology (Zaremba & Keen, 1983). These cages grow even more efficiently on a lipid bilayer supplemented by the adaptor epsin (Dannhauser & Ungewickell, 2012). This is likely due to the liposome providing a nucleation point for clathrin, reducing the area over which it diffuses. These in vitro clathrin-coated structures required on average two epsin molecules to attach one clathrin triskelion. This is in agreement with in vivo total internal reflection fluorescence (TIRF) imaging, where 75% of CCPs were initiated by two AP-2 complexes and 1 triskelion (Cocucci et al., 2012). It was suggested here that AP-2 is the pioneer at CCP sites, transiently sampling the membrane until it is stabilised by binding with clathrin and another AP-2. The CCP then grows from this cluster.
While AP-2 may be the first protein to the site of endocytosis, it depends on PI(4,5)P$_2$ lipids for its localisation. PI(4,5)P$_2$ also binds other adaptor proteins making it a good candidate for the common initiating factor. Ectopic rerouting of inositol 5-phosphatase to the plasma membrane caused dephosphorylation of PI(4,5)P$_2$, resulting in the inhibition of Tf uptake due to loss of clathrin, AP-2, and epsin from the PM (Zoncu et al., 2007). It has been shown that binding of AP-2 to PI(4,5)P$_2$ is necessary to release cargo and clathrin binding sites which are concealed in the AP-2 core when inactive in the cytoplasm (Jackson et al., 2010, Kelly et al., 2014). Whether PI(4,5)P$_2$ can be classified as the initiator will depend on whether it takes an active or passive role in this opening step. What is clear is that initiation needs cooperation between these different components, utilising multiple positive feedback loops. Once AP-2 has bound cargo and clathrin, this AP-2/cargo complex can then upregulate local PI(4,5)P$_2$ production through stimulation of phosphatidylinositol kinase (PIPK) type 1, attracting more AP-2 (Krauss et al., 2006). Cargo binding is also disproportionally increased by upregulation of AP-2 μ2 phosphorylation, mediated by stimulation of adaptor associated kinase 1 by clathrin (Conner et al., 2003).

An alternative hypothesis is that the site is determined by increased density of cargo which subsequently recruits AP-2 (Figure 1.3A). This is supported by experiments in which biotinylated transferrin receptors are clustered by tetrameric streptavidin. This resulted in faster than normal AP-2 recruitment and an increase in CCP initiation (Liu et al., 2010). This cargo-dependent initiation has also been observed with viruses such as influenza, which was observed to recruit clathrin 2-3 min after binding to the cell surface (Rust et al., 2004). Rather than AP-2, it’s thought influenza is recognised by the ubiquitin interacting motifs of epsin (Chen & Zhuang, 2008). In contrast, clustering of TfR by canine parvovirus did not produce de novo CCPs, instead the virus/receptor complex diffused along the cell surface until it encountered a pre-existing site of CME (Cureton et al., 2012). The difference in these observations may be due to the substantially stronger binding observed between TfR and streptavidin compared to parvovirus.

One explanation for the ability of clustered cargo to initiate a CCP is by bending the membrane through protein crowding. Stachowiak et al. (2012) studied this effect using epsin, which is typically thought to induce membrane curvature through insertion of an amphipathic helix from its ENTH domain into the bilayer. However, a
mutant lacking this ‘helix 0’ was able to induce membrane tubulation on giant unilamellar vesicles when covering at least 20% of the surface area. This membrane bending could also be replicated using membrane-bound GFP. Clathrin can be recruited to curved membranes through proteins such as amphiphysin and epsin, both of which have clathrin binding sites and a preference for curved membranes (Peter et al., 2004, Holkar et al., 2015). As an initiation method, protein crowding is only applicable to cargo with large intracellular and small extracellular domains since curvature is towards the side of steric pressure.

It has been proposed by Henne et al. (2010) that membrane curvature itself is the initiating step in endocytosis, through the activity of Fer/Cip4 homology domain-only (FCHo) proteins 1 and 2 (Figure 1.3B). Knockdown of FCHo1/2 with siRNA resulted in a complete loss of CCPs. Tf uptake could not be rescued by FCHo2 carrying a mutation in its F-BAR domain, therefore, its membrane bending activity is fundamental for the initiation of a CCP. The arrival of FCHo is independent of AP-2, as shown by RNAi, and it can still recruit accessory proteins Eps15 and intersectin using its μ homology domain in these AP-2 depleted cells. However, complete block of Tf uptake in FCHo1/2 depleted cells could not be replicated in TALEN-mediated gene edited cells lacking both FCHo 1 and 2 (Umasankar et al., 2014). In these cells, lack of FCHo1/2 caused large, structurally irregular clathrin lattices to form at the plasma membrane. These structures were positive for the common pioneer proteins AP-2, CALM, Dab2, intersectin, Eps15, and NECAP1; this is not consistent with a role for FCHo1/2 as sole initiator. Transferrin uptake was found to be delayed by the stability of these large clathrin-coated structures, and there was a decrease in CCP initiation but it was not blocked completely. Recent research suggests that FCHo1/2 actually has an alternative initiating role, inducing the conformational change of AP-2 through binding of the FCHo1/2 linker domain to the μ2 subunit in association with Eps15/R. FCHo and Eps15 are pushed to the edge of the CCP as it matures, with clathrin-associated proteins (CLASPs) such as CALM and epsin occupying their AP-2 binding sites (Hollopeter et al., 2014, Ma et al., 2016).

From the evidence presented above, the fundamental initiation sequence of constitutive CME is binding of AP-2 to PI(4,5)P₂, which together with a FCHo1/2 and Eps15 complex triggers the conformational change of AP-2. This is stabilised by further binding to PI(4,5)P₂ and cargo sorting motifs, and recruits clathrin to begin
Figure 1.3 Potential mechanisms of CME initiation. (A) Cargo clustering attracts AP-2 followed by clathrin binding. (B) FCHo initiates pit formation through membrane bending, recruiting Eps15 and intersectin followed by AP-2 and clathrin. (C) FCHo/Eps15 complex together with PI(4,5)P₂ and cargo controls the conformational change of AP-2 at the plasma membrane, followed by recruitment of accessory proteins and clathrin.
CCP formation (Figure 1.3C). While this sequence controls the timing of CME, the location of the initiation site must also be determined. The location may be selected by clustering of receptor cargo (Liu et al., 2010), random sampling of the membrane by adaptor proteins (Ehrlich et al., 2004), or highly productive ‘hotspots’ (Gaidarov et al., 1999). In support of the latter, Nunez et al. (2011) found 53 ± 12% of CCPs occurred in an area of repeated nucleation. These hotspots lasted around 160 seconds and had a radius of ~200 nm. Hotspot formation was dependent on an intact actin cytoskeleton. Hotspots do not appear to be more efficient at generating CCPs than random sites, but they are less sensitive to changes in adaptor and PI(4,5)P$_2$ levels. A study of the dynamics of endocytic proteins showed no differences in the recruitment profiles between the inside and outside of hotspots. Although, following scission within hot-spots, higher levels of clathrin-binding proteins remained compared to non-hotspot sites, including Eps15, FCHO1/2, dynamin1/2, μ2 adaptin (Taylor et al., 2011). Ligand-induced CME has also been observed at these hotspots, with β2AR clustering at pre-existing clathrin spots and plaques following stimulation with isoproterenol (Lampe et al., 2014). Further support for this idea has come from using structured illumination microscopy (SIM) to observe CCP initiation at <100 nm resolution. Here, sequential nucleation of CCPs could be observed from a single hotspot, with 3.6% of these sites generating more than 5 CCPs (Li et al., 2015).

1.1.2 Vesicle maturation

Following initiation, the CCP must bend the membrane further and complete the clathrin coat. There is some debate over the order of these two events; early EM imaging showed flat clathrin lattices as an array of hexagons which remodelled into curved structures by adding pentagons to the lattice (Heuser, 1980). However, it has been argued that these flat clathrin plaques do not produce true clathrin-coated vesicles, having different dynamics and internalisation mechanics to isolated CCPs (Saffarian et al., 2009). Clathrin polymerisation during invagination (the canonical model) is also supported by in vitro studies where clathrin was shown to have increased affinity for curved membranes (Pucadyil & Holkar, 2016) and clathrin polymerisation could occur faster and with better thermal stability on polystyrene beads vs flat Formvar grids (Dannhauser et al., 2015). In contrast, significant support for reorganisation of flat clathrin lattices has been generated recently by Avinoam et al. (2015) using correlative light electron microscopy (CLEM). It was shown that the area of the clathrin lattice does not change during invagination, which would not be
the case if clathrin was added during internalisation. Also, the curvature of the CCP increases over time, this would be constant in the canonical model. They used fluorescence recovery after photobleaching (FRAP) experiments to show that these lattices have an exchange of clathrin so would be capable of reorganising to add the pentagons necessary to curve the lattice. A study of CME dynamics by Taylor et al. (2011) suggests both types of event may occur within cells. Isolated events showed the expected clathrin recruitment profile for the canonical model, however the tendency for endocytic events to occur at the edges of plaques supports clathrin remodelling from existing lattices.

Epsin has an important role in CCP growth through membrane bending mediated by its ENTH domain, specifically via an amphipathic helix termed helix zero. This helix is released in response to PI(4,5)P$_2$ binding and inserts into the outer leaflet of the plasma membrane, pushing the head groups apart and reducing the energy required to produce curvature (Ford et al., 2002). Recruitment of clathrin to epsin aids membrane bending further (Holkar et al., 2015). It is thought that the structurally related ANTH domain on the adaptor CALM (and its neuronal homolog AP180) is also capable of bending membranes through a helix zero interaction with the plasma membrane. In CALM depleted cells, an increase in open CCPs with a large diameter was found, which could not be rescued by mutant CALM lacking PI(4,5)P$_2$ binding ability. Therefore, it appears CALM is necessary to ensure correct CCP curvature and size in order for the vesicle neck to be small enough for scission (Miller et al., 2015).

Amphipathic helices do not appear to be the sole mechanism of membrane bending, in vitro liposomes coated in epsin lacking the ENTH domain could be deformed into buds by clathrin alone (Dannhauser & Ungewickell, 2012). It has also since been suggested that this activity was supported by protein crowding of epsin’s disordered C-terminal domain (Busch et al., 2015). In vivo, this method of curvature could be induced by AP-2, because once it is clustered at CCPs AP-2 occupies a similar membrane area to the disordered domains tested. However, this would require cargo to have a comparatively small extracellular domain to prevent counteracting the effect generated by crowding on the intracellular side of the bilayer.

The ENTH domain of epsin is also thought to regulate actin recruitment to CCPs, further contributing to membrane bending via the cytoskeleton. Epsin1/2/3 triple
knockout cells were observed to have a higher density of CCPs but a decreased frequency of internalisation events. There was also a considerable increase in the number of shallow pits that did not recruit dynamin 2 or endophilin, suggesting they had been arrested at an early stage of invagination. Whilst this may have been caused by loss of the membrane bending activity of epsin, these cells displayed defects in the arrangement of the actin cytoskeleton. The ENTH domain of epsin binds the ANTH domain of Hip1R, and together they can bring actin to the CCP through multiple binding sites (Messa et al., 2014).

Unlike endocytosis in yeast, which has an essential role for actin (Engqvist-Goldstein & Drubin, 2003), mammalian cells do not appear to have this dependence during vesicle growth (Fujimoto et al., 2000). However, disruption of the actin cytoskeleton has still been reported to affect a wide range of events, with varying significance. One of its main roles appears to be in spatial organisation of CCPs; treatment with latrunculin or jasplakinolide to disrupt the actin cytoskeleton produced fewer CCPs and those that remained were more clustered. In the same assay no noticeable effect was seen on growth, maturation, or uncoating of the CCV (Boucrot et al., 2006). Actin has also been observed at mobile clathrin-coated structures, which display shorter path lengths after actin depolymerisation (Yarar et al., 2005). Additionally, actin appears to be important to prepare vesicles for scission in cells that have high membrane tension. Latrunculin treatment prevents actin polymerisation and produced wide necked pits that could not recruit dynamin on the apical membrane of polarised MDCK cells, recruitment continued on basolateral membrane which has lower membrane tension. Conversely, jasplakinolide promotes actin polymerisation and increased basolateral CCP lifetime and incidence of arrest in cells mechanically stretched to increase the membrane tension (Boulant et al., 2011). Actin activity at CCPs is regulated by Hip1R, which is recruited by clathrin light chain (CLC). The affinity for actin of Hip1R is negatively regulated through CLC binding, suggesting the clathrin and actin binding events occur sequentially (Wilbur et al., 2008).

1.1.3 Vesicle scission

The core components of scission machinery are dynamin and BAR (bin-amphiphysin-rvs) domain containing proteins. Dynamin is a large GTPase composed of an N-terminal GTPase, a ‘stalk’ region containing GTPase effector domains, a PI(4,5)P₂ targeting domain (PHD) and a C-terminal proline rich domain (PRD). As with AP-2,
dynamin has closed and open conformations to ensure it is only active when needed, this is done through binding of the flexible PHD to the stalk region. Mutation of PHD to keep dynamin in the open conformation resulted in the self-assembly of dynamin in solution into tetramers and higher order oligomers. Membrane binding releases PHD from the stalk, opening the structure and allowing the PHD to insert hydrophobic loops into the bilayer, which themselves support membrane curvature and scission (Srinivasan et al., 2016).

In vitro studies of dynamin-coated membrane tubules have shown that dynamin dimers form a ring around the tubule, connecting at their GTPase domains (Cocucci et al., 2014). On binding of GTP, the layers of this dynamin ring rotate up to 30 times to constrict the tubule. However, this motion was only able to complete scission when under longitudinal tension, this may be provided by actin in vivo (Roux et al., 2006). More recent work suggests the scission process may be in two stages, with dynamin constriction only enough to reach the hemi-fission, or transition, state where the two membranes have fused but not broken. Full scission is achieved through GTPase-dependent conformational changes that loosen the dynamin scaffold, create axial force, and retract PHD helices from the membrane (Mattila et al., 2015).

BAR proteins typically have an SH3 domain that interacts with the proline and arginine rich domain (PRD) of dynamin, and a BAR domain that acts as a scaffold on curved membranes (Daumke et al., 2014). Amphiphysin and endophilin are recruited to the late CCP through their membrane-curvature sensing N-BAR domains, together they regulate and recruit dynamin, promoting scission (Meinecke et al., 2013). Additionally, the N-BAR domains of amphiphysin and endophilin contain an amphipathic helix which can enter the membrane, potentially destabilising the lipid bilayer and facilitating scission (Daumke et al., 2014).

The role of PX-BAR protein sorting nexin 9 (SNX9) is less clear, despite binding clathrin and dynamin, SNX9 RNAi knockdown did not alter dynamin recruitment or uptake of transferrin (Meinecke et al., 2013), this is in line with peak SNX9 recruitment occurring after scission (Taylor et al., 2011). It has been shown that SNX9 has a preference for PI(3,4)P₂ over PI(4,5)P₂, a change mediated by PI(3)K C2α as the vesicle matures, possibly to prepare for fusion with endosomes (Posor et al., 2013). A further F-BAR protein with a role in scission is FBP17, this is involved in the
recruitment of actin to the scission site through the activation of WASP, as well as
dynamin binding activity (McDonald & Gould, 2016).

1.1.4 Vesicle uncoating

Uncoating of vesicles begins with the recruitment of auxilin (in neuronal cells) or its
non-neuronal equivalent cyclin G-associated kinase (GAK). These both have an N-
terminal tensin-like domain which shares homology but not activity with the
phosphatase PTEN, a clathrin-binding domain, and a C-terminal J domain that
interacts with heat shock protein Hsc70 (Greener et al., 2000). The PTEN-like domain
preferentially binds PI-4-P membrane lipids which are synthesised from PI(4,5)P_2 by
phosphatases such as synaptojanin. The appearance of these lipids late in the
endocytic pathway controls the timing of auxilin so it arrives following peak dynamin
activity (Guan et al., 2010). Dynamic light scattering experiments have suggested
uncoating requires one auxilin molecule and three Hsc70 molecules per triskelion,
with the latter arriving in three stages. The first Hsc70/ATP complex binds to the
clathrin cage through the auxilin J domain and clathrin heavy chain C-terminal.
Following ATP hydrolysis, Hsc70 undergoes a conformational change, releasing
auxilin to bind the next Hsc70/ATP and the process repeats (Rothnie et al., 2011).
Hsc70 is not detached immediately from the triskelion after disassembly, this prevents
empty cages forming in the cytoplasm (Schlossman et al., 1984). The catalytic nature
of auxilin is thought to be regulated by clathrin light chain, as the release and recycling
of auxilin was inhibited by removal of CLC (Young et al., 2013). The stoichiometry of
Hsc70/clathrin binding has been challenged by a study using single-particle
fluorescence imaging in which it was observed that the minimal number of Hsc70
required for complete uncoating was 0.5 molecules per triskelion (Bocking et al.,
2011).

Multiple models have been proposed for the mechanical action of Hsc70-induced
uncoating. The power stroke model describes the pulling apart of the oligomeric
substrate through ATP-powered conformational changes in Hsc70 (Glick, 1995).
Secondly, the Brownian or steric wedge model comes from structural studies which
suggest Hsc70 locks the clathrin heavy chains in place during their transient
fluctuation, introducing strain and weakening the cage structure (Xing et al., 2010).
Finally, the collision pressure model uses the collision and repulsion between Hsc70
and the cage wall to provide the necessary force. Recent experiments support this
latter model; adding anti-histidine Fab fragments to histidine-tagged Hsc70 accelerated disassembly, presumably through increased collision from the increased volume. Conversely, moving the Hsc70/clathrin heavy chain binding site further from the cage walls slows disassembly. The steric wedge mechanism would have been more sensitive to the movement of the Hsc70 binding site, with inhibition of disassembly due to the resolution of steric clashes. Finally, the power stroke model was discredited by the successful uncoating of FLAG tagged cages by the binding of a Fab fragment in the place of Hsc70, both around 70 kDa in size (Sousa et al., 2016).

Following uncoating, the vesicles fuse to form early/sorting endosomes; from here they may be recycled or degraded in a process regulated by Rab proteins and their downstream effectors. Recycling to the plasma membrane may occur directly from the early endosome mediated by Rab4a (fast recycling), or after Rab4b-mediated transfer to Rab11 positive recycling endosomes. Both pathways recruit AP-1 and clathrin to form intracellular transport vesicles, with Rab4a also using AP-3 and GGA adaptor proteins (Chamberland et al., 2016). If the cargo is sent to late endosomes, it may go to the trans-golgi network (TGN) or for degradation in lysosomes. Cargo fate is determined by several factors, including the pH at which the ligand dissociates from the receptor (pH decreases in each successive compartment), sorting motifs, and modifications such as ubiquitination (Elkin et al., 2016).

1.2 Clathrin structure

Clathrin is a trimer of three ~190 kDa heavy chains (legs) in a triskelion arrangement, each leg may associate with a short ~25 kDa clathrin light chain (Figure 1.4B). This distinctive shape allows the self-polymerisation of clathrin into cages that completely encase a membrane vesicle (Figure 1.4A). The size of the cage will depend on the size of the cargo, although the soccer ball conformation (60 triskelia) is the smallest cage that will contain a membrane vesicle. The triskelia are arranged in a hexagonal lattice which must contain 12 pentagons in order to form a closed cage, independent of the final size (Fotin et al., 2004). Aside from endocytosis, clathrin triskelia have been proposed to span the gap between microtubules of the mitotic spindle kinetochores, associated with transforming acidic coiled-coil protein 3 (TACC3) and colonic hepatic tumour overexpressed gene (ch-TOG; Cheeseman et al. (2013)).
Figure 1.4 Clathrin structures. (A) Hexagonal barrel clathrin cage (36 triskelia) modelled from EMDataBank entry EM-5119 with one subunit highlighted. (B) Single clathrin triskelion, showing three heavy chains (green) and three light chains (red). Structure from pdb entries 1XI4 and 3LVG. (C) Side view of triskelion hub showing attachment of clathrin light chains and C-terminal tripod structure. Structure from pdb entry 3LVG. (D) N-terminal domain of clathrin showing the seven-bladed β propeller structure and location of the four binding sites. Structure assembled from pdb entries 1UTC, 1C9I and 3GD1.
1.2.1 Clathrin heavy chain

There are two isoforms of clathrin heavy chain (CHC): CHC17 and CHC22, which share 85% sequence homology. The latter is found predominantly in human skeletal muscle where it has a role at neuromuscular and myotendinous junctions. CHC17 is ubiquitously expressed in vertebrate tissues (Wakeham et al., 2005). The CHC leg is about 47.5 nm long, containing an N-terminal β propeller, linker, ankle, distal segment, knee, and C-terminal proximal segment. Excluding the N-terminus, the leg (residues 395-1576) is composed of eight repeats of a ~145 amino acid α helical motif designated clathrin heavy chain repeat (CHCR) 0-7 (Ybe et al., 1999). In the absence of the distal leg section the clathrin cage cannot close, it is thought this is due to the role of the distal leg in fixing the cage vertices. This is achieved through the interaction between two distal and two proximal leg sections of four triskelia, an arrangement which may also inactivate the negative regulation of clathrin assembly by CLC (Greene et al., 2000).

At the C-terminal trimerisation domain, the three CHCs form a tripod of 5 nm alpha helices angled inwards (Figure 1.4C) where they bind a triangle of ankle domains from the CHCs below. The angle at the triskelion vertex does not change in response to different coat sizes, instead it is the angle at which neighbouring CHCs cross that changes (Fotin et al., 2004). Most clathrin/protein interactions occur via the N-terminal domain (Lemmon & Traub, 2012), however there are some exceptions including Hsc70 which binds the C-terminal ‘tripod’ (Xing et al., 2010), and ankle binding proteins TACC3 (Hood et al., 2013) and GGA1 (Knuehl et al., 2006).

1.2.2 CHC N-terminal domain

Clathrin heavy chain N-terminal domain (NTD) is a β propeller made up of seven blades, each with four antiparallel strands (ter Haar et al., 1998). There are four binding sites on this NTD (Figure 1.4D), the first to be identified was the clathrin box motif (CBM) binding site in the groove between blades 1 and 2. This binds the LΦXΦ[DE] consensus motif, which can be found in β-arrestin 1, β2-adaptin hinge, and amphiphysins. The second binding site, the W box motif, is found in the centre of the propeller and binds PWXXW motifs found in amphiphysin and SNX9. The β-arrestin 1L site binds [LI][LI]GXL motifs found in β-arrestin 1L and β2-adaptin and is located in the groove between blades 4 and 5 (Lemmon & Traub, 2012). CHC with
all three of these sites mutated can still support transferrin uptake, whereas deletion of the entire NTD could not, suggesting a fourth site. This was found in a highly conserved region of blade 7, functionality was confirmed by inhibition of CME following mutation of all four sites. All permutations of triple NTD mutants could rescue AP-2/clathrin-dependent endocytosis after CHC RNAi, suggesting that there is considerable redundancy between the sites (Willox & Royle, 2012). This was unexpected because only two of the sites were known to bind AP-2, however recent work used NMR to show that small fragments of β2 adaptin or AP180 could bind promiscuously to all three original sites. They found no evidence for clathrin box binding at the fourth site (Zhuo et al., 2015). Subsequent structural studies from Muenzner et al. (2016) have shown binding of CBM sequences from hepatitis D large antigen proteins at the predicted fourth site (termed the ‘Royle box’) but no consensus motif could be determined. Interestingly, the same study found that a β2 adaptin or amphiphysin fragment containing only the clathrin box motif (LΦXΦ[DE]) could bind both clathrin box and arrestin 1L sites on the CHC NTD but not the W box and with a different structural arrangement to β-arrestin 1L binding.

1.2.3 Clathrin light chain

Clathrin light chain has two isoforms, LCa and LCb, in addition to neuron-specific isoforms. Neither isoform functionally binds CHC22, but they both perform similar functions in the regulation of CHC17. LCa and LCb have different expression patterns in different tissues, suggesting they may provide some specificity to the activity of CHC17 (Wakeham et al., 2005). CLC is not essential for the internalisation of many common CME cargoes including TfR, EGFR, LDLR, and β2 adrenoceptor. Additionally, transgenic mice lacking LCa (dominant isoform in lymphoid tissues) were viable, albeit with a 50% postnatal mortality rate. However, B cells from these animals showed defects in internalisation of TGF β receptor 2 and C-X-C chemokine receptor 4 (Wu et al., 2016). The group of cargoes with the greatest dependence on CLC are those which require actin, as CLC is needed to recruit Hip1 and Hip1R (Wilbur et al., 2008). CLC may also have a role in cargo selection through patterns of phosphorylation. Uptake of GRK2-dependent cargo such as μ opioid and P2Y12 receptors, but not GRK2-independent P2Y1 receptors, was inhibited by a LCb S204A mutation, suggesting phosphorylation of S204 by GRK2. Vesicle uncoating may also be controlled by phosphorylation at this site, with increased auxilin binding to clathrin cages observed with LCb S204A and reduced binding with the phosphomimetic LCb.
S204D (Ferreira et al., 2012). Additionally, CLC has been implicated in the recycling of TfR and β1 integrins, loss of the latter following CLC depletion significantly inhibited cell migration through decreased surface expression of integrins and loss of focal adhesion sites (Majeed et al., 2014).

CLC can also regulate the assembly of clathrin cages, through calcium sensing (Näthke et al., 1990) and control over CHC conformation. The CLC helix is positioned on the hub (knee, proximal leg, trimerisation domain) of the triskelion (Figure 1.4C), and can adopt a compact or extended conformation. The latter allows binding of CLC to the CHC knee near the KR loop, a patch of basic residues that acts as the hinge for the knee. When CLC is bound here, the CHC leg is straight, which is not permissive for assembly of the clathrin lattice. Prior to assembly, the CLC contracts away from the knee allowing it to bend and polymerise. (Wilbur et al., 2010).

1.3 Adaptor proteins

Clathrin adaptor proteins can be broadly split into two structural groups, the five heterotetrameric AP (assembly polypeptide) complexes (Hirst et al., 2011) and monomeric adaptors such as epsin and GGAs. These adaptors are responsible for targeted trafficking of different proteins between different compartments of the cell, e.g. AP-2 is at the plasma membrane, whereas the remaining APs are at the trans-golgi network and endosomes (Figure 1.5). Their activity is controlled by their spatial distribution in the cell and adaptor-specific sorting signals. Cargo-specific adaptors at the plasma membrane may additionally recruit the more versatile AP-2 despite most of them having clathrin binding domains of their own (Robinson, 2004).

1.3.1 AP-2

AP-2 is the most abundant of the clathrin adaptors, and is responsible for CME at the plasma membrane. AP-2 is a tetrameric complex of two large α and β2 adaptin subunits, a medium μ2 subunit, and a small σ2 unit (Figure 1.6A). These are arranged in two hemicomplexes, β2 + μ2 and α + σ, both of which are partially functional alone (Gu et al., 2013). Both α and β2 are mostly within the core of AP-2, however, they also have a globular appendage domain, or ear, that joins to the core through a long, flexible linker. The μ and σ subunits are responsible for cargo binding, recognising YXXΦ and [ED]XXXL[LI] motifs respectively. However, it is estimated that 99.9% of
Figure 1.5 Location of adaptor proteins. AP-2 is the main adaptor involved in CME at the plasma membrane. AP-1 mediates bidirectional transport between endosomes and trans-golgi network (TGN), AP-3 is involved in transport between early endosomes and lysosomes, possibly independently of clathrin. AP-4 can mediate transport between TGN and endosomes, AP-5 is found on lysosomes but the direction of transport is unknown. Neither AP-4 nor AP-5 associate with clathrin. Epsin and GGAs are monomeric adaptors involved in plasma membrane CME and TGN to endosomal transport respectively.
cytoplasmic AP-2 is in a closed conformation where the β2 subunit blocks both sites (Jackson et al., 2010). X-ray crystallography of AP-2 bound to DYQRLN fragment and a myc tag (through a [ED]XXXL[LI] sequence) revealed an open conformation where the C-terminal domain of μ2 was ejected from the AP-2 core. Thus arranging PI(4,5)P₂, YXXΦ, and [ED]XXXL[LI] binding sites co-planar on the AP-2 surface, ideally placed for contacting the plasma membrane (Figure 1.6C). It is thought that the initial contact is made through PI(4,5)P₂ binding sites on α and β2, with basic residues on μ2 C-terminus driving the opening of AP-2 through electrostatic attraction to PI(4,5)P₂. Once open, the conformation is stabilised by cargo binding; it is hypothesised that both types of cargo binding site may be bound at once if the motifs are 65-70 Å apart (Jackson et al., 2010).

Early studies on the AP-2/clathrin interaction identified a region of the β2 hinge (616-674) which was thought to be solely responsible for clathrin binding, based on in vitro binding and cage assembly experiments (Shih et al., 1995). Subsequently, the particular motif responsible for clathrin binding was identified in the β3 hinge. Sequence alignment showed this motif was conserved in the β2 hinge and various other adaptors including β1 adaptin and arrestin (Dell'Angelica et al., 1998). It is now known that this LLNLD clathrin box motif is not the only site on β2 adaptin that can bind clathrin, the hinge contains a second LLGDL box motif first identified in arrestin-2L, that binds a different region of the CHC NTD (Kang et al., 2009). Additionally, the β2 appendage alone can bind clathrin in vitro (Owen et al., 2000). In the closed conformation, these sites sit in the AP-2 core where they are made inaccessible by interactions with α and β trunks, and C-terminal μ2. This is advantageous because it prevents unproductive AP-2-clathrin binding in the cytoplasm. Once AP-2 occupies an open conformation, the movement of μ2 releases β2 hinge and allows clathrin to bind. This was confirmed in vitro by the lack of clathrin assembly with closed FLβ.AP-2 (WT tetramer missing only α hinge and ear) and recovery of function with the addition of YXXΦ motifs to the liposomes or deletion of C-terminal μ2 (Kelly et al., 2014).

The β2 appendage has two sites: a C-terminal platform binding site, which recognises [DE]ₓX₁₋₃FXX[FL]XXXR and DΦ[FW] binding motifs on epsin, ARH, and arrestins, and an N-terminal sandwich binding site that binds clathrin, AP180, and Eps15 (Figure 1.6B). Their activity is disrupted by Y888V and Y815A mutations respectively. Loss
Figure 1.6 Structural details of the AP-2 complex. (A) AP-2 complex showing four subunits: β2 adaptin (dark blue), α-adaptin (pink), μ2-adaptin (light blue), and σ2-adaptin (brown). Structure assembled from pdb files 1B9K, 1E42 and 2VGL. Unstructured hinge regions drawn for illustration purposes only. (B) β2 adaptin appendage and hinge (pdb entry 1E42) showing approximate locations of the clathrin box motif (dark green box), platform binding site (orange) and the sandwich binding site (light green). (C) Cytoplasmic AP-2 is in a locked conformation with both cargo and clathrin-binding sites hidden in the core. Upon binding to PI(4,5)P₂ and cargo proteins, a conformational change releases clathrin binding sites on the β2 hinge and appendage.
of in vitro clathrin binding to β2 appendage following Y815A mutation can be rescued by expanding the fragment to include the LLNLD hinge site; WT β2 appendage is also able to rescue a ∆LLNLD mutant, albeit to a lesser extent. Deletion of LLNLD does not stop β2 hinge + appendage from colocalising with clathrin-coated structures at the plasma membrane, unlike double appendage mutations (Y888V, Y815A) which force β2 hinge + appendage into a diffuse localisation, suggesting different roles for the two binding regions (Edeling et al., 2006). The corresponding Y815 interaction site has been proposed to be in the ankle region of CHC, since mutation of two residues (C682 and G710) prevented β2 appendage binding (Knuehl et al., 2006). However, earlier work supports clathrin binding at the platform domain, rather than the sandwich domain. Following Y888V mutation of the β2 appendage or appendage+hinge, clathrin binding was reduced by ~100% and 50% respectively (Owen et al., 2000). This agrees with work from Schmid et al. (2006) which found only a weak effect on clathrin binding with Y815A in vitro, with Y888V causing a much more significant decrease. However, in a further study, mutation of either the β2 platform site (Y888) or a putative Y888 binding motif (DVF) on CHC ankle had no detrimental effect on β2 appendage binding to clathrin, suggesting this Y888 site is not involved in clathrin binding (Knuehl et al., 2006).

Despite limited similarity between the sequence of α and β2 appendages, they have a similar structure and share binding partners, albeit with different affinities (Schmid et al., 2006). Significantly, amphiphysin and SNX9 have a preference for α, with ARH and β-arrestins having selectivity for the β2 appendage. Clathrin heavy chain does not bind the α appendage (Edeling et al., 2006).

1.3.2 Other tetrameric adaptors

AP-1 is structurally very similar to AP-2, having four subunits, γ, β1, μ1, and σ1 analogous to the α, β2, μ2, and σ2 subunits of AP-2. The binding activity of β1 is also similar to β2, both having a LLNLD clathrin box in the hinge region (Dell’Angelica et al., 1998) and sharing many binding partners, including clathrin, at the appendage domains (Schmid et al., 2006). Larger differences are observed between γ and α, with γ appendage and hinge both able to bind clathrin. The hinge through a LLDLL clathrin box motif and the appendage to a site away from the clathrin NTD. Neither α hinge nor appendage displays clathrin binding activity (Doray & Kornfeld, 2001). Like AP-2, AP-1 is thought to adopt a closed conformation in the cytoplasm, however,
binding to its preferred lipid PI(4)P is not sufficient to induce a conformational change. Instead, AP-1 is recruited by ADP ribosylation factor (Arf) GTPases, particularly Arf1; this recruitment can occur independently of PI(4)P or cargo. Once Arf1 is bound by GTP, it inserts an amphipathic helix into the membrane and releases switch I and II AP-1 binding regions. A further C-terminal binding site allosterically activates AP-1. Once open, cargo binds through YXXΦ and [DE]XXXL[LI] recognition sites on μ1 and γ-σ1 subunits respectively (Ren et al., 2013).

Rapid removal of AP-1 from CCVs by rapamycin-induced rerouting to mitochondrial membranes (‘knocksideways’) depleted vesicles of their accessory proteins and cargo. Mass spectrometry analysis of these vesicles determined that AP-1 is responsible for anterograde transport of lysosomal hydroxylases and retrograde transport of SNARE proteins and receptors such as ATP7A and CIMPR between trans-golgi network and endosomes. Also lost from these vesicles were accessory proteins such as Hip1R, GAK, CALM, and clathrin itself (Hirst et al., 2012). In contrast to AP-2-mediated endocytosis, dynamin is not thought to be needed for scission of AP-1/clathrin vesicles (Kural et al., 2012).

The third family member, AP-3, is involved in the sorting of cargo from endosomes to lysosomes. It has four subunits (δ, β3, μ3, α3) as AP-1 and AP-2, and the YXXΦ, [DE]XXXL[LI], and Arf binding sites are well conserved (Traub & Bonifacino, 2013). The β3 hinge contains a LLDLD binding motif and it has been observed to colocalise with clathrin in fluorescence and electron microscopy images (Dell'Angelica et al., 1998, Kural et al., 2012). However, there is uncertainty over whether clathrin is essential for the activity of AP-3; colocalisation experiments from Peden et al. (2002) showed poor overlap with clathrin, and a β3 mutant lacking the CBM could partially rescue the phenotype of β3-deficient cells.

AP-4 and AP-5 were discovered in silico by sequence similarities to the other APs, and do display the same tetrameric structure. However, neither AP-4 nor AP-5 contain clathrin binding motifs in their β subunits and do not associate with CCVs (Dell'Angelica et al., 1999, Hirst et al., 2011). AP-4 has been shown to be responsible for the trafficking of amyloid precursor protein (APP), through a YKFFE motif binding the μ4 subunit at a site distinct from the YXXΦ binding site. Mutation of this sequence or depletion of AP-4 traps the normally endosomal APP in the TGN suggesting this
adaptor is needed for anterograde transport from TGN to endosomes and potentially the plasma membrane (Burgos et al., 2010). This is the same route taken by AP-1 coated vesicles and despite their differences, AP-4 activity is upregulated to compensate for loss of AP-1 during knocksideways inactivation.

AP-5 is even further removed from AP-1/2/3, with low sequence homology in hinge and appendage regions compared to other family members and no affinity for Arf1. There is no definitive answer on AP-5 cargo, however it tends to be colocalised with LAMP1 on lysosomes, and AP-4 or AP-5 mutations are linked to the hereditary neurological disorder spastic paraplegia (Hirst et al., 2013).

1.3.3 Alternative adaptors

Alternative and cargo-specific adaptors tend to be monomeric, and have binding sites for both membrane (phosphoinositides or Arfs) and cargo (peptide motifs or ubiquitin). Although most have demonstrated direct clathrin-binding ability, they frequently recruit AP-1 or AP-2. This expands the range of cargo that can be recognised by AP-1/2, and can also be used to regulate timing of transport, for example the recognition of phosphorylated GPCRs by arrestins (Traub & Bonifacino, 2013).

Epsin is a monomeric adaptor that contacts the plasma membrane through its N-terminal ENTH domain. In addition to PI(4,5)P₂ binding, it can simultaneously bind the AP-2 α appendage through a series of eight DPW motifs, polyubiquitinated cargo at three ubiquitin-interacting motifs, and the clathrin NTD (Hawryluk et al., 2006). Epsin contains two clathrin box motifs that flank the DPW motifs; both sites act cooperatively suggesting they can bind two separate sites on CHC NTD (Drake et al., 2000). Closely related to epsin are CALM and its neuronal homolog AP180, these adaptors bind α and β2 adaptin appendages and clathrin. At the N-terminus, the ANTH domain simultaneously binds PI(4,5)P₂ and its R-SNARE cargo VAMPs 2, 3, and 8 (Miller et al., 2011, Miller et al., 2015).

The three monomeric GGAs (Golgi-localised, γ-ear-containing Arf-binding proteins) share many functional similarities with AP-1. These include being recruited to the TGN by PI(4)P and Arf1 binding, and recognising DXXLL cargo motifs. Structural similarities are limited to the C-terminal GAE domain, which is homologous to γ adaptin appendage and binds endocytic accessory proteins and clathrin. The major
clathrin binding site is in an unstructured hinge region that also contains a DXXLL sequence that can auto-inhibit GGA through GAE domain binding, or interact with AP-1 through the γ appendage (Bonifacino, 2004). The central GAT domain can bind ubiquitinated cargoes, and the N-terminal VHS domain binds DXXLL motifs, such as those found on mannose-6-phosphate receptor, for transport between TGN and endosomes (Guo et al., 2014).

A further class of adaptor binds the FXNPXY sorting motif through a phosphotyrosine-binding (PTB) domain, this includes Dab2 and ARH. These both bind clathrin and mediate endocytosis of LDL receptors; however, ARH appears to need AP-2 since siRNA knockdown of Dab2 + AP-2 inhibited LDLR uptake. Knockdown of AP-2 and ARH did not affect LDLR uptake, suggesting Dab2 can operate independently of AP-2. It is hypothesised that ARH binds the β2 appendage [DE]nXE12FXX[FL]XXXR site and localises LDLR to pre-existing sites of endocytosis, as observed for arrestins (Keyel et al., 2006).

1.4 Methods of protein dimerisation

Inducible protein dimerisation is typically used to manipulate signalling pathways through relocation or activation of a key component. These methods have also been used instead of RNAi to inactivate proteins because of their rapid onset and option for spatiotemporal control. There are broadly three categories – chemically-induced, light-induced, and a combination of the two (Voss et al., 2015).

1.4.1 Chemically-induced dimerisation

The dimerisation potential of the immunophilin FKBP was first realised by Spencer et al. (1993) using FK1012 (a dimer of the FKBP-binding immunosuppressant FK506) to dimerise FKBP12. This could cluster and activate receptors carrying three FKBP12 domains. Using the related macrolide rapamycin instead of FK1012 allows for heterodimerisation of two different proteins, one tagged with FKBP and one with FRB (FKBP12-rapamycin binding) domains. The first use demonstrated was for stimulation of gene expression through the dimerisation of transcription factor DNA binding and activation domains, restoring the complete complex (Rivera et al., 1996). More recently, a technique has been developed using rapamycin-induced dimerisation of a split intein to link two attached extein proteins directly, termed
conditional protein splicing. Here, the N and C termini of the VMA intein are split and fused to FKBP or FRB, upon addition of rapamycin the intein is reconstituted and the exteins ligated within minutes. This has the advantage of producing protein dimers that once spliced are not linked by FKBP-rapamycin-FRB (Mootz et al., 2003).

The endogenous binding site for rapamycin is the FRB region within mTOR (mammalian target of rapamycin), part of mTOR complex 1 (mTORC1). Rapamycin must first bind to FKBP (specifically the 12 kDa family member FKBP12) and this dimer goes on to allosterically inhibit mTORC1, weakening its interaction with its scaffolding protein raptor (regulatory associated protein of mTOR) and reducing mTORC1 kinase activity. This downregulation results in reduced cell growth and proliferation, and promotion of macroautophagy (Foster & Fingar, 2010). Rapamycin has been shown to up- or down-regulate over 500 genes in a time dependent manner. Although widespread changes were only observed after 12 hours, a small number of changes were observed within 30 min (Peng et al., 2002). These effects are undesirable when using rapamycin for dimerisation, so various rapamycin analogs (‘rapalogs’) have been developed with structural changes that prevent binding to endogenous FRB. Instead, these compounds bind to mutated FRB constructs designed to accommodate the larger rapalogs (Clackson, 2008).

The rapamycin system has been used as an on-demand alternative to RNAi, inactivating AP-1 and AP-2 through relocation to the mitochondria. Tagging of the γ or α adaptins with FKBP was sufficient to reroute the entire tetramer to an FRB domain at the mitochondrial membrane within minutes of rapamycin addition. This has considerable advantages over knockdown with traditional RNAi which takes days to be effective and may induce off-target effects and compensation mechanisms (Robinson et al., 2010).

To minimise off-target effects, a range of alternative dimerising pairs have also been developed, for example, FKBP and SNAP-tag. These dimerise using rCD1, a compound containing a benzylguanine moiety for SNAP-tag binding and SLF (synthetic ligand of FKBP) for FKBP binding. The rate of onset is comparatively slow (~5 min) but it has the benefit of being rapidly reversible upon addition of FK506 to outcompete SLF (Feng et al., 2014). To improve orthogonality further, Liu et al. (2014) used SLF’-TMP (trimethoprim) as a dimeriser of FKBP’ (FKBP F36V) and eDHFR
(E.coli dihydrofolate reductase). SLF'-TMP has ≥1000-fold higher affinity for these proteins than wild type FKBP and mammalian DHFR. This system is again reversible upon addition of TMP alone to compete for eDHFR binding. On the other hand, FKBP-rapamycin binds to FRB with very high affinity ($k_d = 12$ nM) and the rapamycin-FKBP interaction is essentially irreversible, with a $k_d$ of 0.2 nM (Banaszynski et al., 2005) and a $t_{1/2}$ of ~17 h (Hosoi et al., 1999).

### 1.4.2 TULIPs – light induced dimerisation

TULIPs (tunable, light-controlled interacting protein tags) enable protein dimerisation using photostimulation rather than chemicals. They use the light-sensing LOV (light-oxygen-voltage) domain from an A.sativa phototrophin. Upon exposure to <500 nm wavelength blue light, the N-terminal Jα helix is released from the core of LOV, and is then able to bind its ligand, PDZ. The affinity of LOV for an engineered erbin PDZb domain could be altered by adding protein epitopes to the Jα helix; the most favourable configuration was termed LOVpep. The dark and light dimerisation kinetics of LOVpep/PDZb could be tuned through LOVpep mutations that changed the docking affinity of Jα helix. This light-based method offers several advantages over a chemically-induced system, it is rapidly reversed upon cessation of illumination and can undergo multiple activation cycles without any off-target effects from the dimerisation process. Most importantly, it offers spatial control of dimerisation at a sub-cellular level (Strickland et al., 2012).

The TULIP constructs have been used for a broad range of applications, including reversible, spatiotemporal control of organelle positioning through the rerouting of motor proteins to organelles such as endosomes and peroxisomes. By using plus- or minus-end directed motors, the direction of travel can be decided, and was shown to override the normal transport pattern of the organelle (van Bergeijk et al., 2015). Optogenetics can also be used to locally activate proteins, for example, targeted activation of RhoA at the plasma membrane induced cleavage furrow formation in mitotic and rounded interphase cells. This manipulation was not limited to inducing normal cell behaviour, the furrow could be induced at the spindle poles and at multiple locations at once (Wagner & Glotzer, 2016).

### 1.4.3 Chemical and optical dimerisation

Combining the two dimerisation stimuli introduces spatial control without the need for
constant illumination. This can be achieved with a photocaged derivative of rapamycin, pRap, here the caging group is removed from the active site in response to 365 nm UV light. An altered form of FKBP (iFKBP) can distinguish between the caged and uncaged varieties, delaying dimerisation until UV exposure. The disadvantages of this technique are residual binding to endogenous FKBP and diffusion of the activated pRap from the illuminated area (Karginov et al., 2011). Photochemical uncaging of an immobile target can resolve the problem of diffusion, as demonstrated in the cTMP-Htag system. Here, a target with limited short-term mobility (tested with centromeres, kinetochores, and mitochondria) is tagged with Halotag and a second protein with eDHFR. The dimeriser, cTMP-Htag, contains a chlorohexane group (Htag) which binds covalently to Halotag immediately upon addition, fused with a TMP domain photocaged by 6-nitroveratryl carbamate. Following illumination with 365-420 nm light, the TMP is released and binds with eDHFR to complete the dimerisation. This can be achieved with subcellular spatial control, and is reversible upon addition of free TMP (Ballister et al., 2014).
1.5 Aims of this thesis

In order to study the events and interactions that must occur for successful initiation of clathrin-coated endocytosis, it is desirable to have control over the timing and location of the event. This is a criterion that is only partially met using ligand-stimulated CME, as the location is still undetermined and the effect of activating endogenous receptors cannot be ignored.

The aims of this work are:

1. To create and refine a system of CME with spatially and temporally controlled initiation, that functions without detriment to normal cell behaviour.

2. To examine the requirements for successful CME by introducing changes to the synthetic system or cell environment.

3. To explore the minimum requirements for functional \textit{in vivo} clathrin binding by its adaptor proteins.
2. Materials and Methods

2.1 Cell culture

HeLa cells (HPA/ECACC #93021013) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) plus 10% foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (pen/strep) at 37°C and 5% CO₂. HeLa FCHO1/2 double knockout cells (designated clone #64/1.E), a kind gift from Linton Traub, were maintained under the same conditions.

Primary neuronal cultures were prepared from day-old mice, culled by decapitation. The brains were kept in ice cold dissection medium (0.01 M HEPES (pH 7.4) and 1% pen/strep in Earle’s balanced salt solution) and hippocampi isolated with fine forceps. The homogenate was made by digestion of the hippocampi with 10 U/ml papain solution in dissection medium for 5 min at 37 °C. The papain was inactivated by centrifugation (400 x g for 2 min) and the pellet resuspended in 10 ml cold Hippofood (0.01 M glucose, 1% pen/strep, 1 mM sodium pyruvate, 0.025 M HEPES (pH 7.4), 1% N2 supplement and 10% heat inactivated horse serum in MEM). This was centrifuged again (400 x g, 2 min) before resuspension in 2 ml of cold Hippofood. Glass Pasteur pipettes were used to homogenise the hippocampi and quantified to give 2.5 x 10⁵ cells/ml extract, 150 μl of this extract was put on poly-D-lysine and laminin coated 35 mm fluorodishes. Cells were allowed to attach for two hours before 2 ml warm Hippofood was added.

2.2 Molecular biology

CD8-β2-mCherry fusion protein was made by insertion of CD8-8A into β2-mCherry at EcoRI and BamHI. β2-mCherry was previously prepared from PCR of GST-β2 adaptin (616-951) (Hood et al., 2013) inserted into pmCherry-N1. The non-fluorescent variant was produced by replacing β2-mCherry with β2 only at BspEII and NotI sites. CD8-FRB was made by PCR amplification of CD8-8A and subcloning into pMito-FRB at AgeI and EcoRI; CD8-mCherry-FRB was made by inserting CD8 into pMito-mCherry-FRB at the same sites. CD8-P2X2-FRB was made from PCR of a 120
residue fragment of the C-terminal tail of P2X2 inserted into CD8-FRB at AgeI and BamHI sites. CD4-mCherry-FRB was made by overlap extension PCR of CD8-mCherry-FRB and CD4-FRB (the latter synthesised by IDT); GAP43-FRB-RFP and pMito-PAGFP-FRB were available from previous work. Dark versions of mCherry constructs (dCherry) were made using a site-directed mutagenesis (SDM) method based on the QuikChange protocol (Wang & Malcolm, 1999) to introduce a K70N mutation (Subach et al., 2009).

FKBP-β2-mCherry was made by inserting an FKBP PCR product into β2-mCherry at Acc65I and XhoI sites, this was then modified by subcloning of GFP to make FKBP-β2-GFP. Mutations were again made by SDM, Y-A by mutation of β2 adaptin residue Y815 and ΔCBM by deletion of region 627-635 (LLGDLNLD). The double mutant (ΔCBM/Y-A) was created by replacing the β2 adaptin fragment containing the Y-A mutation for the same region in ΔCBM construct using PspOMI and AgeI. Additional FKBP constructs were made by inserting a PCR product in the place of β2 in FKBP-β2-GFP: mouse α adaptin 1 (region 740-977) and mouse epsin (region 144-575) were inserted at Acc65I and AgeI, human β1 adaptin (region 617-949) was added at AgeI and EcoRI, and β2 hinge (region 616-704) was added at BamHI and AgeI. FKBP-β3-GFP and FKBP-β3hinge-GFP were made by insertion of human β3 hinge and appendage (region 702-1094) or β3 hinge (region 702-859) into FKBP-β1-GFP at PspOMI and AgeI. FKBP-β2-β3-GFP and FKBP-β3-β2-GFP were made by insertion of appendage-GFP fragment into the appropriate FKBP-hinge-GFP construct at SalI (site previously added at the GFP end of the hinge during PCR of FKBP-hinge-GFP constructs) and NotI. GFP-FKBP and GFP-LCa were available from previous work (Cheeseman et al., 2013).

FKBP-GTSE1-GFP was made by PCR of GTSE1 (638-720) and insertion into FKBP-β2-GFP at BamHI and BspEl/AgeI. Clathrin box motif mutations were added by SDM using three primer pairs to mutate CBM1&2 (644-651), CBM3 (669-672), and CBM4&5 (694-704) to alanines. FKBP-CBM-Bub1-CBM-GFP was made by PCR of Bub1 (146-377), using primer overhangs to add clathrin box motifs (LLNLD) on both ends. This PCR product was then inserted into FKBP-β2-GFP at BamHI and AgeI.

CD8-mCherry-LOVpep and its constitutively active version were made by PCR and subcloning of LOVpep and LOVpep CA (Addgene #34971 and #34966) into CD8-
mCherry at NotI and XbaI. CD8-pTagRFP657-LOVpep(T406A,T407A,I532A) was made by ligation of LOVpep(T406A,T407A,I532A), ordered as a custom gene from IDT, and pTagRFP657 (Addgene #31959). This product was then inserted into CD8-mCherry at EcoRI and AgeI sites. ePDZb-β2-GFP and ePDZb1-β2-GFP were made by PCR of ePDZb (Addgene #34980) or ePDZb1 (Addgene #34981), inserted into FKBP-β2-GFP at XhoI and BamHI, replacing FKBP. ePDZb1-β2-mCherry and ePDZb1-β2-mCherry were made by subcloning of ePDZb/ePDZb1 from ePDZb-β2-GFP or ePDZb1-β2-GFP and inserting into FKBP-β2-mCherry at BamHI and XhoI sites. ePDZb1-mCherry was made by PCR of ePDZb1 from ePDZb1-GFP inserted into pmCherry-N1 at BamHI and AgeI.

2.3 DNA and siRNA transfection

DNA transfections were performed with GeneJuice (MerckMillipore, UK) according to the manufacturer’s instructions. In summary, cells were plated at 50-80% confluency in a 6 well plate and transfected with 1-1.5 μg plasmid DNA and 3 μl Genejuice per μg DNA, incubated in 100 μl serum-free DMEM for 15 min. This mix was added dropwise to 3 ml complete DMEM per well. Transfection mixture was removed after a minimum of six hours and cells were imaged or fixed 2 days after DNA transfection. siRNA transfections with made using Lipofectamine 2000 (Life Technologies, UK) in a “two hit” protocol with transfection at 2 and 4 days prior to use. Briefly, 10 μl siRNA was incubated with 15 μl Lipofectamine in 500 μl Opti-MEM (Gibco) for 15 min and added to the cells in 1.5 ml DMEM + 10% FBS overnight. Sequences targeting μ2 (μ2-2) or clathrin heavy chain (chc-2) were as described previously (Motley et al., 2003).

Hippocampal neurones were transfected one day after extraction using 4 μl Lipofectamine 2000 and 4 μg DNA per fluorodish, incubated for 20 min in Opti-MEM. Prior to transfection, 1 ml (50%) of the Hippofood per well was removed and replaced with 1 ml new Hippofood. The removed medium was kept at 37 °C for use as pre-conditioned medium. The transfection mixture was kept on the cells overnight then replaced with 2 ml of pre-conditioned medium.

2.4 Immunofluorescence

For live immunolabelling of CD8-β2-mCherry in HeLa cells, 1:100 Alexa 488-
conjugated mouse anti-CD8 (AbD Serotec) was added at 4 °C or 37 °C for 40 min, diluted in DMEM + 10% FBS. The cells were then placed on ice, washed with PBS + 1% BSA and the surface fluorescence was quenched with rabbit anti-Alexa 488 (Invitrogen) before relabelling of primary antibody with anti-mouse Alexa 633-conjugated secondary antibody at 1:500 (Life Technologies). The cells were then fixed in 3% paraformaldehyde (PFA) + 4% sucrose in PBS for 15 min, washed, and mounted in Mowiol + DAPI. This protocol was adapted for CD8-β2 and transferrin uptake in AP-2 knockdown cells with the addition of a 30 min incubation in serum-free DMEM before the primary CD8 antibody was added for 40 min, also in serum-free medium. Additionally, 100 μg/ml of Alexa 568-conjugated transferrin was added for the final 10 min of anti-CD8 incubation.

Live labelling of the rapamycin-inducible system was performed in HeLa cells transiently transfected with CD8-mCherry-FRB and FKBP-β2-GFP (or mutant variants) or GFP-FKBP. Live cells were incubated with untagged anti-CD8 (AbD Serotec) at 1:1000 at 37 °C for 40 min; 200 nM rapamycin was then added for 30 min before cells were put on ice and washed with PBS + 1% BSA. Alexa 647-conjugated anti-mouse secondary was added in complete DMEM at 1:500 for 1 h before cells were fixed with 3% PFA + 4% sucrose and permeabilised with 0.1% Triton X-100 in PBS. Finally, 1:500 Alexa 568-conjugated secondary antibody was added in complete DMEM for 1 hour before washing in PBS and mounting with Mowiol + DAPI.

For analysis of transferrin uptake alongside inducible endocytosis, HeLa cells transfected with FKBP-β2-GFP ± CD8-mCherry-FRB were serum-starved for 20 min in no additions DMEM then exposed to 200 nM rapamycin or ethanol vehicle for 20 min, with Alexa 647-conjugated transferrin (Invitrogen) added for the final 10 min before fixing in 3% PFA + 4% sucrose. All dilutions were performed in serum-free media. A similar protocol was used when both transferrin and CD8 uptake were required in μ2 adaptin or CHC knockdown cells. Firstly, anti-CD8 was added for 40 min followed by 30 min 200 nM rapamycin or ethanol vehicle treatment, both in serum-free DMEM. Alexa 647-conjugated transferrin was applied for the last 10 min of rapamycin treatment before cells were moved to ice. Unconjugated goat anti-mouse antibody was added for 30 min on ice to block surface anti-CD8. Cells were then fixed, permeabilised, and incubated with Alexa 568-conjugated secondary antibody for 1 hour to target internalised CD8.
Materials and Methods

Fixed cell staining of CD8 anchors was performed in HeLa cells grown on coverslips and fixed in 3% PFA + 4% sucrose, permeabilised with 0.1% Triton X-100 and blocked for 1 h with blocking solution (3% BSA + 5% goat serum in PBS). Primary anti-CD8 and Alexa 488-conjugated secondary antibody were added sequentially for 90 min each in blocking solution. Coverslips were washed in PBS and dH$_2$O before mounting with Mowiol. Clathrin staining in CD8-mCherry-FRB + FKBP-β2-GFP cells was performed similarly using X22 primary antibody, Alexa 568-conjugated secondary, and an additional 30 min rapamycin incubation step before fixing.

2.5 Light microscopy

For live-cell imaging of rerouting experiments, HeLa cells were transfected with CD8-mCherry-FRB, CD4-mCherry-FRB, pMito-PAGFP-FRB or GAP43-FRB-mRFP with FKBP-β2-GFP (or mutants), FKBP-β1-GFP, FKBP-α-GFP, FKBP-β3-GFP, FKBP-β2hinge-GFP, FKBP-β3hinge-GFP, FKBP-β2-β3-GFP, FKBP-β3-β2-GFP, FKBP-epsin-GFP, FKBP-CBM-Bub1-CBM-GFP, FKBP-GTSE1-GFP (or mutants), or GFP-FKBP. For experiments where the red channel was needed for imaging something other than CD8, non-fluorescent CD8-dCherry-FRB was used. Cells were imaged in glass-bottomed fluorodishes (WPI) with Leibovitz L-15 CO$_2$-independent medium (Sigma) supplemented with 10% FBS and kept at 37 °C. Imaging was performed using a spinning disc confocal system (Ultraview Vox, PerkinElmer) with a 100x 1.4 NA oil-immersion objective. Images were captured every 5, 20, or 30 s using a dual camera system (Hamamatsu ORCA-R2) after excitation with 488 nm and 561 nm lasers; 200 nM rapamycin (Alfa Aesar) in Leibovitz medium was added after 60 s (300 s for mitosis experiments).

Light-induced dimerisation experiments were performed similarly, HeLa cells transfected with CD8-mCherry-LOVpep or CD8-mCherry-LOVpep CA and ePDZb- or ePDZb1-β2-GFP were imaged with 488 nm and 561 nm lasers at 5 s intervals. Localisation activation was performed in HeLa transfected with CD8-TagRFP657-LOVpep (T406A, T407A, I532A) and ePDZb1-mCherry, ePDZb1-β2-mCherry or ePDZb-β2-mCherry. Photoactivation was done with a 488 nm laser at <20% maximum power in a defined ROI using PerkinElmer PhotoKinesis FRAP module. Photoactivation was performed after 561 nm and 640 nm only imaging of 12 x 5 s frames; for reversible activation, 488 nm illumination was followed by 30 x 1 s frames imaged at 561 nm, as indicated in Figure 5.3B. For continuous rerouting,
photoactivation and imaging were interleaved, with 488 nm photoactivation and imaging at 561 nm and 640 nm both occurring within 5 s frames, as indicated in Figure 5.4B. All HeLa imaging performed in Leibovitz medium + 10% FBS at 37 °C. Neuronal growth cone imaging was performed in NES buffer (in mM: 136 NaCl, 2.5 KCl, 10 HEPES, 1.3 MgCl₂, 10 glucose, 2 CaCl₂, 0.01 CNQX and 0.05 DL-APV, pH 7.4) supplemented with 1 mM glucose. Background of 6 x 30 s frames was recorded before illumination, followed by interleaved localised 488 nm activation and global 561 nm and 640 nm imaging at 30 s intervals.

Single time point imaging of CD8-β2-mCherry + GFP-LCa and fixed cell immunostaining experiments was performed using the same Ultraview spinning disc confocal at 100x, z-slices were taken at 0.5 μm intervals.

2.6 Correlative light-electron microscopy (CLEM)

HeLa cells transfected with CD8-dCherry-FRB and FKBP-β2-GFP or GFP-FKBP were imaged in gridded glass bottomed dishes (P35G-2-14-CGRD, MatTek Corp, Ashland, MA) following incubation with anti-CD8 (1:1000) for 30 min and then Alexa 546 FluoroNanoGold-anti-mouse Fab' (1:200) for 10 min. Using the photo-etched coordinates on each grid, the cell location was recorded using brightfield illumination at 20x on a Nikon Ti epifluorescence microscope equipped with a Coolsnap Myo camera (Photometrics). Following Rapamycin addition, GFP and mCherry fluorescence was observed at 100x using the same equipment. Once a sufficient response had been observed, the sample was immediately fixed with 3% glutaraldehyde and 0.5% paraformaldehyde in 0.05M phosphate buffer pH 7.4 for 2 hours. Remaining aldehydes were quenched in 50 mM glycine and cells were washed in dH₂O. Gold enhancement was then performed for 3 min as per manufacturer’s instructions (GoldEnhance-EM, Nanoprobes, Inc.). Cells were washed twice in 0.3% Na₂S₂O₃ and then dH₂O. Gold-enhanced samples were postfixed in 1% OsO₄ for 60 min, rinsed with dH₂O and then stained with 0.5% uranyl acetate in 30% ethanol for 60 min. Cells were dehydrated in increasing concentrations of ethanol for 10 min each (50%, 70%, 80%, 90%, 100%) before being embedded in epoxy resin (TAAB) and left to polymerise for 48 h at 60 °C. Each cell of interest was identified by correlating the grid reference on the surface of the polymerised block with previously acquired brightfield images. 70 nm sections were collected on formvar coated copper grids.
using an EM-UC6 ultra-microtome (Leica Microsystems) and contrasted with saturated aqueous uranyl acetate and Reynolds lead citrate. Sections were imaged on a Jeol 1400 TEM at 100 kV.

### 2.7 Biochemistry

Extent of μ2 adaptin knockdown following μ2 or GL2 (control) siRNA treatment in HeLa was assessed by western blot of cell extracts from non-transfected cells or cells expressing CD8-mCherry-FRB and FKBP-β2-GFP ± 30 min rapamycin. Cell extraction was performed on ice using 100 μl lysis buffer (RIPA buffer + 0.2 mM PMSF) per 35 mm well. Cells were transferred to 1.5 ml tubes and centrifuged at 14000 rpm for 15 min at 4 °C and the supernatant collected. A Bradford assay was performed to calculate the protein concentration in the cell lysates. 40 μg of cell extract was prepared in 30 μl Laemmli buffer, denatured at 95 °C and run on a 4-15% Mini-PROTEAN TGX precast gel (Bio-rad). Semi-dry transfer was performed using Trans-Blot Turbo system (Bio-Rad), followed by 1 h block in 5% milk. The membrane was cut at the 37 kDa protein marker and the large molecular weight membrane section incubated overnight at 4 °C with anti-μ2 (BD Bioscience) at 1:500 and the lower molecular weight with anti-GAPDH (Sigma) at 1:5000. These were recognised by 1:10000 anti-mouse HRP and anti-rabbit HRP respectively. All antibody dilutions performed in 2.5% milk. GAPDH was developed using standard ECL and μ2 adaptin using ECL Prime (GE Healthcare) for 4 min. Overexposure for 15 min was used to confirm the complete absence of μ2 in the μ2 siRNA condition.

### 2.8 Image analysis

For analysis of triggered endocytosis, the green channel was first corrected for photobleaching using the simple ratio method in Fiji. To examine rerouting kinetics, the mean pixel density within a cytoplasmic ROI was measured for all GFP frames in the movie. Next, a binary threshold was applied to the green channel which isolated any newly formed vesicles due to their relatively high intensity and excluded background signal from other structures such as the plasma membrane. The raw integrated density (number of pixels over threshold * 255) within an ROI containing the cell was measured for all frames in the movie. These values for all cells were fed into Igor Pro 7 and a series of functions custom written by Stephen Royle processed the data (available at https://github.com/quantixed/PaperCode/). The raw integrated
densities were aligned to time 0, defined as the point at which the mean pixel density of GFP in the cytoplasmic ROI fell by 50%. These time-aligned traces were normalised by the size of the ROI and baseline subtracted. This gave a good approximation of the number of internalised vesicles as defined by bright spots of GFP-hook-FKBP fluorescence. For light-activated endocytosis the same method was used with no time alignment. The raw integrated density was extracted in the same way except measurements were taken within the illuminated ROI and an equivalent, non-exposed area of the same cell. Due to variability in image capture, time-stamps of image capture were extracted from the mvd2 library using IgorPro. These were used for interpolation to calculate precise averages in IgorPro.

For analysis of antibody feeding experiments, four z slices from the centre of the stack were thresholded in both red and green channels to isolate bright vesicular structures. Using Fiji and the ‘analyse particles’ plugin, a mask showing only particles of 0.03-0.8 μm and circularity of 0.3-1.0 was created. Particles present in both channels were determined using the AND function in Fiji image calculator and a third mask created. The pixels over threshold within each cell mask were measured for red+green and for red alone, and the values combined from each of the four z slices. The ratio of red+green/red pixels represents the proportion of red (CD8) puncta which were GFP-positive, discarding structures containing only CD8. Transferrin uptake was measured using the same analyse particles technique and parameters, without considering CD8-mCherry-FRB colocalisation.

Figures were made in Fiji, Igor Pro, Photoshop and assembled in Illustrator. Statistical tests between two groups was done using Student’s t-test, three or more groups were tested with one-way ANOVA and Tukey’s post-hoc test using Igor Pro or Minitab.
# Materials and Methods

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Table 1.1 DNA constructs used in this study  Cloning information or source for all plasmids used for imaging or cloning.
## Materials and Methods

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### Table 1.2 Oligonucleotides used in this study

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### Table 1.3 Antibodies used in this study

All primary and secondary antibodies used, their working concentrations and source.
3. Development of an inducible model of clathrin-mediated endocytosis

3.1 Introduction

Recruitment of clathrin to the site of endocytosis at the plasma membrane requires multiple upstream events. Most importantly, the AP-2 complex must bind to cargo proteins and undergo a conformational change that releases its β2 adaptin hinge and appendage (Kelly et al. (2014); Figure 3.1A). It is this region that contains the clathrin binding sites. The difficulty this presents when exploring the initiation of clathrin-mediated endocytosis (CME) is knowing when and where this will occur. Ideally these variables need to be controlled experimentally to make the initiation event more predictable. Timing, but not sub-cellular location, may be controlled through the activation of an endogenous receptor such as G protein-coupled receptors by its ligand. However, activated receptors may additionally recruit non-AP-2 adaptors and activate pathways that alter other aspects of the cell’s behaviour (McPherson et al., 2001).

Assuming clathrin only binds AP-2 via the β2 adaptin hinge and appendage, this fragment of AP-2 should be sufficient to recruit clathrin to the plasma membrane. The aims of this chapter are to explore this hypothesis and determine if this method of clathrin recruitment is adequate to support an endocytic event. Furthermore, combining this minimal system of clathrin recruitment with the dimerisation properties of rapamycin (Rivera et al., 1996, Robinson et al., 2010) to develop a synthetic tool to initiate endocytosis on-demand with minimal impact on normal cell function.

3.2 Membrane anchored β2 adaptin is constitutively internalised

In order to successfully initiate CME, the interaction between β2 adaptin and clathrin must take place at the plasma membrane. This was arranged through the fusion of
Figure 3.1 β2 adaptin fusion protein is constitutively internalised. (A) Structure of open conformation AP-2 at the plasma membrane, showing binding of cargo to μ2 subunit and exposed hinge and appendage regions of β2. (B) Fusion of β2 to the α chain of CD8, labelled with mCherry. (C) Antibody uptake protocol, Alexa 488-conjugated antibody identified surface CD8 receptors prior to internalisation. Fluorescence from receptors remaining at the surface after 40 min was quenched and relabelled with Alexa 633. (D) Live cell antibody uptake, as B, constitutive internalisation is observed at 37 °C. Scale bar = 10 μm (E) Quantification of internalisation, bars show mean ± 1 s.d, p = 0.0067.
β2 hinge + appendage to the α chain of CD8, a single pass membrane transmembrane protein (Figure 3.1B). CD8 was chosen because it is incapable of binding AP-2 and undergoing endocytosis (Kozik et al., 2010, Fielding et al., 2012) and the extracellular domain can be easily labelled by antibodies. The first test was to simply observe whether this β2 fragment now allows CD8 to undergo constitutive endocytosis. The internalisation was tracked using two colour live immunolabelling to distinguish the surface and internalised populations. Alexa 488-labelled CD8 primary antibody was added to live, intact cells to label CD8 at the cell surface. After 40 min, plasma membrane fluorescence from any labelled CD8 receptors not internalised was quenched using an antibody against the Alexa 488. These primary CD8 antibodies at the cell surface were then relabelled with a further Alexa 633-conjugated secondary antibody (Figure 3.1C).

Initial observations of CD8-β2-mCherry localisation suggest a largely cytoplasmic distribution in puncta of varying sizes. Antibody uptake in cells incubated at 37 °C showed a strong fluorescence signal from internalised antibody, predominantly localised to these presumably vesicular structures. Negative control cells placed at 4 °C to inhibit endocytosis had very little detectable intracellular antibody (Figure 3.1D, E). Any Alexa 488 signal in these control cells colocalises with surface Alexa 633 antibody, indicating incomplete quenching rather than internalisation. The merit of this live uptake system is that it confirms that at least some of the cytoplasmic puncta observed with CD8-β2-mCherry are the product of endocytosis at the plasma membrane rather than non-functional aggregates. It also gives a measure of the efficiency of endocytosis as all green vesicles must have been internalised between primary antibody addition and fixation.

To test the assumption that internalisation was dependent on the interaction with β2 adaptin and clathrin, CD8-β2-mCherry and GFP-tagged clathrin light chain (GFP-LCa) were simultaneously expressed in HeLa. A considerable number of intracellular CD8-β2-mCherry puncta were also positive for GFP-LCa, supporting the role of clathrin in this constitutive internalisation (Figure 3.2).

To ensure clathrin was not being recruited by endogenous AP-2, HeLa cells were treated with siRNA against μ2 adaptin subunit, depleting the cell of functional AP-2. Using the same antibody uptake assay in these cells, CD8-β2 showed robust
Figure 3.2 CD8-β2-mCherry vesicles colocalise with clathrin. Representative confocal images of live cells showing strong colocalisation of CD8-β2-mCherry and GFP-LCa suggesting the constitutive internalisation of the β2 adaptin fusion protein is clathrin-mediated. Scale bar = 10 μm.
Figure 3.3 Establishing AP-2 independent uptake of CD8-β2 fusion. Live immunolabelling of CD8β2 showing uptake following GL2 or AP-2 μ2 siRNA. Simultaneous measurement of transferrin uptake was used as a marker of successful AP-2 depletion. Scale bar = 10 μm.
internalisation in both negative control (GL2 firefly luciferase) and μ2 siRNA conditions (Figure 3.3). Functional knockdown of AP-2 was confirmed by concurrent addition of fluorescently labelled transferrin, which depends entirely on AP-2 for its internalisation, and remained on the cell surface in all μ2 siRNA treated cells. Interestingly, while untransfected cells in the GL2 condition have strong transferrin uptake, this appears to be inhibited in cells with CD8-β2 uptake, possibly due to competition for endocytic machinery. AP-2-independent internalisation supports the hypothesis that all components needed for synthetic endocytosis can be recruited directly or indirectly by the β2 hinge and appendage alone.

3.3 In vivo dimerization adds temporal control

Following successful proof of principle, the next step was to change this constitutive internalisation system into one that could be triggered on demand. By supplementing the C terminus of CD8 with FRB and the N terminus of β2-GFP with FKBP, these two fragments can be dimerised upon addition of rapamycin. This is predicted to reroute β2 to the plasma membrane. Once in place, clathrin can bind and endocytosis can proceed (Figure 3.4). In this way, the timing of endocytosis is controlled by addition of rapamycin.

Initially some difficulties were encountered in the expression of CD8-FRB, which showed significant intracellular accumulation, presumably due to problems in protein folding or transport during synthesis. It was hypothesised that this processing problem may be caused by the proximity of CD8 and FRB domains. This was tested by adding a protein spacer, which can help with folding, and therefore correct the mislocalisation. The first spacer tested was the unstructured C terminus of P2X2 receptor, chosen for its flexibility and lack of endocytic activity; this CD8-P2X2-FRB construct displayed the same localisation issues. The next spacer tested was the highly structured mCherry fluorophore which improved the plasma membrane localisation significantly, although it didn't completely remove intracellular aggregates (Figure 3.5).

Prior to the addition of rapamycin, no colocalisation of CD8-mCherry-FRB (the plasma membrane ‘anchor’) and FKBP-β2-GFP (the clathrin ‘hook’) was observed, with FKBP-β2-GFP having mostly a diffuse cytoplasmic signal with some evidence of interaction with clathrin plaques at the plasma membrane. Upon addition of 200 nM
Figure 3.4 Proposed mechanism of action for rapamycin-triggered endocytosis. Hetero-dimerisation of FRB and FKBP by rapamycin controls the rerouting of β2 hinge and appendage to a CD8 plasma membrane anchor. Subsequent interaction of CD8-β2 construct and clathrin initiates downstream CME processes culminating in vesicle internalisation.
Figure 3.5 Development of a superior CD8 membrane anchor. Localisation of three membrane anchors differing only in the region linking CD8 and FRB, as determined by staining with anti-CD8, showing improved plasma membrane localisation using mCherry as a linker. Scale bar = 10 μm.
rapamycin there was a rapid and substantial rerouting of FKBP-β2-GFP to the plasma membrane and to intracellular CD8-mCherry-FRB aggregates (Figure 3.6). Within minutes, small bright FKBP-β2-GFP positive puncta could be clearly seen, first at the plasma membrane then in the cytoplasm. These new puncta could be distinguished from intracellular aggregates by their smaller size, intensity of GFP fluorescence, and shape as FKBP-β2-GFP surrounding larger aggregates appears as a ring. It is later shown that these bright green FKBP-β2-GFP puncta are vesicular.

The area contained within these green puncta was measured for each frame during the rerouting process to extract the dynamics of vesicle production. Additionally, the drop in cytoplasmic GFP fluorescence intensity was measured in a cytoplasmic ROI in order to assess the rerouting efficiency. It is clear that the majority of the vesicles are formed within the first 90 s after rerouting, and indeed can even be observed before the rerouting process has completed (Figure 3.7). Following this initial burst of activity, the increase in number of observed puncta stabilises, presumably due to saturation of either the membrane anchors and β2 hooks or CME machinery. Also shown in Figure 3.7 is the rerouting profile of GFP-FKBP; despite successful rerouting of >80% of the GFP construct, there was no corresponding increase in cell puncta, thus confirming the dependence of puncta formation on the β2 hinge and appendage.

3.4 Uptake can be replicated with various membrane anchors

So far, all experiments have used a modified CD8 receptor as the plasma membrane anchor. To ensure this synthetic endocytosis is not dependent on any property of CD8, two other membrane proteins were tested: CD4 and a fragment of GAP43 (1-20). CD4 is also a single pass transmembrane receptor but contains a significantly larger N terminal domain than CD8 and does not dimerise, unlike CD8. CD4-mCherry-FRB was engineered to contain only the signal sequence and transmembrane domain of CD4, with the N-terminal domain largely replaced by a myc tag. The C-terminal AP-2-binding dileucine motif is absent from this construct so it is unable to undergo CME alone. GAP43 is considerably different to both CD4 and CD8, being a palmitoylated peripheral membrane protein. When observed in live cells, CD4-mCherry-FRB and GAP43-FRB-mRFP displayed similar activity to CD8-mCherry-FRB, supporting vesicle production with FKBP-β2-GFP but not GFP-FKBP (Figure 3.8). Considering the substantial structural differences between these anchors and the robust response observed with all, it appears the anchor properties
Figure 3.6 Rerouting of FKBP-β2-GFP to plasma membrane induces CME. Sample frames from live cell confocal recording of rapamycin-induced rerouting of FKBP-β2-GFP to CD8-mCherry-FRB with subsequent production of vesicles. Time shown as min:sec, rapamycin added at -0:05 (filled orange bar); scale bar = 10 μm.
Figure 3.7 Dynamics of synthetic vesicle formation. Change in cytoplasmic puncta over time (coloured line, right axis) in representative FKBP-β2-GFP and GFP-FKBP control cells (note change in scale between these two conditions). Also shown is the decrease in cytoplasmic GFP signal (grey line, left axis) representing the rerouting of GFP constructs to the plasma membrane.
Figure 3.8 A range of plasma membrane anchors support synthetic endocytosis. Comparison of the activity of three membrane anchors following rerouting of FKBP-β2-GFP or GFP-FKB. Despite structural differences all were functionally similar. Shown are stills from live cell imaging experiments before rapamycin at 0 s and after rapamycin at 665 s (empty and filled orange bars). Scale bar = 10 μm
do not influence the endocytic response. Importantly, these alternative anchors do not offer any advantages over CD8, having similar expression profiles and rerouting behaviour. For this reason, CD8 is kept as the membrane anchor for the remainder of this work.

3.5 Vesicles cannot be produced from mitochondrial membrane

Clathrin-mediated endocytosis is dependent on various properties of the plasma membrane, predominantly the presence of PI(4,5)P$_2$ to trigger the conformational change of AP-2. Since this synthetic endocytosis system is independent of AP-2, it may be possible to trigger endocytosis at any membrane, even one with atypical properties. One such membrane is the mitochondrial membrane, which is composed of two layers with significantly altered lipid composition compared to the plasma membrane, lacking in supporting sphingolipids and sterols (van Meer et al., 2008). This organelle was chosen to test the synthetic endocytosis system because it does not participate in endocytosis and it has been established as a successful target for rapamycin-induced rerouting. FKBP-β2-mCherry showed comprehensive rerouting to an FRB domain targeted to the outer mitochondrial membrane upon addition of rapamycin, but was not able to produce vesicles (Figure 3.9). This suggests that some property of the mitochondrial membrane makes it unsuitable for making vesicles. Most likely a difference in the membrane composition or physical property of the membrane, such as the double membrane arrangement.

3.6 Study of vesicle morphology using CLEM

In order to confirm that the GFP puncta that form in response to rerouting of FKBP-β2-GFP to CD8-mCherry-FRB are vesicular and compare their morphology to typical clathrin-coated vesicles, correlative light-electron microscopy (CLEM) was used. CD8-dCherry-FRB (where dCherry is a non-fluorescent variant of mCherry) was tagged with Fluoronanogold targeted to anti-CD8. Each Fluoronanogold Fab' fragment is conjugated to Alexa 546 and a 1.4 nm gold nanoparticle allowing the internalised antibody to be visualised by light and electron microscopy in the same cell. Labelling was done before rapamycin addition in live, intact cells to maximise surface labelling. Fluorescence imaging during the rerouting process showed high levels of antibody uptake in cells expressing FKBP-β2-GFP but not in those with GFP-
Figure 3.9 Rerouting of FKBP-β2-mCherry to mitochondria does not produce vesicles. Frames from live cell confocal imaging of FKBP-β2-mCherry showing rerouting to the mitochondria but no subsequent vesicle budding. Time shown as min:sec, rapamycin added at 0:00, scale bar = 10 μm. Inset shows 2x enlargement of boxed area.
Figure 3.10 Synthetic endocytosis occurs via clathrin-coated vesicles. (A) Fluorescence images comparing Alexa 546 labelled anti-CD8 uptake in FKBP-β2-GFP and GFP-FKBP cells. Scale bar = 10 μm (B) CLEM images from the same cells, CD8-containing vesicles were identified by the presence of dense clusters of NanoGold. Scale bar = 100 nm (C) Quantification of vesicle diameter, bars indicate mean ± 1 s.d.
FKBP, as expected (Figure 3.10A). Of note, qualitative observations suggest the addition of FluoroNanogold does not hinder uptake compared to primary anti-CD8 alone.

Following appropriate fixation, gold enhancement and sectioning, the same cell observed using light microscopy was then imaged using transmission electron microscopy. Clathrin coated vesicles were identified by the electron dense coat on the outside of the membrane. Many of these vesicles in FKBP-β2-GFP expressing cells contained large, electron dense objects, marking them as those which contained the FluoroNanogold probe and therefore the anchor/hook complex. The large size of these gold accumulations compared to labelled receptors at the surface suggests that the CD8 receptors are clustered during internalisation. In contrast, cells expressing GFP-FKBP showed only small patches of gold at the plasma membrane and no intracellular vesicles, coated or not, contained gold particles (Figure 3.10B).

Comparison of the diameter of the FKBP-β2-GFP vesicles containing gold and those in GFP-FKBP cells showed an increase from 91.0 ± 2.2 nm to 114.7 ± 3.8 nm (Figure 3.10C), which was principally due to an increase in the thickness of the coat (31.2 ± 1.1 nm vs 46.1 ± 2.0 nm). These results support a clathrin-dependent mechanism as the method of internalisation and suggest that while the average diameter is significantly increased \( p = 2.1 \times 10^{-6} \), it is not out of the range of endogenous vesicles (Ehrlich et al., 2004) and no other superficial changes could be detected.

### 3.7 Synthetic CME operates orthogonally to endogenous CME

Ideally this system of induced endocytosis would have minimal impact on the endogenous endocytic activity of the cell. As shown in section 3.2, the expression of the fusion construct prevents transferrin uptake. It was hoped that the inducible version would not have this effect as CD8 internalisation was triggered for periods of minutes, versus ~2 days of constitutive internalisation. There was also a concern that the expression of cytoplasmic β2 hinge + appendage in cells could sequester clathrin before rerouting, and inhibit non-synthetic endocytosis (Laporte et al., 2002). In a transferrin uptake assay, expression of FKBP-β2-GFP had no effect on the amount of fluorescent transferrin internalised, and this was not changed after 30 min of rerouting to CD8-mCherry-FRB (Figure 3.11A). In each condition tested, there was no quantitative difference in transferrin uptake \( p = 0.103 \) even with significant CD8-
Figure 3.11 Synthetic endocytosis is orthogonal to endogenous CME. (A) Representative images showing transferrin uptake in cells expressing FKBP-β2-GFP ± CD8-mCherry-FRB following treatment with rapamycin or vehicle. (B) Quantification of internalised puncta of transferrin and FKBP-β2-GFP, with or without CD8-mCherry-FRB. Condition with 200 nM rapamycin indicated by filled orange circles. Scale bar = 10 μm.
mCherry-FRB uptake (Figure 3.11B). These results show that neither simple expression of the FKBP-β2-GFP construct nor 30 min CD8-mCherry-FRB internalisation has a detrimental effect on transferrin uptake, supporting the orthogonality of the inducible system.

3.8 Synthetic endocytosis is independent of AP-2 but not clathrin

To test whether the inducible system of endocytosis is AP-2 dependent, uptake of CD8 antibody in cells treated with siRNA against μ2 adaptin was recorded. Following 30 min of rapamycin, CD8 uptake appeared to be very similar between wild-type and AP-2 depleted cells (Figure 3.12A). The average number of puncta containing both CD8 and FKBP-β2-GFP was not significantly changed in μ2 siRNA treated cells vs GL2 control cells (Figure 3.12B). A limitation of this assay is that it only shows the system is capable of operating without AP-2, but it does not exclude the possibility that AP-2 is involved in synthetic endocytosis. AP-2 depletion was very good, as shown by western blot (Figure 3.12C) and transferrin uptake, which was inhibited only in cells treated with μ2 adaptin siRNA.

As final confirmation that synthetic endocytosis is clathrin mediated, cells were depleted of clathrin heavy chain using siRNA. As expected, in cells sufficiently depleted to prevent fluorescent transferrin uptake, internalisation of CD8-mCherry-FRB and FKBP-β2-GFP was completely inhibited, confirming the indispensable role of clathrin in this system (Figure 3.13).

3.9 Conclusions

Together these data support the hypothesis that β2 appendage and hinge recruitment to the plasma membrane is sufficient to induce endocytosis. Fusion of this β2 fragment to membrane-bound CD8 produced constitutive endocytosis that was dependent on clathrin, but could function independently of AP-2. This suggests that this construct is a good mimic of the open conformation of AP-2 and that all necessary machinery for vesicle formation can be engaged downstream of AP-2 recruitment. There are limitations to this fusion system however, there is no control over initiation and sustained internalisation was detrimental to endogenous endocytosis.
Figure 3.12 Cells depleted of AP-2 still support synthetic endocytosis. (A) Representative images of live cell immunolabelling of CD8 and transferrin uptake in cells treated with GL2 or AP-2 μ2 siRNA, rapamycin added in both conditions (filled orange bars). Scale bar = 10 μm. (B) Quantification of proportion of total vesicles that contain both CD8-mCherry-FRB and FKBP-β2-GFP in siRNA treated cells, with or without rapamycin (filled and empty orange circles). (C) Western blot of whole cell extract from cells harvested from the same experiment showing decrease in μ2 expression, detected with anti-μ2. GAPDH was used as the loading control.
Figure 3.13 Knockdown of CHC disrupts synthetic and endogenous endocytosis. Live immunolabelling of CD8 uptake in cells treated with GL2 or CHC siRNA. Loss of transferrin uptake was used as a marker of functional knockdown. Cells fixed 30 min after rapamycin treatment and after 10 min transferrin uptake. Scale bar = 10 μm.
These problems can be overcome by chemical dimerisation of CD8 and β2 hinge + appendage to reconstitute the fusion protein on demand. Prior to rapamycin addition, CD8 remains at the cell surface, rerouting of FKBP-β2-GFP to the plasma membrane induces rapid endocytosis of the CD8-mCherry-FRB anchor. This activity is not dependent on the type of membrane anchor, with the peripheral membrane protein GAP43 performing as well as the transmembrane CD8. The type of membrane is important however, as endocytosis could not be induced on the mitochondrial membrane. As with the constitutively active version, this on-demand synthetic endocytosis could occur independently of AP-2 but was dependent on clathrin. Finally, presumably due to the short-term nature of chemically-induced internalisation, this system could operate without detriment to endogenous endocytosis. This orthogonality and the temporal control offered by this system make it ideal for examining the early stages of CCV formation.
4. Examining the molecular details of CME

4.1 Introduction

Having established a system of inducible clathrin-mediated endocytosis, it provided an ideal opportunity to study the details of clathrin recruitment in living cells. Importantly, the measured output (appearance of clathrin-coated vesicles) requires the interaction between clathrin and synthetic hook to be functional. It has been proposed that clathrin binds β2 adaptin at two sites, one in the hinge region (Dell'Angelica et al., 1998), and the other in the appendage (Owen et al., 2000, Edeling et al., 2006). In vitro, either site can partially compensate for the loss of the other (Edeling et al., 2006). AP-1 found at endosomes and the TGN shows a similar pattern of clathrin binding at the β1 hinge and appendage (Schmid et al., 2006). The functional interaction between AP-3 and clathrin is more controversial, the β3 adaptin hinge contains a clathrin box motif (Dell'Angelica et al., 1998) but colocalisation of AP-3 and clathrin in vivo is variable (Peden et al., 2002, Kural et al., 2012). Epsin is an alternative adaptor that recognises ubiquitinated cargo and contains two clathrin binding motifs, LADVF and LVDLD, thought to bind at two distinct sites on the clathrin N-terminal domain (Drake et al., 2000). A fragment of epsin (residues 144-575) containing these two sites has been shown to be able to initiate clathrin-coated vesicles in vitro from liposomes (Dannhauser & Ungewickell, 2012). The first aim of this chapter is to look at the molecular interactions of clathrin and adaptor fragments to determine whether the prevalence of two clathrin binding sites is significant.

This system is also suitable for studying the impact on CME of whole cell changes. It has been widely reported that cells in mitosis show inhibition of CME (Schweitzer et al., 2005, Fielding et al., 2012). This is potentially caused by increased plasma membrane tension that would be typically overcome using actin, however actin is unavailable during mitosis due to remodelling of the actin cytoskeleton (Kaur et al., 2014). This idea has been previously challenged (Boucrot & Kirchhausen, 2007), with the most recently published data suggesting a significant, but incomplete, reduction in newly formed clathrin-coated pits of 75% (Aguet et al., 2016). The second aim of
this chapter therefore is to explore the strength of this inhibition and whether it may be overcome by triggering of synthetic endocytosis.

Initiation of CME requires the early arrival of pioneer proteins; one such protein is FCHo. It has been proposed that FCHo is the first to arrive at the site of CME and primes the site through its membrane bending activity (Henne et al., 2010). Although, it has been shown to be non-essential (Cocucci et al., 2012, Umasankar et al., 2014) and it may instead, together with Eps15, facilitate the conformational change of AP-2 (Ma et al., 2016). The final aim of this chapter is to look at the dependence of synthetic endocytosis on FCHo. Together, these experiments will explore the effect of synthetic endocytosis on changes at a molecular and whole cell level.

4.2 Testing functionality of adaptin-based clathrin hooks

The system of synthetic endocytosis was designed with β2 adaptin since AP-2 is the most abundant adaptor on endocytic CCVs (Borner et al., 2012) and therefore the most relevant for inducing CME. The model proposed so far suggests clathrin recruitment is the key requirement for this on-demand system; therefore, any clathrin-binding adaptor should be just as effective. This hypothesis was tested through rerouting using rapamycin of the hinge and appendage regions of β1 and β3 adaptin to CD8-mCherry-FRB as previously demonstrated for FKBP-β2-GFP. Additionally, AP-2 α hinge and appendage was used as a negative control, having no known clathrin binding activity (Shih et al., 1995); as anticipated, no vesicles were observed in this condition. FKBP-β1-GFP was the only tested construct capable of inducing endocytosis, as defined by the appearance of bright green puncta (figure 4.1A). FKBP-β3-GFP was not able to initiate vesicle formation. β1 hinge and appendage appeared to be less efficient than the equivalent β2 region in this synthetic system, with a reduced rate of response in the initial stages (Figure 4.1B). This lower efficiency is reflected in the mean number of puncta after 5 minutes of rapamycin, with a reduction of almost 50% compared to FKBP-β2-GFP (Figure 4.1C), potentially due to reduced compatibility with the plasma membrane environment. However, this reduction between β1 and β2 adaptin constructs is not statistically significant (p=0.1222). In contrast, the response of α and β3 constructs was indistinguishable from control GFP-FKBP. The success of β1 adaptin is unsurprising because it shares 84% homology with β2 adaptin (Hirst & Robinson, 1998) and contains a LLDLD CBM in its hinge region (Dell'Angelica et al., 1998). The inability of β3 adaptin to induce
Figure 4.1. β adaptin subunits are not functionally equal. (A) Representative images from live cell rerouting of β1, α, or β3 hinge + appendage to CD8-mCherry-FRB. Time points at 0 and 15 min are shown, with 200 nM rapamycin added after 1 min. Before and after rapamycin represented by open and filled orange bars respectively. FKBP-β1-GFP is the only construct capable of supporting endocytosis. Scale bar = 10 μm. (B) Quantification of the number of newly-formed GFP puncta detected over time, compared to FKBP-β2-GFP and GFP-FKBP controls. Mean traces aligned to rapamycin addition at 0 s. (C) Increase in puncta after 5 min of rapamycin, each dot indicates one cell, bars represent mean ± 1 s.d.. n_{cell} = 8-15.
endocytosis was harder to predict as it contains a CBM motif but AP-3 does not reliably interact with clathrin (Peden et al., 2002). These results suggest AP-3 has a different mechanism of action to AP-1 and AP-2, which may be independent of clathrin.

To explore whether the ineffectiveness of FKBP-β3-GFP could be due to a lack of clathrin recruitment, a clathrin colocalisation study was performed with the adaptin hooks. After 30 min incubation with rapamycin, bright puncta containing CD8-mCherry-FRB and FKBP-β2-GFP or FKBP-β1-GFP could be seen. These vesicles showed substantial colocalisation with clathrin heavy chain, as detected by X22 antibody. In contrast, FKBP-β3-GFP and FKBP-α-GFP again did not produce any vesicles, and there is no evidence of clathrin colocalisation, despite successful rerouting of the FKBP construct to the plasma membrane (Figure 4.2). These results further support clathrin as an indispensable component of this synthetic endocytosis system, with insufficient clathrin binding key to the inactivity of FKBP-β3-GFP. Colocalisation was specific to vesicular structures and there did not appear to be any large scale changes in clathrin distribution within this time frame, supporting the previous observation that synthetic endocytosis did not disrupt endogenous CME. Interestingly, very few FKBP-β2-GFP positive puncta were found without clathrin, suggesting clathrin may not be able to dissociate from the hook, preventing vesicle uncoating and downstream processing to endosomes.

To test the idea that clathrin binding to β2 hinge and appendage is dependent on two sites, β2 hinge CBM and appendage Y815 site were disrupted through targeted mutation. The ΔCBM construct is lacking both the canonical clathrin box (LLNLD), and the β-arrestin 1L site binding motif (LLGDL); since these sites overlap, they have been treated as one site for the purposes of this study (Figure 4.3D). Initial observations suggest Y815A appendage mutation (Y-A) does not completely inhibit CME, with a small number of GFP-positive vesicles created in these cells. FKBP-β2-GFP ΔCBM is also capable of supporting a low level of synthetic endocytosis, unlike the double mutant which shows no detectable puncta (Figure 4.3A). The lack of even plasma membrane puncta suggests the inhibition is at the initiation stage of CME. Moreover, these two hinge and appendage sites make up the whole, or an essential part of the clathrin-binding ability of β2 adaptin. Despite these observations, mean puncta formed after five minutes in both single and double mutants was statistically
Figure 4.2 Synthetic endocytic vesicles colocalise with clathrin. After 30 minutes of rapamycin, only FKBP-β2-GFP and FKBP-β1-GFP produced vesicles. The majority of these inducible vesicles were positive for clathrin, identified by X22 and Alexa647-conjugated antibodies. Scale bar = 10 μm.
Figure 4.3 β2 hinge + appendage clathrin-binding site mutations disrupt synthetic endocytosis. (A) Representative images of live cell rerouting of FKBP-β2-GFP carrying mutations in hinge CBM and/or appendage Y815 clathrin-binding sites. Images taken 1 min before, or 14 min after 200 nM rapamycin addition (empty and filled orange bars); scale bar = 10 μm. (B) Quantification of average GFP puncta formation over time, aligned to rapamycin addition at 0 s. Mutants compared to WT FKBP-β2-GFP and GFP-FKBP (both previously shown in Figure 4.1). All mutants were severely compromised in their ability to induce endocytosis. (C) Increase in GFP puncta observed after 5 min, represented as mean ± 1 s.d., all groups significantly reduced compared to WT, p<0.0001, n_{cell} = 11-15.
equivalent to GFP control, possibly due to the necessary stringency in the analysis in order to avoid the detection of false positive events (Figure 4.3B, C).

To verify the level of CD8-mCherry-FRB internalisation after rerouting of mutant hooks, an antibody feeding assay was performed. In this protocol unlabelled anti-CD8 is added to intact cells, so will only be internalised if synthetic endocytosis is successful. The surface and internalised populations are distinguished by two different secondary antibodies, one added before and the other after fixation and permeabilisation of the cells (Figure 4.4B). After 30 minutes of rapamycin, wild type FKBP-β2-GFP cells showed a large number of GFP puncta, which were also positive for internalised anti-CD8 as identified by Alexa 568-conjugated secondary antibody. These results support the live cell observations, with single Y-A or ΔCBM mutations unable to completely prevent rapamycin-induced anti-CD8 uptake and no detectable uptake with the double mutant (Figure 4.4A). Quantification of vesicles produced was performed by identifying circular puncta that were <0.8 μm² and were positive for both GFP and Alexa 568; results are shown as the proportion of Alexa 568 positive puncta that also contained GFP (Figure 4.4C). Both single mutants perform similarly with mean vesicle formation halved compared to WT; this activity is halved again in the double mutant. This suggests a co-operative effect of the two sites, where one may compensate slightly for the loss of the other.

This live antibody uptake protocol together with recording puncta number at a later time point after rapamycin appears to be superior compared to live cell analysis for determining the relationship between the mutants. There are some limitations however; despite searching for puncta of defined characteristics, a small number of non-vesicular red and green positive structures are recorded, such as filopodia. This gives a deceptively high GFP-FKBP response compared to no rapamycin control. Additionally, there was some unwanted labelling of surface CD8 with Alexa 568; fortunately, these surface populations are easily identified by colocalisation with Alexa 647, which was added before cell permeabilisation.

One possibility for the lack of activity with FKBP-β3-GFP is that it only contains the hinge CBM and is lacking the critical second clathrin binding site in the appendage. This is supported by the lack of clathrin interaction shown previously (Figure 4.4). However, the β2 hinge + appendage carrying the Y-A mutation retains some
Figure 4.4 Live cell CD8 antibody uptake confirms inhibitory effect of β2 hinge + appendage mutations. (A) Representative images from WT and mutant FKBP-β2-GFP before or after 30 min 200 nM rapamycin (empty and filled orange boxes respectively). Rerouting was to CD8-dCherry-FRB with dual colour anti-CD8 feeding to distinguish internalised and surface CD8. (B) Schematic of 2 step antibody labelling protocol. (C) Proportion of puncta that contain both FKBP-β2-GFP and CD8-dCherry-FRB, bars show mean ± 1 s.d. n_{cell} = 8-14. All mutants are significantly inhibited compared to WT (p = 9.75 x 10^{-7})
functionality with only the hinge CBM suggesting that this single β3 hinge site should be sufficient. To ascertain whether the β2 appendage has further roles, a truncation mutant was made, which contained only the β2 adaptin hinge. Neither this construct nor β3 hinge alone could successfully initiate endocytosis (Figure 4.5A) despite both containing canonical clathrin box motifs. The further reduction in CME initiation with FKBP-β2hinge-GFP vs FKBP-β2-GFP Y-A may be caused by the loss of further interactions with accessory proteins or clathrin itself.

Assuming the CBMs on β2 hinge and β3 hinge are functionally equal, fusion of β2 appendage to the β3 hinge should be expected to be functional for synthetic endocytosis. This hypothesis was tested by live rerouting of this chimera, FKBP-β3-β2-GFP, to CD8-mCherry-FRB. A small number of vesicles were observed with this construct but not the β3 appendage containing FKBP-β2-β3-GFP (Figure 4.5A). This is confirmed by the quantification, in which FKBP-β3-β2-GFP is the only construct to produce a notable response (Figure 4.5B, C), albeit at a considerably reduced level compared to FKBP-β2-GFP. This suggests that the β2 appendage is essential for endocytosis and has activity that the β3 ear does not. It appears that the FKBP-β3-β2-GFP response is not consistent between cells, with approximately half the cells imaged having no response. Together with the reduced number of puncta compared to FKBP-β2-GFP, this suggests that the β2 and β3 hinges are not functionally identical, or a precise arrangement between β2 hinge and appendage is required for optimal activity.

4.3 Monomeric clathrin hooks

As the initiation of synthetic endocytosis is dependent on clathrin recruitment, it is reasonable to expect other clathrin-binding proteins to be functional as hooks. For example, the monomeric adaptor epsin, which contains two clathrin NTD binding domains. The ability of epsin to initiate synthetic endocytosis was tested using live cell rerouting of FKBP-epsin-GFP to CD8-mCherry-FRB, the epsin fragment used (144-575) was lacking the membrane-binding ENTH domain. This proved to be a potent clathrin hook, with a considerable increase in intracellular puncta observed after rapamycin addition (Figure 4.6). To better understand whether two clathrin-binding sites are the only requirement for CME, a hook was designed to contain two LLNLD motifs (β2 adaptin CBM) separated by 232 amino acids to mimic the CBM arrangement within epsin (Figure 4.6D). The flexible linker between the CBMs was
Figure 4.5 Two clathrin binding sites per clathrin hook are needed for endocytosis. (A) Representative images from live cell rerouting of indicated constructs to CD8-mCherry-FRB. Images shown were taken at 0 min and 15 min, with 200 nM rapamycin added after 1 min. Filled orange bars represent post-rapamycin condition, scale bar = 10 μm. (B) Increase in puncta shown as mean values over time, aligned to rapamycin addition at 0 s (C) Change in puncta after 5 min, bars show mean ± 1 s.d. n_cell = 7-16.
Figure 4.6 Synthetic endocytosis using monomeric clathrin hooks. (A) Confocal images from live cell rerouting of epsin (144-575) and an engineered hook containing two CBMs linked by an unstructured region of Bub1. Images before and after 15 min of rapamycin (empty and filled orange bars) are shown; scale bar = 10 μm. (B) Change in GFP puncta area over time. (C) Increase in FKBP-epsin-GFP but not FKBP-CBM-Bub1-CBM-GFP puncta observed after 5 min, bars show mean ± 1 s.d. n_cell = 8-16. (D) Sequence of epsin and Bub1 hooks, clathrin binding sites underlined.
taken from the disordered region of Bub1 kinase, which contains no known clathrin-binding motifs. Following rerouting to CD8, no vesicles were observed with this construct (Figure 4.6). This may be due to the use of identical clathrin-binding sites, whereas epsin uses two different clathrin box motifs (LADVF and LVDLD); this may prevent FKBP-CBM-Bub1-CBM-GFP from binding twice to the same clathrin heavy chain, weakening the interaction. Additionally, even with successful clathrin binding, Bub1 may be lacking the ability to recruit endocytic accessory proteins that can be recruited to synthetic endocytosis by β2 hinge + appendage or epsin.

In order to find the minimal hook that can induce endocytosis, the two properties of clathrin binding and accessory protein recruitment by the hook need to be separated. This was achieved using monomeric G2 & S phase-expressed protein 1 (GTSE1), a protein found together with clathrin and TACC3 at the mitotic spindle (Hubner et al., 2010). It is ideally suited for use as a hook since it is already known to bind clathrin but it is not an endocytic protein (Borner et al., 2012) so is unlikely to attract endocytic accessory proteins. The C-terminal domain (638-720) contains five clathrin binding motifs of the form LI[DQ][FL] arranged into three groups. Rerouting of this C-terminal fragment to CD8-mCherry-FRB was sufficient to induce vesicle formation in live cells (Figure 4.7A), suggesting that clathrin alone at the plasma membrane can recruit all essential endocytic accessory proteins.

All other functional clathrin hooks interact with clathrin at only two binding sites. To test the level of redundancy between the five GTSE1 CBMs, each site was sequentially inactivated by mutation to alanines and the live cell rerouting assay repeated. Due to the proximity of CBMs 1 and 2, and 4 and 5, these pairs were each treated as one site; the three groups of clathrin binding sites are highlighted in Figure 4.7D. Initial observations show that disruption of CBM 1&2 or CBM 4&5 resulted in complete inhibition of synthetic CME, whilst FKBP-GTSE1-GFP ΔCBM 3 was capable of producing a very small number of GFP puncta in some of the cells imaged (Figure 4.7A). The inconsistency of the response between cells prevents FKBP-GTSE1-GFP ΔCBM3 from showing an increase in mean puncta compared to the other mutants (Figure 4.7B,C). This suggests that for this type of clathrin-binding motif, at least three regions of binding are required for significant activity. Two sites are sufficient for low level activity providing they have a suitable sequence and/or they are a suitable distance apart (40 residues in this example).
Figure 4.7 Wild-type GTSE1 can produce synthetic vesicles. (A) Representative images at 0 min (empty orange bars) and 20 min (filled orange bars), with rapamycin added after 1 min. Scale bar = 10 μm. (B) Quantification of average GFP puncta number over time, aligned to rapamycin addition at 0 min. (C) Mean GFP puncta after 20 min of rerouting, WT GTSE1 activity decreases following clathrin binding site mutation, n_cell = 5-22. (D) Sequence of GTSE1 N-terminal domain used in construct with 3 groups of CBMs highlighted.
4.4 Inhibitory effect of mitosis

Cells in mitosis have been shown to have considerably reduced constitutive CME, as quantified by measurement of transferrin uptake (Fielding et al., 2012). By attempting to override this block by inducing synthetic endocytosis during metaphase, the extent of inhibition can be observed. Cells transfected with CD8-mCherry-FRB and FKBP-β2-GFP were treated with rapamycin during metaphase; despite successful rerouting, no vesicles were observed in these cells. At late telophase bright GFP spots, presumably CCPs could be seen on the plasma membrane; intracellular puncta did not appear until after cytokinesis, which occurred >30 min after rerouting (Figure 4.8). The late appearance of CME showed that the chosen cell was ultimately capable of endocytosis but was being delayed until some unknown cue. That endocytosis cannot be forced during mitosis points to a comprehensive inhibition of all CME. Curiously, rapamycin-induced endocytosis was inhibited until cytokinesis in all cells (n = 7), whereas transferrin uptake typically resumes in late anaphase (Fielding et al., 2012). As synthetic endocytosis bypasses many of the initial stages of CME, it might be expected to resume earlier; this suggests there may be other mechanistic differences between the initiation of endogenous and synthetic endocytosis. That the cells could still go through cytokinesis is further evidence of the orthogonality of the synthetic endocytosis system, as spatially-targeted endocytosis is critical for successful cytokinesis (Schweitzer et al., 2005).

4.5 FCHO1/2 and initiation

FCHO1/2 arrives during the early stages of CCP formation (Taylor et al., 2011) and its membrane bending ability has been implicated as the initiating factor for CME (Henne et al., 2010). If FCHO1/2 is needed to define the site of CME before the arrival of AP-2, it will also be required for initiation of synthetic endocytosis. To test this, HeLa cells depleted of FCHO1 and FCHO2 by Umasankar et al. (2014) using transcription activator-like effector nuclease (TALEN)-mediated gene editing, designated clone #64 1.E, were used as the host for the inducible CME system. Vesicle production after rerouting of FKBP-β2-GFP to CD8-mCherry-FRB did not appear to be hindered by the loss of FCHO1/2, with a puncta density similar to that of WT cells (Figure 4.9). This is in agreement with a role of FCHO1/2 in inducing the conformational change of AP-2 (Hollopeter et al., 2014) since this initiating step is not required for synthetic endocytosis.
Figure 4.8 Synthetic endocytosis is inhibited during mitosis. A time series of rerouting of FKBP-β2-GFP during mitosis; rapamycin is added at time -0:05 during metaphase. Vesicle production is delayed for >40 min until the cell has passed through cytokinesis. Time shown as min:sec, scale bar = 10 μm.
Figure 4.9 Rerouting of FKBP-β2-GFP can produce vesicles in FCHO1/2 knockout cells. Time series of FKBP-β2-GFP rerouting in FCHO1/2 depleted cells, vesicle production is not blocked in these cells. Time shown as min:sec, rapamycin added at time 0:00, scale bar = 10 μm.
4.6 Conclusions

In this chapter, a system of synthetic endocytosis has been used to explore endocytosis initiation. These experiments have shown that proteins other than β2 adaptin are capable of inducing endocytosis once at the plasma membrane, providing they have sufficient clathrin binding activity. The closely related β1 adaptin was an effective initiator, whereas β3 adaptin was not. A lack of clathrin colocalisation and endocytic activity suggests any clathrin interactions with β3 adaptin are not sufficient to be functional at the plasma membrane. The importance of clathrin in the initiation of synthetic endocytosis was confirmed by siRNA knockdown of CHC and mutation of β2 appendage and hinge clathrin binding sites. It required mutation of both CBM and Y815 sites to bring hook activity down to GFP control levels, supporting the idea that functional clathrin binding requires two binding sites.

The initiation of endocytosis with FKBP-epsin-GFP and, to a lesser extent, FKBP-GTSE1-GFP ΔCBM3, agrees with the two-site hypothesis as both constructs contain two regions of clathrin binding. The lack of activity with other GTSE1 mutants suggests that there is a minimum distance required between the two sites in order for functional clathrin binding. This is likely to correlate with the spacing between the complementary binding sites on the clathrin triskelion. The residual endocytic activity with the single mutants of β2 hinge and appendage may be due to a third binding site such as Y888 (Schmid et al., 2006), or high efficiency of the Y815 and CBM binding sites allowing them to function as single sites. Variation in the level of endocytic response between different hooks may be explained by the involvement of different clathrin binding motifs which interact with clathrin in different ways. In vitro experiments examining the dominant negative effect of clathrin fragments on adaptor-mediated cage formation suggest clathrin-adaptor interactions are limited to primarily the CHC NTD and potentially the distal leg. AP-2 interaction at the NTD was critical for aligning the triskelia such that the clathrin cage could close at physiological pH. The importance of this arrangement suggests all adaptors will bind this region of clathrin to preserve this organisation activity. (Greene et al., 2000).

The inactivity of FKBP-CBM-Bub1-CBM-GFP compared to epsin suggests that the two clathrin binding sites need to be different, presumably to bind the same clathrin molecule twice. Another explanation for the lack of endocytosis with this construct is
that it is not capable of binding endocytic accessory proteins. FKBP-GTSE1-GFP functions in this system without recruiting accessory proteins but the most potent hooks, β1/β2 hinge + appendage and epsin, can all bind to other proteins involved in the CME process. Further evidence for the assistance of accessory proteins comes from the complete inhibition of endocytosis following the removal of the β2 appendage, this region is known to bind to a number of accessory proteins such as CALM, epsin, and Eps15 (Edeling et al., 2006). In addition to clathrin, the binding of these accessory proteins to β2 adaptin is inhibited by mutation of the Y815 site, which provides an alternative interpretation of the FKBP-β2-GFP Y-A results. The decreased CME initiation observed with this mutant may be caused by a disruption in its ability to recruit accessory proteins, rather than solely its clathrin binding activity. The β2 appendage is further shown to be indispensable for endocytosis by its ability to confer endocytic activity to the β3 hinge fragment.

Despite the obvious differences in initiation of synthetic endocytosis compared to endogenous CME, this inducible system is still sensitive to some of the same environmental changes. For example, endogenous CME is inhibited during mitosis and synthetic endocytosis cannot overcome this block. There is no delay in rerouting, however FKBP-β2-GFP remains with CD8-mCherry-FRB at the plasma membrane until the cell has completed cytokinesis. This suggests that the point of inhibition is common between synthetic and endogenous CME and begins/persists at a stage later than adaptor recruitment. This agrees with previous work suggesting CME inhibition during mitosis is due to increased membrane tension and unavailability of actin preventing membrane deformation and CCP formation (Kaur et al., 2014).

Finally, synthetic endocytosis was shown to function in cells depleted of FCHo1/2, suggesting that its proposed role in preparing the site of endocytosis through F-BAR-mediated membrane bending (Henne et al., 2010) is dispensable in this system. A role for FCHo in AP-2 activation is not discredited by these observations as this would not be expected to affect synthetic endocytosis. The limited effect of FCHo1/2 depletion is consistent with an early role for FCHo1/2 since synthetic endocytosis bypasses events prior to clathrin binding.
5. Spatial control using optogenetics

5.1 Introduction

A disadvantage of chemically-induced dimerisation is a lack of spatial control. Single cell activation is possible if the cells are isolated, for example with microfluidics, but diffusion of the dimeriser prevents more localised activation. Tunable light-inducible dimerisation tags (TULIPs) can provide this control at a whole cell or subcellular level. Exposure of LOVpep, a modified phototrophin 1 light oxygen voltage (LOV) domain from *A. sativa*, to light of wavelength <500 nm causes unfolding of its Jα helix from the protein core. This exposes the binding site for Erbin PDZ domain, triggering dimerisation of any two proteins carrying these two sequences (Strickland *et al.*, 2012). Using spatially-defined illumination, photoactivation and dimerisation are limited to a small sub-section of the cell. A further advantage of this optogenetics system over rapamycin-induced dimerisation is the rapid reversibility in the absence of photoactivation, which allows on/off control of dimerisation. The aim of this chapter is to use this optogenetic system to add spatial control to the synthetic endocytosis system.

The impact of localised triggering of endocytosis is best observed in a cell type that naturally displays polarised endocytosis. Asymmetrical CME is implicated in the directional movement of neuronal growth cones, which turn towards the side with lower endocytic activity (Tojima *et al.*, 2010, Tojima *et al.*, 2014). Whether this turning behaviour can be replicated or modulated by localised synthetic endocytosis is explored in this chapter.

5.2 TULIPs dimerisation can induce endocytosis

The system of chemically-inducible endocytosis was adapted for photoactivation by replacement of FRB with LOVpep and FKBP with ePDZb1 (Figure 5.1A). As it is LOVpep that responds to photoactivation, this was fused to the CD8 anchor so that endocytosis could be limited to discrete subsets of the plasma membrane. To determine whether TULIPs would be suitable for inducing synthetic endocytosis, a
Figure 5.1 Constitutively active dimerisation of LOVpep and ePDZ induces endocytosis. (A) Schematic of clathrin hook and plasma membrane anchor system which has been altered for light-induced dimerisation. (B) Localisation of ePDZb1-β2-GFP without CD8 anchor. (C) Constitutively active form of CD8-mCherry-LOVpep induces endocytosis without the need for photoactivation using clathrin hooks with either low (ePDZb) or high (ePDZb1) affinity for LOVpep. Scale bar = 10 μm.
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constitutively active variant of LOVpep which contains a ΔK533 mutation that prevents Jα helix docking was tested for constitutive endocytosis. In the absence of CD8-mCherry-LOVpep, ePDZb1-β2-GFP had a largely diffuse localisation reminiscent of FKBP-β2-GFP (Figure 5.1B). However, when expressed with CD8-mCherry-LOVpep CA, it was found in small intracellular vesicles that were present from the first frame imaged, so was unrelated to 488 nm exposure. Internalisation was also observed for the lower affinity variant ePDZb-β2-GFP (Figure 5.1C). This constitutive internalisation served as proof of principle that this alternative dimerisation technique could function to induce endocytosis.

The first test for the photoactivatable system was to confirm its suitability for controlling the timing of CME initiation by inducing global endocytosis following whole cell illumination. Prior to illumination, inactive CD8-mCherry-LOVpep shows plasma membrane localisation with ePDZb1-β2-GFP diffuse, comparable to expression in the absence of CD8. Upon imaging of GFP at 488 nm, whole cell rerouting occurs within the five seconds between first and second frames. Vesicles begin to appear within the first minute and increase in number, reaching a peak around 15 min (Figure 5.2). As before, aggregates of the CD8 anchor are present, but newly formed vesicles are easily distinguished from these by their intense GFP signal. The similarities in behaviour between light-induced and chemically-induced endocytosis support TULIPs as an effective method for triggering CME. Additionally, they validate the internalisation seen with rapamycin-inducible system as a reaction to β2 rerouting and not a side effect of activating pathways downstream of rapamycin.

5.3 Rerouting is reversible and repeatable

LOVpep activation in the above whole cell illumination experiments persisted due to the repeated 488 nm exposure (0.2 frames/s), this was necessary for GFP imaging and LOVpep activation was unavoidable. This fluorophore setup would make local activation impossible, so ePDZb1-β2-GFP was changed to an mCherry tag, and LOVpep labelled with far red TagRFP657. An additional change from this point forward is the use of a mutated LOVpep (T406A, T407A, I532A) which has reduced baseline dark activation compared to wild type LOVpep (Strickland et al., 2012). In order to determine the photoactivation rate necessary to maintain dimerisation, fast imaging of ePDZb1-mCherry lacking β2 hinge and appendage hook was performed following one photoactivation event, repeated for three cycles (Figure 5.3B).
Figure 5.2 Global illumination triggers synthetic endocytosis throughout the whole cell. Rerouting of PDZb1-β2-GFP to CD8-mCherry-LOVpep occurs immediately following imaging with 488 nm laser and vesicles can be seen within minutes, with the maximum response at around 15 min. Scale bar = 10 μm.
ePDZb1-mCherry was used because it would not produce any vesicles that could complicate the analysis. Photoactivation was performed on only one edge of the cell using the same 488 nm imaging laser, this produced immediate rerouting that occurred within the one second between frames. Detachment of ePDZb1-mCherry from the membrane is fairly rapid without 488 nm illumination, with reduced intensity observed within 10 s and sustained decrease in plasma membrane fluorescence throughout the 30 s recovery period (Figure 5.3A). The cytoplasmic fluorescence increases proportionally during this time so the plasma membrane decrease is not due entirely to bleaching of the fluorophore. These dynamics were remarkably consistent through two further rounds of photoactivation and recovery, with little change in the peak response or speed of deactivation (Figure 5.3C). It is unknown how long it would take to reach pre-activation levels as the quantification shows that within 30 s only 67% of the ePDZb1-mCherry has been released, with the release rate slowing. These results suggest that pauses in photoactivation of less than 10 seconds will be required for optimum dimerisation and endocytic initiation.

5.4 Localised dimerisation triggers localised endocytosis

To combat the dissociation of ePDZ and LOVpep over time, a pattern of targeted illumination was determined where photoactivation would occur between each frame imaged at 0.2 frames/s (Figure 5.4B). Illumination resulted in precise and persistent ePDZb1-β2-mCherry recruitment to CD8-TagRFP657-LOVpep with a clear increase in mCherry fluorescence in that region only. Again, vesicles were initiated within the first minute, increasing in number before stabilising (Figure 5.4A). Very few vesicles were found outside of the activation zone, this may be due to limited mobility of the new vesicles or the breakdown of the vesicle through loss of LOVpep and ePDZb1 dimerisation in the absence of photoactivation. Quantification of newly formed puncta reveals a similar level of response between the two variants of ePDZ, with the lower affinity ePDZb still maintaining sufficient dimerisation at this rate of activation. The initial sharp drop in cytoplasmic fluorescence at a site distant from illuminated ROI suggests recruitment of ePDZb1-β2-mCherry occurs from the whole cell, not just the activated region (Figure 5.4C).

5.5 Inducing CME in neuronal growth cones

Neuronal growth cones exhibit polarised endocytosis to control their direction of
Figure 5.3 Rerouting of PDZb1-mCherry to CD8-TagRFP657-LOVpep is reversible and repeatable. (A) Rerouting occurs immediately after photoactivation but rapidly declines once stimulation ends. This pattern of recruitment was reproducible for multiple cycles. Time shown as min:sec, scale bar = 10 μm, blue dashed lines mark the illumination area. (B) Experimental procedure, 2 s illumination followed by 30 s imaging, repeated three times. (C) Intensity profile of plasma membrane (purple) and cytoplasm (grey) during stimulation (blue arrows) and recovery.
Figure 5.4 Local endocytosis can be triggered within a small region of photoactivation. (A) Exposure to 488 nm light within the indicated area results in localised ePDZb1-β2-mCherry rerouting to CD8-TagRFP657-LOVpep and vesicle initiation in this region only. Time shown as min:sec, scale bar = 10 μm. (B) Experimental protocol, repeated photoactivation was needed to keep PDZb1-β2-mCherry recruited to the membrane. (C) Vesicles were produced with both variants of PDZ, the higher affinity PDZb1 giving the largest increase in puncta (purple line), loss of cytoplasmic fluorescence also shown (grey line). Line shows mean ± s.e.m.
growth. An increase in clathrin-mediated endocytosis or decrease in exocytosis on one side is associated with the growth cone turning away from that side (Tojima et al., 2014) so it would be anticipated that localised synthetic endocytosis could reproduce this behaviour. To test this idea, primary cultured mouse hippocampal neurones were transfected with CD8-TagRFP657-LOVpep and ePDZb-β2-mCherry and imaged at 2 frames/min with localised photoactivation before each frame. The response rate was slow, with a significant number of bright puncta only appearing after more than 10 minutes of imaging. The triggering of endocytosis appeared to induce changes in the growth cone morphology, with increased spreading and membrane ruffling in the illuminated area. After 60 minutes the cone had curved slightly away from the photoactivated side (Figure 5.5), but doesn’t show the directed migratory behaviour that has been previously reported. This shift away from the side of photoactivation was observed in 2/5 growth cones that displayed rerouting of ePDZb-β2-mCherry or ePDZb1-β2-mCherry. None of the cells transfected with ePDZb-mCherry control showed morphological changes in response to localised rerouting.

5.6 Conclusions

The optogenetic system of dimerisation is as effective as rapamycin-induced dimerisation for inducing endocytosis using rerouting of β2 adaptin, and has several advantages. Firstly, the rerouting is reversible, unlike rapamycin-induced dimerisation which has a separation $t_{1/2}$ of 17 h (Hosoi et al., 1999) so is not reversed within the experimental timescale. This allows the effect of inducing multiple rounds of endocytosis to be observed, and may potentially provide a more realistic model of the later stages of CME by allowing uncoating of the vesicles. Secondly, this method removes the use of chemical dimerisers which may have unwanted effects on cell behaviour. Most importantly, this technique provides the spatial control to initiate endocytosis at a defined time and place within the cell.

Neuronal growth cones were previously shown to turn in response to increased local endocytosis induced by a regional increase in cytosolic Ca$^{2+}$ levels with simultaneous inhibition of calcium-induced calcium release (to prevent upregulation of exocytosis) or directional application of monodansylcadaverine (MDC) a non-specific inhibitor of endocytosis (Tojima et al., 2014). Light-induced synthetic endocytosis provides a more direct and spatially accurate method of inducing localised endocytosis.
Figure 5.5 Vesicles can be induced in neuronal growth cones. Localised 488 nm illumination (within blue dashed lines) at 30 s intervals beginning at 0:00 of a mouse hippocampal growth cone produced PDZb-β2-mCherry-containing vesicles and induced slight morphological changes. Time shown as min:sec, scale bar = 10 μm.
Localised photoactivation was performed in mouse hippocampal cells, which displayed a regional increase in bright puncta, resembling those seen in HeLa cells. This verifies the usefulness of the synthetic endocytosis system in alternative cell types, including primary cultured cells. The effect on growth cone migration was lower than expected, which could be due to the small size of the growth cones making it difficult to only activate only a sub-cellular region. Also, the turning behaviour of neuronal growth cones is thought to be dependent on uptake of β₁-integrins (Hines et al., 2010), which may not be included in the CD8 vesicles. Further work is needed to explain these preliminary results.
6. Discussion

This thesis describes the development of novel methods to induce clathrin-mediated endocytosis on demand. Initiation of endogenous CME requires multiple steps prior to clathrin recruitment, not least the release of β2 hinge + appendage from the core of the AP-2 complex (Kelly et al., 2014). Recruitment of a clathrin-binding protein (primarily the β2 hinge + appendage) direct to a plasma membrane cargo protein was shown to induce CME that was dependent on clathrin recruitment, but not AP-2. These observations suggest that the pre-clathrin stages of CME are not essential for endocytosis, other than to recruit clathrin.

As a tool to study CME, this system of synthetic endocytosis is superior to initiation through activation of endogenous receptors by addition of their cognate ligand. Firstly, synthetic endocytosis is not known to activate any downstream signalling pathways. Secondly, the mechanisms involved are less complex since the cargo and ‘adaptor’ are defined. Finally, it can be used to study constitutive AP-2/clathrin-mediated internalisation, the most common form of CME. Additionally, the orthogonality of the inducible system allows endogenous endocytosis to continue unaffected. Most importantly, both chemically-induced and light-induced synthetic endocytosis offer refined control over timing, with the latter also providing subcellular spatial control.

Synthetic endocytosis has been used in this thesis to examine the nature of the interaction between adaptors and clathrin. The benefit of this system over traditional in vitro binding assays is that it reveals whether an interaction is functional or not through the formation of CCVs. This distinction was particularly noticeable when using β3 hinge + appendage as the clathrin hook; despite previous work showing clathrin binding to a CBM in the hinge region (Dell'Angelica et al., 1998), β3 hinge + appendage was not functional for endocytosis suggesting AP-3 normally has a clathrin-independent mechanism of action. Interestingly, endocytic activity could be induced with this hook if the β3 appendage was replaced with the appendage from β2 adaptin. This change adds a second clathrin binding site which appears to be significant because the most successful hooks based on β2/β1 adaptin and epsin all
contain two binding sites. Mutation of hinge (CBM) or appendage (Y815) binding sites in β2 adaptin caused a significant reduction in synthetic endocytosis but complete inhibition required the mutation of both sites. This might suggest that one clathrin binding site is sufficient, however, complete removal of the appendage, leaving just the hinge site blocked all activity. Further work is required to identify whether this can be explained by a third site on the appendage that might fulfil the requirement for two clathrin binding sites in the single mutants. This seems reasonable, as there is in vitro evidence for clathrin binding at a second site (Y888) in the appendage (Schmid et al., 2006).

A caveat of the two site hypothesis is that the two sites must be of the correct type. An engineered hook containing two identical CBMs was not able to induce endocytosis, despite the length of the Bub1 spacer being modelled on the successful epsin hook. All other natural hooks that function for synthetic endocytosis contain dissimilar clathrin binding motifs. Additionally, not all clathrin binding sites are functionally equal, demonstrated by the GTSE1 hook which was functional but at a considerably lower level than β2 hinge + appendage, despite containing five CBMs. This agrees with recent work from Pucadyil & Holkar (2016) who observed significant variation in rates of clathrin assembly between four adaptors that each contained 2 CBMs of different sequences, including β1 adaptin and epsin.

Synthetic endocytosis has also proven to be useful to study the relationship between CME and the cell. For example, it was not possible to induce endocytosis in mitotic cells, providing compelling evidence for robust shutdown of CME during mitosis, previously a matter of some debate (Fielding et al., 2012, Tacheva-Grigorova et al., 2013). Furthermore, the method of inhibition must be present throughout metaphase to late telophase because synthetic endocytosis is only paused and can begin following cytokinesis. Finally, it was demonstrated that the optogenetic version of synthetic endocytosis can be used to induce localised endocytosis, providing a unique tool to examine polarised CME, for example during cell migration.

There is considerable potential to expand these techniques, for example by modification of the plasma membrane anchor to test the physical limits of endocytosis or force the cell to take up foreign material such as nanoparticles. Continuing the work of this thesis using engineered clathrin hooks could provide the template for the
minimum requirements of *functional* clathrin recruitment by an adaptor, regarding the number, arrangement, and sequence of the binding motif(s). Pairwise mutations of the clathrin hook and clathrin itself will be able to produce a map of the corresponding binding sites on both proteins. Overall, this technique of synthetic endocytosis provides a unique opportunity to influence all aspects of endocytosis, from the molecular interactions to the time and place of initiation.
7. Bibliography


