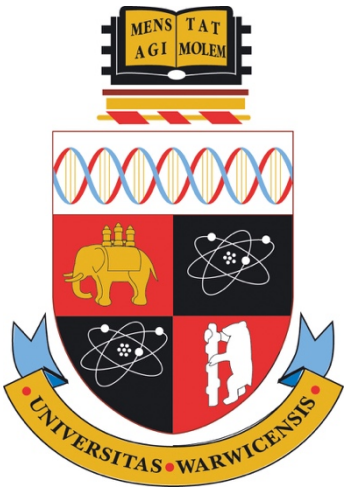
The Assembly and Disassembly of Clathrin Cages

Michael Baker



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iv. Declaration

The work conducted and presented in this thesis is original and was conducted by the author unless otherwise stated. Where work was conducted in collaboration with Joseph Jones (MOAC, University of Warwick) on dynamic light scattering this is indicated (in Chapters 4 and 8) and the work is presented with his permission.

None of this work has been submitted previously for another degree at the University of Warwick or any other institution.

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Signature: ……

Michael James Baker September 2016

v. Summary

Clathrin mediated endocytosis (CME) is an integral process in eukaryotic cells and governs a wide range of processes in higher organisms including neurotransmitter release and cell signalling, to development and cell polarity. Due to its wide ranging roles this mechanism has been implicated in various disease states such as Huntington's disease and various cancers as well as being a method of entry into cells for many viruses and bacteria. The process of CME involves the formation of a clathrin coat, primarily consisting of the protein clathrin, that drives uptake of cargo at the plasma membrane. These interactions are facilitated through a large family of proteins known as adaptor proteins, which drive the process of CME through specific interactions with clathrin, cargo, the plasma membrane and the cytoskeleton. Once the cargo has been endocytosed the process must be reversed through the actions of the disassembly proteins auxilin/GAK and Hsc70. A number of questions remain as to how adaptors promote assembly and how auxilin and Hsc70 drive disassembly through interaction with clathrin and potentially through interactions with the adaptor proteins.

By purifying adaptor proteins and clathrin I have used various biochemical and biophysical techniques to investigate these interactions *in vitro*. Using a novel assembly assay based on dynamic light scattering I have shown that it is possible to measure the effect of adaptors on clathrin cage size distribution during assembly. In disassembly I have shown how mutations in the disassembly protein auxilin affect its ability to catalyse the disassembly of clathrin cages and how the presence of various adaptor proteins alters the ability of auxilin and Hsc70 to disassemble these structures. Finally, I demonstrate an inhibitory effect on disassembly by the adaptor protein epsin and propose a mechanism of interaction with clathrin that can be disrupted through mutations to epsin clathrin-binding motifs and discuss the implications of this effect for the role of adaptors *in vivo.*

vi. Abbreviations

AAK - AP2 Associated Kinase

ADP - Adenosine Diphosphate

AEBSF - 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride

α HA - α adaptin Hinge Appendage domain

ANTH - AP180 N-Terminal Homology domain

AP180 - Adaptor Protein 180

AP(1-5) - Adaptor Protein complex (1-5)

APS - Ammonium per sulphate

Arf6 - ADP-ribosylation factor 6

ARH - Autosomal Recessive Hypercholesterolemia protein

Arp2/3 - Actin Related Proteins 2/3

ATP - Adenosine Triphosphate

β2 HA - β2 adaptin Hinge Appendage domain

BAR - Bin/Amphiphysin/Rvs domain

βME - Beta-2-Mercaptoethanol

BSA - Bovine Serum Albumin

CALM - Clathrin Assembly Lymphoid Myeloid leukemia protein

Cbox - Clathrin box

CC - Coiled Coil domain

CCP - Clathrin Coated Pit

CCV - Clathrin Coated Vesicle

CD - Circular Dichroism

CD4 - Cluster of Differentiation 4

ch-TOG - Colonic and hepatic Tumor Overexpressed Gene protein

CHC - Clathrin Heavy Chain

CLASP - Clathrin Coat Associated Sorting Proteins

CLC - Clathrin Light Chain

CME - Clathrin Mediated Endocytosis

DAB - Disabled protein

DEPOL - Depolymerisation buffer

DLS - Dynamic Light Scattering

DMSO - Dimethyl Sulphoxide

DNA - Deoxyribonucleic Acid

DTT - Dithiothreitol

EDTA - ethylene-diamine-tetraacetic acid

EGFR - Endothelial Growth Factor Receptor

EM - Electron Microscopy

ENTH - Epsin N-Terminal Homology domain

Eps15 - Epidermal growth factor receptor substrate 15

ESCRT - Endosomal Sorting Complexes Required for Transport

EGTA - ethylene-glycol-bis(β aminoethyl ether)-tetraacetic acid

Fab - Functional antibody

FCHo - Fer/CIP4 Homology domain only

GAK - cylin-G-Associated Kinase

GPCR - G-Protein Coupled Receptor

GSH - Glutathione

GST - Glutathione S-Transferase

GTP - Guanosine Triphosphate

Hip1 - Huntingtin Interacting Protein 1

Hip1R - Huntingtin Interacting Protein 1 Related

HIV - Human Immunodeficiency Virus

HKM - Hepes, Potassium acetate, Magnesium Acetate buffer

HRP - Horseradish Peroxidase

Hsc70 - Heat Shock Cognate protein 70 kDa

IPTG - Isopropyl β-D-1-thiogalactopyranoside

ITC - Isothermal Titration Calorimetry

KD - Knock Down

KO - Knock Out

LB - Lysogeny broth

LRP6 - Low Density Lipoprotein Receptor 6

MES - 2-(N-morpholino)ethanesulfonic acid

MOI - Multiplicity of Infection

MOPS - 3-*N*-morpholinopropanesulfonic acid

NECAP - adaptin Ear-binding Coat-Associated Protein

NEF - Negative Regulatory Factor or Nucleotide Exchange Factor

NTD - Nucleotide binding Domain

N-WASP - N-Wiskott–Aldrich Syndrome Protein

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PIP - Phosphatidylinositol Phosphate

POL - Polymerisation buffer

PTB - Phsophotyrosine Binding domain

PRD - Proline Rich Domain

RNAi - Ribonucleic Acid interference

RTK - Receptor Tyrosine Kinase

Rh - Hydrodynamic radius

SBD - Substrate Binding Domain

SDS - Sodium Dodecyl Sulphate

SDS PAGE - Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis

Sf9 - Spodoptera frugiperda 9 cell line

siRNA - small interfering RNA

SNARE - Soluble NSF Attachment Protein Receptor

SPR - Surface Plasmon Resonance

TAAC3 - Transforming Acidic Coiled Coil Protein

TD - Terminal Domain

TEA - Triethanolamine

TEMED - Tetramethylethylenediamine

TFB - Transformation Buffer

TGN - Trans-Golgi Network

THATCH - Actin Tethering C-terminal Homology domain

UIM - Ubiquitin Interacting Motif

VEGFR2 - Vascular Endothelial Growth Factor Receptor 2

Chapter 1: Introduction

'The beauty of a living thing is not the atoms that go into it, but the way those atoms are put together' Carl Sagan, Cosmos

1.1.1 Overview

In this chapter I introduce the topic of clathrin mediated endocytosis in the context of cellular trafficking with an overview of the process given. I explore the structure and function of the major proteins involved in this process with particular emphasis on those proteins actively studied during this project. Finally, I introduce the aims of the investigation and the subsequent structure of the thesis.

1.2.1 Endocytosis and membrane trafficking

Cells of all types require the turn over, uptake and release of materials at the plasma membrane. Proteins and nutrients must be transported across the membrane in order for the cell to interact with its environment. The protein and lipid components of the membrane itself must also be regulated and recycled through constant re-modelling of the composition of the protein and lipid content of the membrane. In addition, certain cells require the ability to take up and engulf large cargos including other cells. These broad functions are termed exocytosis for the release of cargo and endocytosis for the uptake of cargo.

Endocytosis can be broadly categorised into 3 major categories: phagocytosis, pinocytosis and receptor mediated endocytosis (Doherty and McMahon, 2009). Phagocytosis, or cell eating, is the uptake of large cargos such as bacteria in the case of white blood cells, utilising the cytoskeleton to engulf the target. By contrast, pinocytosis or cell drinking facilitates the uptake of small fluid cargo from the extracellular space. Receptor mediated endocytosis functions at the intermediate scale between these two extremes and can be categorised further into clathrin dependent (clathrin mediated), clathrin independent and caveolae dependent endocytosis (McMahon and Boucrot, 2011; Grant and Donaldson, 2009; Mayor *et al*. 2014). Caveolae dependent endocytosis is characterised by the caveolin family of proteins that preferentially bind to membranes with high levels of cholesterol and glycolipids

and has been implicated in a wide range of functions from mechano-sensing and modulation of the extracellular matrix to modulating lipid content at the plasma membrane (Parton and del Pozo 2013). Other clathrin independent pathways are less well characterised but are often involved in the endocytosis of specific cargos such as internalisation of major histocompatibility complexes mediated through G-proteins such as Arf6 (Maldonado-Baez *et al*. 2013). The best studied of the endocytic systems, and the topic of this thesis, is clathrin mediated endocytosis (CME).

1.3.1 Clathrin mediated endocytosis

Clathrin mediated endocytosis or CME is characterised by the presence of the protein clathrin which associates with adaptor proteins to facilitate the uptake of cargo and receptors. CME was implicated in the uptake of the low density lipoprotein receptor by Brown and Goldstein which exhibits defective internalisation in Familial Hypercholesterolemia (Goldstein *et al*. 1985). Since then this process has been shown to be integral to multiple cellular processes including: synaptic vesicle recycling, cell polarity, cell signalling, development, membrane protein recycling, and nutrient uptake. In addition, it provides a route of entry into the cell for some viruses and bacteria (Pizarro-Cerda *et al*. 2010). Therefore characterizing the mechanism of CME is crucial for our understanding of cell function.

Although questions remain to be answered as to the precise temporal and spatial organisation of CME a number of studies have monitored the activity and recruitment of proteins at the plasma membrane to produce a detailed picture of the process as outlined below and summarised in Figure 1.01 with an interactome diagram grouping the major components shown in Figure 1.02 (Mettlen *et al*. 2009; Saffarian *et al*. 2009; Mattheyses *et al*. 2011; Taylor *et al*. 2011; Traub *et al.* 2011).

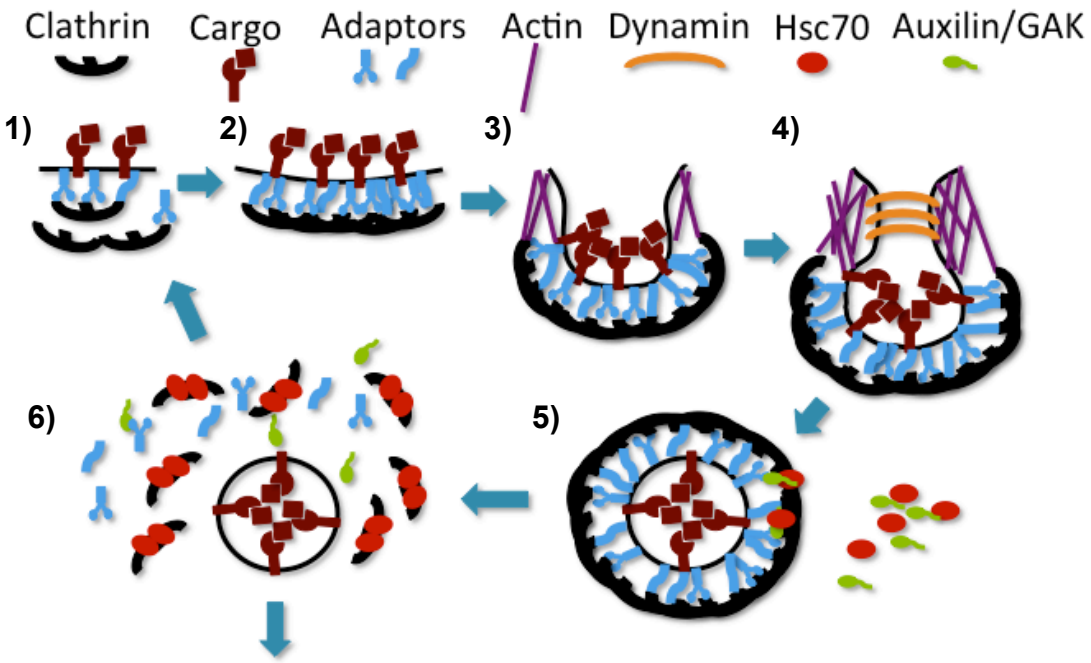

Figure 1.01: Representation of the main stages of clathrin mediated endocytosis. *1) The endocytic cargo recruits adaptors such as AP2 to the plasma membrane, which in turn recruit clathrin. 2) The subsequent recruitment of clathrin and adaptors causes curvature of the membrane. 3) Further clathrin/adaptor recruitment along with cytoskeletal components results in the invagination of the membrane. 4) Recruitment and activation of the GTPase dynamin results in scission from the membrane and the formation of a clathrin coated vesicle (CCV). 5) CCV un-coating is co-ordinated by the recruitment and activation of ATP hydrolysis of Hsc70:ATP by auxilin/GAK. 6) The vesicle is able to fuse with its target compartment and endocytic components are recycled.*

1.3.2 Cargo and adaptor recruitment

Endocytic cargo is selected for internalisation through the presentation of various signals or motifs that facilitate interactions with adaptor proteins (Traub 2009). AP2 is is the primary adaptor required for CME and is able to interact with the plasma membrane, clathrin, cargo and other adaptors to promote the formation of the clathrin coat. The two canonical internalisation motifs recognised by AP2 are the YXXΦ (where X is any amino acid and Φ is any bulky hydrophobic amino acid) and the [DE]XXXL[LIM] motif (Di-leucine motif) which bind to the μ2 and the σ2-α subunit interface respectively (Owen and Evans 1998), (Kelly *et al*. 2008). The [FY]XNPX[YF] motif is recognised by the phosphotyrosine binding domain (PTB) of adaptors ARH, DAB and Numb (Wolfe and Trejo 2007). Other adaptors detect modifications to transmembrane proteins such as serine/threonine phosphorylation of GPCRs, which is detected by β-arrestin and is essential for the internalisation of this class of protein (Laporte *et al*. 2000; Kim and Benovic 2002). Ubiquitination of proteins targeted for proteasomal degradation interact with epsin and members of the eps15 protein family which contain ubiquitin interacting motifs (UIM) (Traub 2007).

In addition to cargo recognising adaptors, adaptors such as the BAR domain (Bin/amphiphysin/Rvs) (Liu *et al*. 2015) protein FCHo may be present to initiate recruitment of other adaptors. FCHo senses low membrane curvature (Cocucci *et al*. 2012) and has been implicated in recruiting and activating AP2 (Hollopeter *et al*. 2014) potentially through interactions with epsin and eps15 (Ma *et al*. 2016).

1.3.3 Maturation of the clathrin coat

Once cargo and adaptors have been selected clathrin can be recruited through interactions with adaptor proteins that facilitate polymerisation of the clathrin coat. Budding can occur either with clathrin recruitment or from flat clathrin plaques (Saffarian *et al*. 2009; Taylor *et al*. 2011). Membrane bending and invagination occurs through the interaction of clathrin and the membrane binding regions of adaptors. The ANTH/ENTH domains of adaptors such as AP180/CALM and epsin bind to phosphatidyl-inositol 4,5 bis-phosphate (PI(4,5)P2) (Ford *et al*. 2001; Mao *et al*. 2001; Ford *et al*. 2002) and (in the case of the ENTH domain) contain amphipathic helices that insert into the membrane and alter membrane fluidity thereby making the membrane more conducive to bending (Ford *et al*. 2002; Hom *et al*. 2007; Lai *et al*. 2012). These adaptors are important recruiters of clathrin through their unstructured C-terminal domains which help to regulate the dimensions of the growing pit and ultimately the vesicle size (Morgan *et al*. 2000; Kalthoff *et al*. 2002; Meyerholz *et al*. 2005; Jakobsson *et al*. 2008; Petralia *et al*. 2013; Holkar *et al*. 2015). They can also actively contribute to membrane bending (Busch *et al*. 2015). Continual recruitment and remodelling of the coat occurs throughout the maturation of the coated pit, possibly through the action of auxilin and Hsc70 that may facilitate the continual addition and removal of clathrin (and possibly adaptors) from the clathrin coat (Yim *et al*. 2005; Avinoam *et al*. 2015).

1.3.4 Elongation and scission

The structure is now referred to as a clathrin coated pit (CCP). The neck of the CCP is now extended and narrowed through the action of BAR domain proteins such as endophilin and amphiphysin that in turn recruit the GTPase dynamin to the neck of the budding vesicle (Verstreken *et al*. 2003; Mettlen *et al*. 2009; Neumann and Schmid 2013). Dynamin in conjunction with adaptor proteins forms a ring around the contracting vesicle with GTP hydrolysis driving contraction of the ring, eventually

resulting in scission from the membrane (Loerke *et al*. 2009; Schmid and Frolov 2011). Although actin is often present at endocytic sites (Collins *et al*. 2011; Shevchuk *et al*. 2012) to drive the elongation and scission of the neck it is not essential in higher eukaryotes (Fujimoto *et al*. 2000) but is in yeast being likely required to overcome local membrane tension (Mishra *et al*. 2014). The cytoskeleton in mammals is likely only required for specific cargo or to overcome regions of membrane tension (Saffarian 2009; Kaur *et al*. 2014). The process is mediated by the Hip1 and Hip1R proteins that can interact with actin (Brett *et al*. 2006; Wilbur *et al*. 2008) as well as the plasma membrane (Hyun *et al*. 2004; Hyun and Ross 2004) and other actin regulating proteins such as cortactin (Le Clainche *et al*. 2007) which regulate actin assembly. This process may also require motors such as myosin to facilitate closure of the neck (Chandrasekar *et al*. 2014).

1.3.5 Uncoating of the clathrin coated vesicle

Once the vesicle has undergone scission from the membrane the clathrin coat must be removed in order to facilitate fusion of the vesicle to its target organelle, which is mediated by the interactions of adaptors such as CALM and EpsinR that facilitate interactions with SNARE proteins that allow vesicle fusion (Miller *et al*. 2011; Hirst *et al*. 2004). In addition, recycling of clathrin and adaptors must occur to facilitate further rounds of endocytosis. The removal of clathrin from the coat occurs through the recruitment of the chaperone protein Hsc70 by the J-domain containing protein cyclin-G-associated kinase GAK (Greener *et al*. 2000; Umeda *et al*. 2000) or its neuronal homologue auxilin (Ahle and Ungewickell 1981; Ungewickell *et al*. 1995). The J-domain of auxilin/GAK binds to clathrin and recruits Hsc70 under the vertex of the trimerisation domain where it binds to a QLMLT motif on the clathrin C-terminus (Rapoport *et al*. 2008; Xing *et al*. 2010). ATP hydrolysis to ADP stimulated by the J-domain induces tight binding to clathrin which destabilises triskelia interactions through a collision induced mechanism (Sousa *et al*. 2016).

As for the removal of adaptor proteins from the coat the mechanism is not as well understood. As auxilin/GAK contains many of the same binding motifs contained in adaptors it has been suggested that auxilin may actively work to compete for binding to clathrin and AP2 (Scheele *et al*. 2003; Smith *et al*. 2004). In addition, changes in phosphorylation of PIP have been implicated in regulating the timing of disassembly.

The phosphatase synaptojanin is recruited just prior to vesicle scission (Taylor *et al*. 2011) through interactions with amphiphysin (Verstreken *et al*. 2003) and endophilin (Milosevic *et al*. 2011) and dephosphorylates PI(4,5)P2 to PI(4)P which is hypothesised to reduce the affinity of many adaptors for the membrane and thereby facilitate their release (Cousin *et al*. 2001; Stefan *et al*. 2002; Stefan *et al*. 2005). An increase in PI(4)P may also improve auxilin binding due to its higher affinity for this phospholipid (Guan *et al*. 2010).

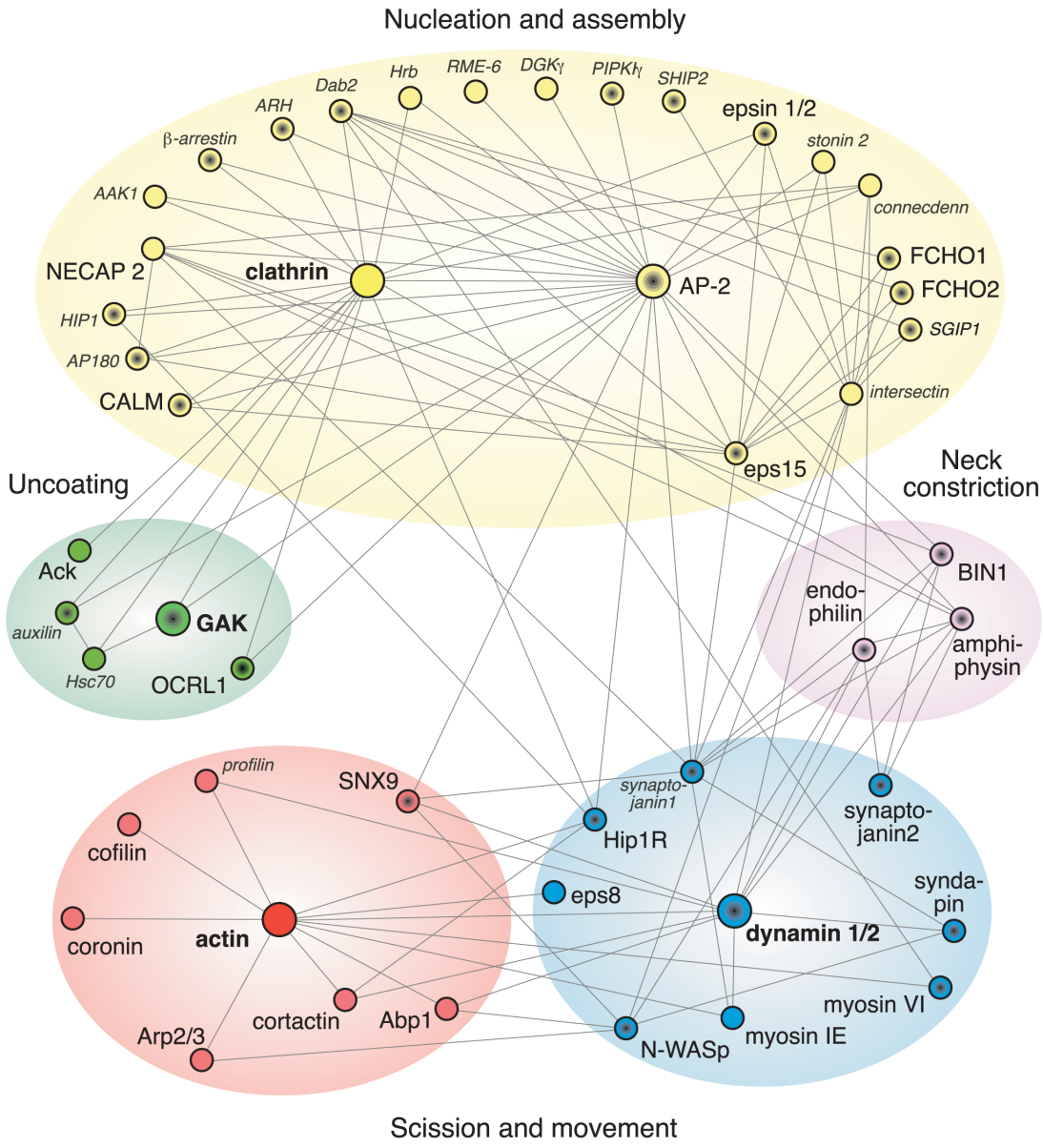

Figure 1.02. The clathrin-adaptor interactome network in vertebrates. *A hub and spoke diagram of many of the proteins involved in CME grouped according to their proposed functions and recruitment profiles during endocytosis. Hubs with dark circles indicate proteins that bind to phospholipids. Reproduced from (Traub 2011).*

1.4.1 Protein structure & function in clathrin mediated endocytosis

CME is a highly complex process involving the interactions of multiple proteins. In this section I will focus on the the structure and function of the proteins investigated in this thesis: clathrin, AP2, Hip1, Hip1R, epsin, auxilin and Hsc70 and their role in CME.

1.4.2 Clathrin

Clathrin was first defined as a major component of coated vesicles by Barbra Pearse in 1975 (Pearse 1975). The name derives from the word clathrate, used in chemistry to define a compound that encloses another compound which is itself derived from the Latin word *clathratus* meaning lattice. Clathrin is therefore a highly appropriate name for a protein which forms a cage lattice structure, enclosing a protein-lipid cargo.

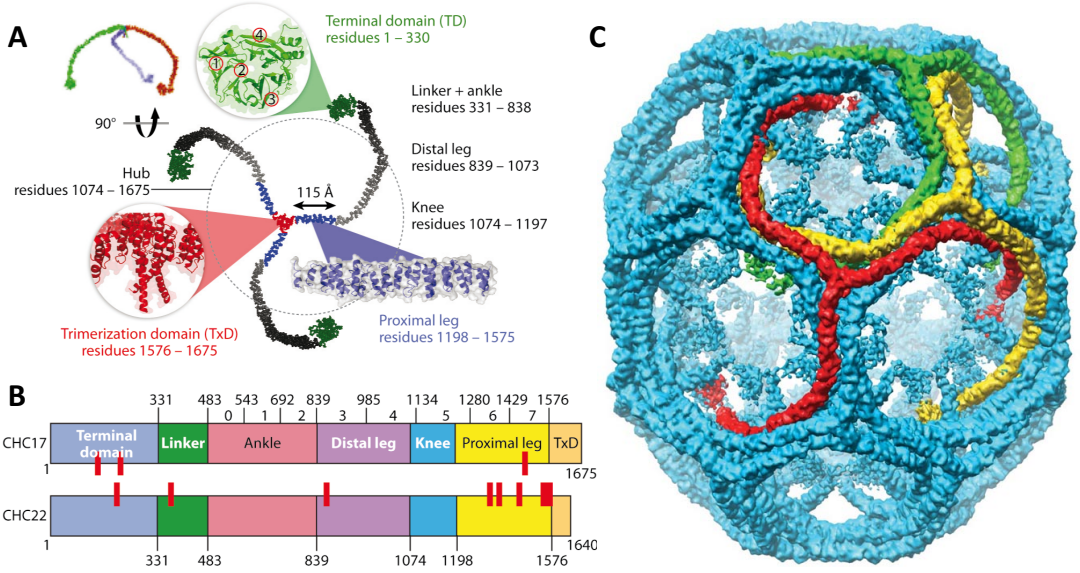

Figure 1.03 Structure of the CHC and its structure in the triskelion and clathrin cage. *A) The CHC interacts to form a trimer called the triskelion which is the functional unit of clathrin. Domain regions and their residues are indicated with the 4 numbers on the highlighted terminal domain domain representing the 4 binding sites for clathrin binding proteins. B) A linear representation highlights the domains of the two isoforms of CHC 17 and 22 with red bars indicating conserved differences between the two isoforms. C) The structure of the D6 barrel as determined by (Fotin et al. 2004b) with 3 triskelia highlighted within the structure. This diagram is adapted from (Brodsky 2012) (A and B) and (Fotin et al. 2004b) (C).*

Clathrin is capable of self-assembling into cage structures in the presence or absence of lipids and adaptor proteins. The clathrin monomer was identified in 1981 by Ungewickell and Branton who revealed the three legged structure which was named the triskelion (Ungewickell and Branton 1981). The triskelion consists of a trimer of

~180 kDa heavy chains (CHC) linked at a trimerisation hub with a clathrin light chain (CLC) associated with the C-terminus of each CHC.

1.4.3 Clathrin heavy chain

The CHC is divided up into 7 distinct regions (see Figure 1.03). From the N-terminus residues 1-330 form the terminal domain (TD) which consists of a 7-bladded β propeller structure (ter Haar *et al*. 1998). The terminal domain is the primary binding site for adaptor proteins containing up to 4 different sites for proteins containing clathrin binding motifs (Lemmon and Traub 2012; Willox *et al.* 2012; Zhuo *et al*. 2015). In the cage structure this domain is closest to the membrane and hence is best located to interact with adaptors proteins in the layer between the membrane and clathrin itself. In addition to the 4 binding sites on the terminal domain an additional binding site that interacts with AP2 is present in the ankle domain (Knuehl *et al*. 2006; Edeling *et al*. 2006a). Whilst the terminal domains are not required for assembly *in vitro* (Greene *et al*. 2000) they are essential for the function of clathrin *in vivo* (Willox and Royle 2012). It has been proposed that linking of terminal domains by adaptor proteins facilitates the polymerisation of the clathrin coat and leads to adaptor control of vesicle size (Drake *et al*. 2000; Greene *et al*. 2000; Kalthoff *et al*. 2002; Morgan *et al.* 2000).

The rest of the structure consists of a long α-solenoid region consisting of 8 clathrin heavy chain repeat motifs with each motif consisting of 10 stacked α-helix-hairpin connected by loops (Ybe *et al*. 1999). Interactions between the proximal and distal regions of the CHC facilitate interactions between triskelia and explain the pH sensitivity of clathrin assembly *in vitro*. Sequence analysis indicated several histidines that could potentially form salt bridges with glutamates in neighbouring triskelia legs (Ybe *et al*. 1998) which was confirmed through mutational studies that significantly reduced the ability of clathrin to assemble (Bocking *et al*. 2014).

Trimerisation of the clathrin heavy chains is mediated by the C-terminal domain (Nathke *et al*. 1992; Liu *et al*. 1995) which are stabilised by cysteine residues that may contribute to the formation of bridges between the neighbouring triskelia (Ybe *et al*. 1998; Ybe *et al*. 2003). The C-terminal domain forms 3 helices that extend below the trimerisation hub (Fotin *et al*. 2004b; Wilbur *et al*. 2010) with the final residues

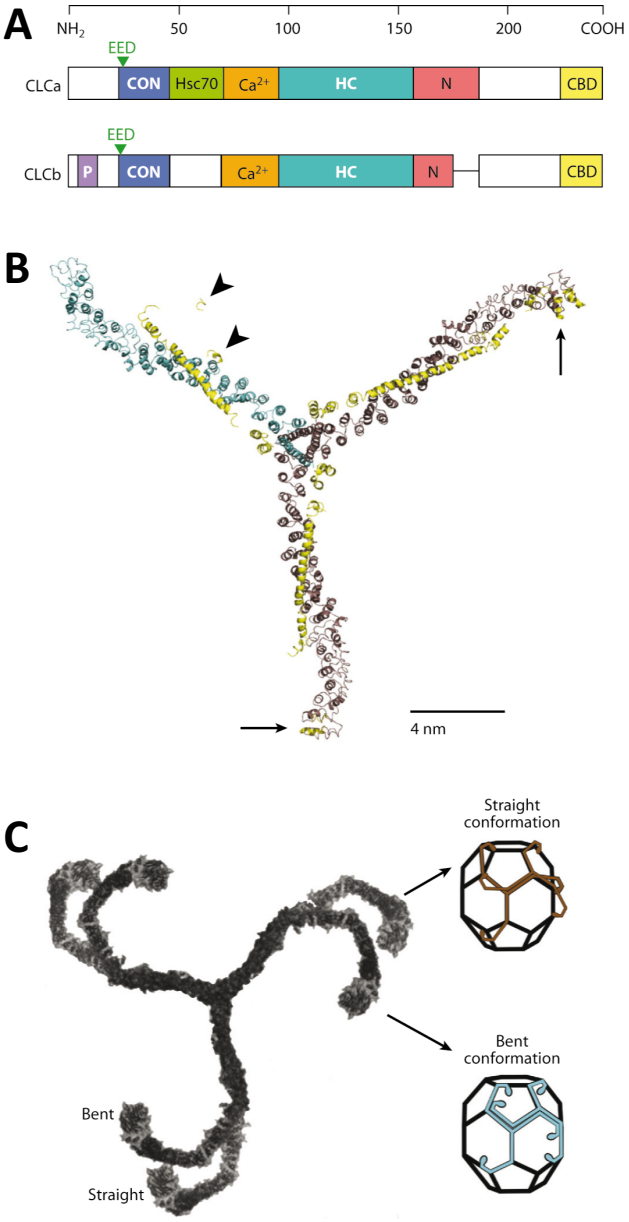

Figure 1.04 Structure of the CLC and functional interactions in the triskelion. *A) The domain structures of CLCa and CLCb with domains and interactions highlighted. Acidic EED motif are implicated in the regulation of clathrin assembly and the CON sequence allowing Hip1/1R CC binding. Both proteins contain Ca^{2+} binding and CHC (HC) binding sites as well as a calmodulin binding site (CBD). CLC a and b differ through the presence of an Hsc70 interacting site in CLC a and a phosphorylation site in CLC b. Both proteins contain variable neuronal splice sites (N). B) Composite structure of the triskelion hub showing the straight (brown) and bent (blue) conformations of the CHC with the associated interactions of the CLC in yellow. In the straight conformation the CLC interacts with the knee but in the bent conformation this interaction is lost with differences in interactions indicated by arrows. C) This change in interaction of the CLC mediates the conformational switch from straight to bent CHC with the bent conformation being more conducive to clathrin cage assembly into clathrin cages. Figure reproduced and adapted from Brodsky (2012).*

forming an unstructured tail that contains the binding motif for Hsc70 (Rapoport *et al*. 2008).

1.4.4. Clathrin light chain

The CLC is an important regulator of clathrin assembly which is implemented through its interactions with the CHC and appears to associate with the CHC at a 1:1 ratio (Girard *et al*. 2005). The CLC binds along the length of the proximal region of the CHC (Chen *et al*. 2002) and interacts with the trimerisation domain and the knee facilitating both increased stability of the triskelion (Ybe *et al*. 2007b) but also indicates the regulatory mechanism of the light chain on assembly (Wilbur *et al*. 2010). Significant amounts of the light chain are not assigned in crystal and EM density maps, suggesting that much of the protein is free in solution (Fotin *et al*. 2004b; Wilbur *et al*. 2010). The CLC consists of two isoforms CLCa and CLCb along with numerous splice-forms. Whilst sharing 60% identity CLCa contains a site that stimulates Hsc70 activity (De Luca-Flaherty *et al*. 1990) where as CLCb contains a

phosphorylation site (Brodsky *et al*. 1991). CLCb is a negative regulator of assembly whose negative effect on assembly can be reversed through interactions with Ca^{2+} and Mg^{2+} (Liu *et al*. 1995). Both CLCs have been shown to interact with the knee of the CHC through a patch of acidic residues and it is proposed that this interaction mediates CLC regulation of assembly (Ybe *et al*. 2007b). Crystal structures obtained by Wilbur *et. al.* of the hub region of the triskelion associated with CLC showed how the CLC adopts an extended and retracted conformation (Wilbur *et al*. 2010). The extended conformation interacts with the knee of the CHC which results in a 'straight leg' conformation which is not-conducive to cage formation and loss of this interaction with the knee results in the 'bent leg' conformation that is more conducive to cage formation (see Figure 1.04). The role of the CLC in regulating triskelia interactions is also shown by how the presence of the CLC is important for regulating the assembly of triskelia on surfaces and for regulating the stiffness of the lattice (Dannhauser *et al*. 2015a).

Whilst deletion or knock down (KD) of the CHC17 gene is lethal KD of CLC by RNAi appears to effect cargo uptake differentially with no affect on transferrin or EGF uptake (Huang *et al*. 2004; Poupon *et al*. 2008) but does negatively affect the uptake of GPCRs (Ferreira *et al*. 2012). In addition, clathrin coats can be disassembled in the absence of CLC by Hsc70 and auxilin (Ungewickell *et al*. 1995). Given the role of the CLC in regulating the assembly of clathrin it seems reasonable to suggest that the light chain is required for regulation of assembly with specific cargo. This is also supported by the interaction observed of the adaptor proteins Hip1 and Hip1R with the CLC which is likely to play a role in adaptor interactions (Metzler *et al*. 2001; Legendre-Guillemin *et al*. 2002; Hyun *et al*. 2004) and actin polymerisation (Engqvist-Goldstein *et al*. 1999) respectively during endocytosis. This may be particularly pertinent to Hip1/Hip1R interactions as actin is not essential for CME but is important for regulating some endocytic events. Although why actin is required at some but not all sites is not known its involvement may be related to specific cargos. If this is the case the light chain may be required to facilitate regulation of actin assembly though Hip1R (Saffarian *et al*. 2009; Collins *et al*. 2011; Shevchuk *et al*. 2012). Work by Majeed *et al.* (2014) lends support for this by showing that deletion of the CLC impedes cell migration by reducing the amount of gyrating or G-clathrin that is implicated in the trafficking of β-integrins which are crucial in cell migration. G-clathrin has been

implicated in the recycling of receptors such as transferrin receptor from the endosome to the plasma membrane and loss of CLC in this study also inhibits the recycling of this protein. In addition, the loss of CLC in these cells leads to an increase in the amount of abnormal actin structures. Taken together it seems likely that CLC is required for processes involving the recruitment of actin via Hip1/1R.

1.4.5 Clathrin cage structure

The architecture of the clathrin cage structure has been investigated primarily using cryo-EM due to the large size and heterogeneity of the complex making it unsuitable for crystallisation. Early low resolution studies were able to image clathrin cages and vesicles to determine that clathrin formed an outer layer with adaptors forming an inner layer between clathrin and the membrane (Vigers *et al*. 1986). A subsequent 21Å structure of clathrin and AP2 by Smith *et al.* (1998) revealed how the triskelia legs interact as well as confirming the location of AP2 in the centre of the cage structure. To date the highest resolution structure of a clathrin cage from Fotin *et al.* (2004) where a 7.9 Å map of the clathrin cage confirmed the location of the ankle and terminal domain suspended below the outer cage lattice as well as revealing in greater detail the interactions between the helices which form the 'helical tripod' at the trimerisation domain and heavy chain proximal-distal CHC interactions (See Figure 1.03) (Fotin *et al*. 2004b). Subsequent structures have also been solved with clathrin in complex with auxilin and Hsc70, (Fotin *et al*. 2004a; Smith *et al*. 2004; Xing *et al*. 2010; Young *et al.* 2013; Sousa *et al.* 2016) helping us to understand the mechanism by which auxilin and Hsc70 disassemble clathrin cages.

1.5.1 The adaptor proteins

At least 40 proteins have been implicated in the function of CME in higher eukaryotes (excluding cargo) (Traub 2011) and these form a highly complex network of interactions (see Figure 1.02). The investigations in this project are aimed at dissecting the interactions that occur between four key adaptor proteins and clathrin. These proteins are able to bind cargo, other adaptors and clathrin through a number of binding motifs. Figure 1.05 summarises the structure of the adaptor proteins investigated during this project (with the addition of AP180) and the location and number of the adaptor motifs and their locations on clathrin and AP2 and are detailed in this section.

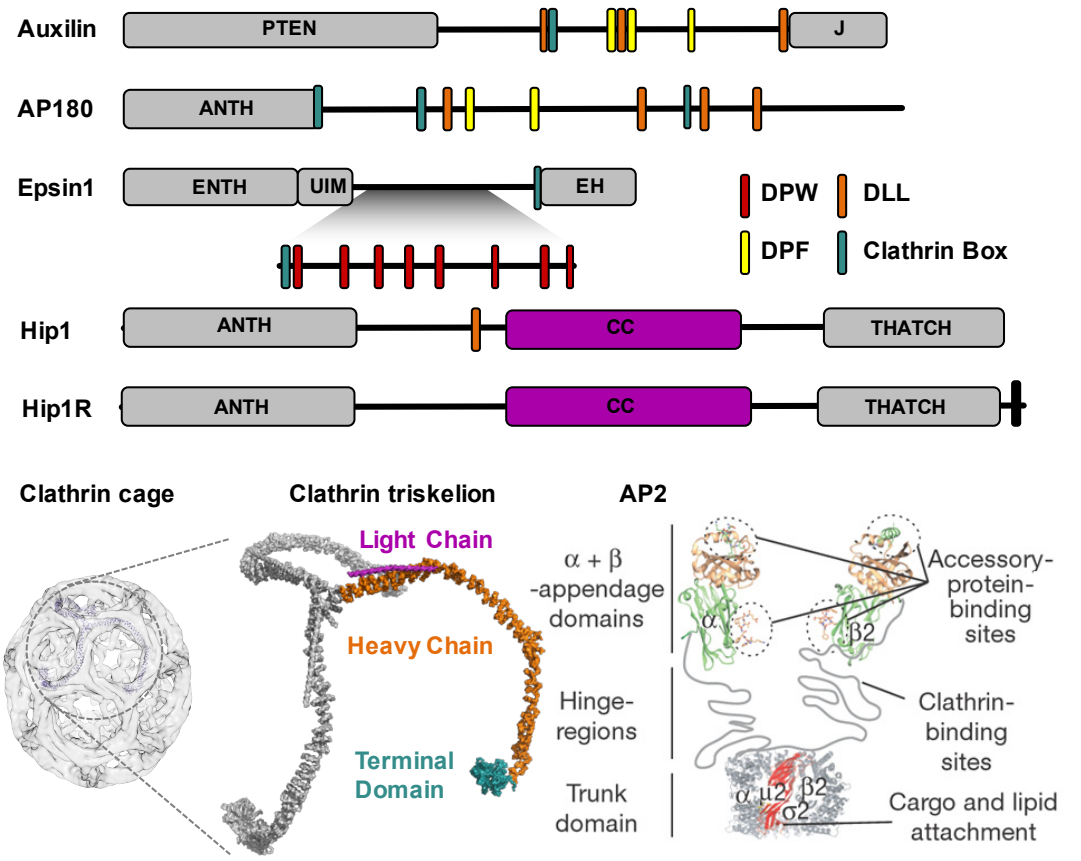

Figure 1.05 Structure of adaptor proteins used in this investigation and their interactions with clathrin and the AP2 complex. *Adaptor proteins interact with the membrane through various phospholipid binding domains with the PTEN domain in auxilin which preferentially interacts with PI(4)P and the ANTH/ENTH domains found in epsin AP180, Hip1 and Hip1R which preferentially interact with PI(4,5)P2 and PI(3,4)P2 respectively. Adaptors interact with clathrin primarily through clathrin box motifs (cyan) that interact with the clathrin terminal domain or through DLL (orange) motifs that interact with the clathrin terminal domain and potentially other regions on the CHC. Hip1 and Hip1R CC domains (purple) interact with the CLC. The DPW (red) and DPF motifs (yellow) interact primarily with the α appendage (ear) domain of the AP2 complex but also with the clathrin terminal domain and potentially other regions on the CHC. AP2 contains multiple adaptor/clathrin binding sites on the ear/appendage domains with the primary clathrin binding site found in the unstructured hinge-region of β2. The trunk domain of AP2 contains phospholipid and cargo binding sites. Clathrin structure created from structure 3IVY courtesy of Kyle Morris and AP2 structure adapted from Schmid and McMahon (2007).*

1.6.1 The AP2 complex

AP1 and AP2 were the first adaptor proteins identified in clathrin coats (Keen *et al*. 1987; Pearse and Robinson 1990) having been isolated from mixed coat proteins stripped from clathrin coated vesicles. Along with clathrin, AP2 is one of the essential components of CME as loss of this protein is shown to be lethal (Mitsunari *et al*. 2005) with knock down by siRNA reducing the number of coated pits formed in cells (Mettlen *et al*. 2009; Loerke *et al*. 2009). The reason for this is that AP2 is a major interaction hub able to interact with a wide range of cargo, adaptors, the plasma membrane and clathrin itself (Schmid and McMahon 2007). Therefore, it is implicated

in a broad range of cellular functions and disease states with brief examples explored here.

AP2 is present through out all stages of endocytosis (Mettlen *et al*. 2009; Saffarian *et al*. 2009; Taylor *et al*. 2011). The recruitment of AP2 is implicated as a major regulator of maturation of sites of endocytosis as the absence of AP2 or clathrin inevitably leads to failure of endocytosis where as almost all sites where both clathrin and AP2 are present mature to vesicles (Honing *et al*. 2005). Regulation of vesicle size and number is a function of many adaptors including AP2. AP2 is able to promote the formation of clathrin cages *in vitro* (Greene *et al*. 2000; Morgan *et al*. 2000) and CCPs/CCVs *in vivo* (Morgan *et al*. 2000; Miller *et al*. 2015) and binding to the other adaptors such as AP180, epsin, CALM and NECAP can enhance assembly of the clathrin coat (Hao *et al*. 1999; Morgan *et al*. 2000; Ritter *et al*. 2013; Miller *et al*. 2015).

AP2 has been implicated in the trafficking of a wide range of cargos including receptor tyrosine kinases (Crupi *et al*. 2015), glutamate receptors (Garafalo *et al*. 2015) and endothelial growth factor receptor (Fortian *et al*. 2015) to name a few. Interactions of AP2 with other adaptors are also important for the trafficking of a wide range of cargos. The AP2-β arrestin complex is essential for internalisation of GPCRs (Kim and Benovic 2002) and AP2 can also interact with the adaptors DAB and numb in the internalisation of integrins (Chao and Kunz 2009; De Franceschi *et al*. 2016). Interactions with the HIV protein NEF reduce cell surface expression of the CD4 receptor and hence enable the virus to avoid the host immune system (Craig *et al*. 1998; Zhang *et al*. 2011).

AP2 is also important for modulating signalling pathways and degradation of protein components associated with disease. The LRP6 signalosome formation is dependent on clathrin and AP2 with this compartment forming an important component in the Wnt signalling pathway, a signalling cascade implicated in carcinogenesis and development (Kim *et al*. 2013). Interactions between AP2 and CALM have also been shown to be important in autophagy of β-amyloid particles which are implicated in Alzheimer's disease (Tian *et al*. 2013). Taken together these studies, and many others, highlight the many important roles that AP2 plays in CME.

1.6.2 Structure and composition of AP2

AP2 is one of the five tetrameric adaptor protein complexes that are involved in membrane trafficking in higher eukaryotes and is specific to endocytic events at the plasma membrane (Edeling *et al*. 2006b; Canagarajah *et al*. 2013). The different AP complexes fulfil specific roles in trafficking: AP1 functions at the TGN-endosome phase (Edeling *et al*. 2006b; Kural *et al*. 2012), AP3 in lysosomal transport (Kural *et al*. 2012) and AP4 and AP5 do not interact with clathrin and play other roles in membrane trafficking (Hirst *et al*. 2011). These adaptors consist of two ~100 kDa subunits, a ~50 kDa subunit and a ~20 kDa subunit. The combination of different forms of these subunits determines the role of the overall complex in trafficking. For example the AP1 complex contains the γ1 and β1 ~100 kDa subunits along with the μ1 and σ1 ~ 50 kDa and ~20 kDa subunits respectively. In contrast AP2 contains the α, β2, μ2 and σ2 subunits (Paczkowski *et al*. 2015) (see Figure 1.05). The α and β2 domains of AP2 form the core of the protein with extended hinge/ear domains attached to the core by the flexible unstructured hinge regions. The α domain of AP2 binds to PI(4,5)P2 (Beck and Keen 1991) as does the μ2 subunit with the μ2 and σ2 subunits contain or shield the YXXΦ and dileucine cargo binding sites respectively (Owen and Evans 1998; Kelly *et al*. 2008). The hinge/ear domains of α and β2 provide interactions sites for adaptors and clathrin (Traub *et al*. 1999; Owen *et al*. 1999; Owen *et al*. 2000; Edeling *et al*. 2006a; Schmid *et al*. 2006).

1.6.3 AP2 conformational changes regulate its interactions

The ability of AP2 to bind to the membrane, cargo, adaptors and clathrin is regulated by a number of conformational changes that control temporal and spatial recruitment of AP2 and its binding partners (see Figure 1.06). Initial recruitment to the membrane is facilitated through interactions with other adaptors with weak binding to the membrane through a PIP(4,5)P2 binding site on the base of the α subunit (Collins *et al*. 2002). The phosphorylation of Thr156 by AAK1 (Olusanya *et al*. 2001; Ricotta *et al*. 2002) or GAK (Umeda *et al*. 2000; Korolchuk and Banting 2003) triggers the release of the μ2 subunit which exposes the YXXΦ binding site as well as further PIP(4,5)P2 binding sites to enhance recruitment to the membrane (Jackson *et al*. 2010). This conformational change also enhances clathrin binding by releasing the β2 hinge/appendage from a binding pocket which protects the primary clathrin binding

site on the β2 hinge prior to phosphorylation and membrane binding (Kelly *et al*. 2014).

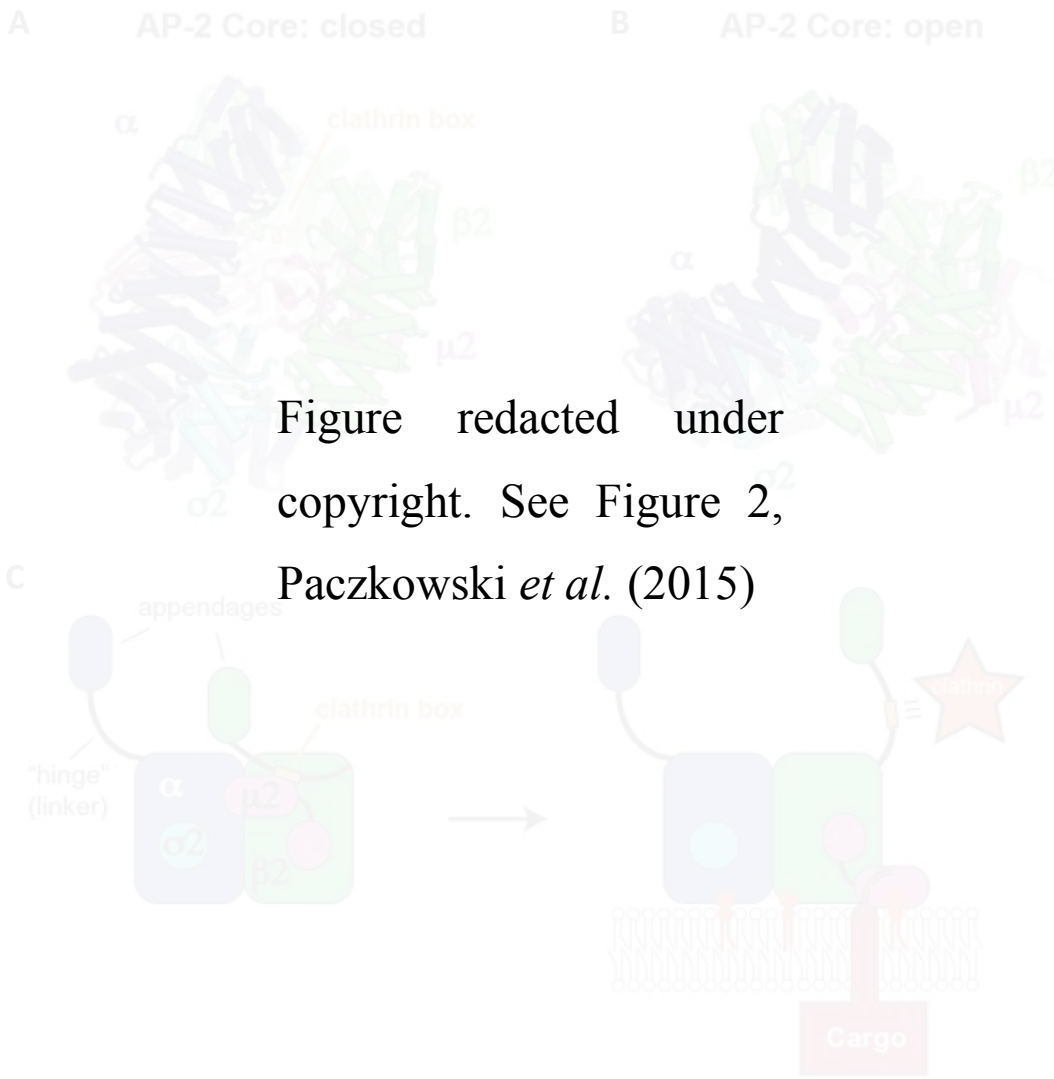

Figure redacted under copyright. See Figure 2, Paczkowski *et al.* (2015)

Figure 1.06 Conformational changes in AP2 drive interactions with clathrin, adaptors, cargo and the plasma membrane. *A) Structure of the closed core conformation of AP2 hides the cargo binding sites of the μ2 subunits σ2 and the clathrin box binding site on the hinge of the β2 subunit. B) In the open conformation the clathrin binding site is freed and cargo binding sites exposed. C) The switch from the closed conformation of AP2 is triggered by phosphorylation of Thr156 of μ2 leads to enhanced binding to PI(4,5)P2 with exposure of cargo binding sites and clathrin box motif, allowing recruitment of clathrin and adaptors to the plasma membrane. Figure adapted from Paczkowski et al. (2015).*

Whilst these conformational changes are needed for the enhancement of binding it is clear that AP2 can bind clathrin in the absence of lipids (Smith *et al*. 1998; Morgan *et al*. 2000; Fotin *et al*. 2004b; Boecking *et al*. 2011) and clathrin and AP2 appear to be recruited as partners during *in vivo* tracking experiments (Cocucci *et al*. 2012). Therefore it is possible that this recruitment is facilitated through interactions with the α ear domain, that shows weak clathrin binding but remains accessible in the closed

conformation of AP2 (Kelly *et al*. 2014) or that the complex can switch between the closed and open conformation in solution.

1.6.4 α and β2 Hinge/Appendage Domains are Adaptor and Clathrin Interaction Hubs

The ear domains (also referred to as the appendage domains) and the hinge regions of α and β2 adaptin are important for the interaction of AP2 and adaptors (see Figure 1.05 and Figure 1.06). The β2 hinge contains two clathrin box motifs (LLNLD and LLGDL) in the unstructured region of the hinge that facilitates interaction with the clathrin terminal domain and is the primary interaction site for β2 with clathrin (Shih *et al*. 1995; Clairmont *et al*. 1997; Owen *et al*. 2000). This motif is important for the promotion of assembly as a peptide containing the clathrin box motif inhibits assembly *in vitro* and peptides containing clathrin box motifs block coated pit formation in synapses (Morgan *et al*. 2001). The β2 and α ears also contain another clathrin binding site that interacts with the ankle domain of the CHC (Edeling *et al*. 2006a; Knuehl *et al*. 2006). It is this interaction that has been proposed to explain the ability of AP2 to promote clathrin cage formation in that the two binding sites could potentially interact with neighbouring ankle and terminal domains and thus stabilise cage interactions (Greene *et al*. 2000; Knuehl *et al*. 2006).

Both the α and β2 ears are the primary interaction hubs for adaptor proteins with competition and sequential binding of adaptors to these domains and clathrin is likely to play an important role in the maturation of the CCPs and CCVs (Schmid and McMahon 2007). Both of the ear domains of AP2 contain a top or 'platform' binding site and a second side site or 'β sandwich' site (Owen *et al*. 1999; Traub *et al.* 1999, Owen *et al*. 2000; Edeling *et al*. 2006a; Schmid *et al*. 2006). Edeling *et al.* (2006) showed how in the β2 ear domain the adaptor epsin preferentially binds to the platform site whereas eps15, amphyphysin and AP180 preferentially bind to the side site. Interestingly the platform site also binds to the ankle domain of clathrin and mutation of Y815 in this binding site reduces binding to both clathrin and these adaptors (Knuehl *et al*. 2006). From these observations it was concluded that AP2-clathrin interactions may displace adaptors from AP2. This is supported by the observation by Edeling *et al.* (2006) and others that eps15 preferentially localises to the edge of coated pits (Cupers *et al*. 1997; Edeling *et al*. 2006a; Saffarian *et al*. 2009). Eps15 lacks

clathrin binding where as other adaptors such as AP180 and epsin can bind directly to clathrin and so may not be displaced from the growing coat (Kalthoff *et al*. 2002).

In contrast the α hinge/ear lacks the clathrin box motif but does show some affinity for clathrin, potentially through binding through to the ankle of the CHC in the same manner as the β2 ear (Knuehl *et al*. 2006). This domain shows affinity for many adaptors that contain FxDxF/W or WVxF motifs such as epsin, AP180, eps15, amphyphysin and auxilin (Traub *et al*. 1999; Owen *et al*. 2000; Brett *et al*. 2002; Praefcke *et al*. 2004; Walther *et al*. 2004; Olesen *et al*. 2008). Like the β2 ear domain the α ear side and platform site show preferences for different binding motifs with the platform site allowing general binding of FxDxF/W motifs (e.g. amphyphysin) whereas the side site shows preferential binding of DPW/WVxF motif proteins such as epsin, eps15 and synaptojanin (Brett *et al*. 2002; Praefcke *et al*. 2004; Schmid *et al*. 2006). This generalised and specific site may allow specific recruitment of different adaptors during different phases of pit maturation. This is demonstrated in the case of synaptojanin which contains both the FxDxF and WVxF motifs located close together spatially in the structure. Praefcke *et al*. (2004) note that these motifs are too close in space to be able to interact with the platform and side site simultaneously, suggesting that one site may be preferentially occupied at different stages during endocytosis (Praefcke *et al*. 2004).

1.7.1 Hip1 and Hip1R

The Hip1 and Hip1R proteins are unusual among the adaptor proteins due to their unique binding interaction with clathrin. Hip1 was initially discovered as a binding partner with Huntingtin through yeast 2 hybrid (Wanker *et al*. 1997) and hence was named Huntingtin Interacting Protein 1 (Hip1) and was additionally shown to have reduced affinity with Huntingtin with the longer polyQ forms of Huntingtin associated with Huntington's Disease (Kalchman *et al*. 1997). Hip1R in contrast was identified on its sequence homology with Hip1 but lacks the ability to bind Huntingtin (Gottfried *et al*. 2010). Both proteins were subsequently implicated in CME (Engqvist-Goldstein *et al*. 2000; Waelter *et al*. 2001; Mishra *et al*. 2001) with overexpression of Hip1R leads to build up of CLC in the cytosol (Engqvist-Goldstein *et al*. 2001) and KD of Hip1R reducing the rate of pit maturation (Mettlen *et al*. 2009). In addition to their

roles in CME both Hip1 and Hip1R have been variously implicated as oncogenes and are involved in activation of apoptosis (Hyun and Ross 2004; Gottfried *et al*. 2010).

1.7.2 Structure of the Hip1 protein family

The Hip1 family of proteins including the yeast homologue Sla2p share a similar structure (see Figure 1.05 and Figure 1.07). The N terminus contains the membrane ANTH binding domain that binds phospholipids as seen in many adaptors (Legendre-Guillemin *et al*. 2004). Interestingly the ANTH domains in this family bind in preference to PI(3,4)P2 in contrast to the majority of phospholipid binding proteins with ANTH/ENTH domains involved in CME which preferentially bind PI(4,5)P2 (although these proteins can bind to this isoform) (Hyun *et al*. 2004).

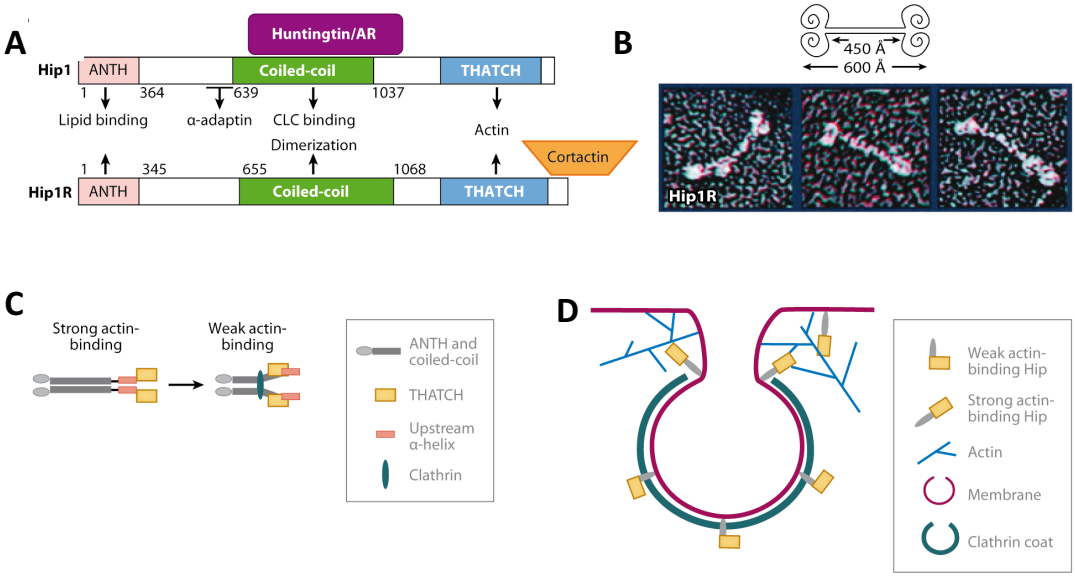

Figure 1.07 Structure and function of the Hip1/1R proteins. *A) Domain structure of Hip1/1R with regions implicated in interactions highlighted. B) EM images of the Hip1R dimer 'dumbbell' structure. C) Conformational change in the Hip1/1R proteins on CLC binding induces shielding of the actin binding region of the THATCH domain thereby inhibiting actin binding. D) Proposed interactions of Hip1/1R with actin in the budding vesicle with binding of Hip1/1R to CLC inhibiting actin binding. At the neck, membrane binding and loss of interaction with CLC allows interaction with clathrin and regulation of actin polymerisation at the neck of the vesicle. Reproduced from Brodsky (2012).*

The central coiled-coil (CC) domain (Niu and Ybe 2008) serves as both the region for dimerization (Legendre-Guillemin *et al*. 2002; Legendre-Guillemin *et al*. 2005; Repass *et al*. 2007; Wilbur *et al*. 2008) and as the primary site for interaction with the CLC (Engqvist-Goldstein *et al*. 2001; Legendre-Guillemin *et al*. 2002; Legendre-Guillemin *et al*. 2005; Chen and Brodsky 2005). This binding to the CLC is unusual for adaptors which primarily contact the CHC. CC binding to CLC has been shown to promote the formation of clathrin cages *in vitro* (Engqvist-Goldstein *et al*. 2001;

Legendre-Guillemin *et al*. 2002; Legendre-Guillemin *et al*. 2005; Chen and Brodsky 2005) which is likely to occur through the neutralisation of the regulatory acidic region on the CLC via basic patches found in the CLC binding region of Hip1 (Legendre-Guillemin *et al*. 2005; Ybe *et al*. 2007a; Ybe *et al*. 2010). Evidence for homo or hetero dimerization of Hip1/R is mixed although most of the evidence suggests that these proteins exist as homodimers and are therefore likely to perform specific functions in the cell (Legendre-Guillemin *et al*. 2002; Legendre-Guillemin *et al.* 2005; Repass *et al*. 2007; Wilbur *et al*. 2008).

The N-terminus of the structure contains an upstream helix (USH) connected to a Talin like domain (THATCH) which binds to F-actin (Brett *et al*. 2006). Binding to actin is regulated by the USH which, when the CC domain is interacting with the CLC, causes the THATCH domain to fold back along the length of the protein and thereby blocking actin interactions (Engqvist-Goldstein *et al*. 2004; Senetar *et al*. 2004; Wilbur *et al*. 2008) (see Figure 1.07). EM images of Hip1R have shown a dimer 'dumbbell' structure with the region suspected to be the THATCH domain folded back on the protein (Engqvist-Goldstein *et al*. 2001). This has lead to the hypothesis that the Hip1 proteins are recruited to the plasma membrane by clathrin where they preferentially bind to the membrane and lose the interaction with the CLC. The loss of CLC binding removes the steric hindrance on the THATCH domain, allowing the proteins to interact with actin and regulate its assembly (Senetar *et al*. 2004; Wilbur *et al*. 2008). This evidence is support by EM images localising Hip1, actin and clathrin which show no co-localisation of actin and Hip1 with clathrin except at the neck of the vesicle (Wilbur *et al*. 2008) and by experiments with the yeast Sla2p homologue that shows that the CLC negatively regulates Sla2p-actin interaction *in vivo* (Boettner *et al*. 2011).

1.7.3 Differences in Hip1/1R function

Both structural differences and differences in phenotypes on genetic manipulations of Hip1 and Hip1R expression suggest different functions of the proteins. RNAi of Hip1R results in the formation of stable actin structures associated with CCPs and an inhibition in transferrin uptake (Engqvist-Goldstein *et al*. 2004). Further evidence for the potential role of Hip1R in actin regulation is its ability to interact with cortactin via a C-terminal proline rich domain (PRD) (Le Clainche *et al*. 2007). This interaction by Hip1R is able to cap actin polymers with cortactin and prevent cortactin from

stimulating actin polymerisation by interacting the the N-WASP-Arp2/3 complex. This capping is proposed to regulate actin polymerisation to the neck of the vesicle which otherwise stalls the CCP and prevents budding. More recently Hip1R has been shown to be crucial in cells where actin is required to overcome local membrane tension (Boulant *et al*. 2011).

Interactions with the ENTH domain of the adaptor protein epsin are also crucial for Hip1R recruitment and actin organisation and lends support to the role of Hip1R in actin regulation (Hyun *et al*. 2004; Repass *et al*. 2007; Brady *et al*. 2010). In addition, both functional and recent cryo-EM structures have shown how the ANTH and ENTH domains of the yeast homologues of Hip1R and epsin (Sla2 and Ent1) form an ordered structure around tabulated vesicles, and are important in transferring the force of the actin cytoskeleton to the budding vesicle (Skruzny *et al*. 2012; Skruzny *et al*. 2015). Although actin polymerisation is essential for endocytosis in yeast to overcome the higher membrane tension (Mishra *et al*. 2014) and hence this interaction plays a more important role in yeast it is possible that a similar action may be required through epsin and actin in higher eukaryotes. In conclusion it seems that Hip1R and actin recruitment are required only in situations where membrane tension is high. Hip1R may also be important in forming coated plaques (Saffarian *et al*. 2009).

The role of Hip1 in cells is less clear. Hip1 also contains a clathrin box (LMDMD) along with FxDxF and DPW motifs that can interact with the clathrin terminal domain and the α ear of AP2 respectively where as Hip1R lacks these binding motifs (Metzler *et al*. 2001; Legendre-Guillemin *et al*. 2002; Hyun *et al*. 2004). Gottfried *et al.* (2009) showed that endocytic sites positive for Hip1 fluorescence had a longer average lifetime of internalisation, suggesting that Hip1 plays a role in stabilising CCPs (Gottfried *et al*. 2009). In addition, Hip1 has also been shown to act as a pro-apoptotic protein by interacting with the Hip1 interacting protein (HIPPI) that then interacts with caspase-8 to induce apoptosis (Gervais *et al*. 2002). Hip1 mutants lacking the ANTH domain can also induce apoptosis, suggesting that miss-localisation of the protein from the membrane induces apoptosis (Rao *et al*. 2003). Therefore, Hip1 may play multiple subtle roles in cells.

1.8.1 Epsin

Epsin belongs to the general class of adaptors known as CLASPs (Clathrin coat Associated Proteins) to distinguish themselves from the AP complexes (Traub 2007). Epsin1 was discovered in 1998 as a component of CME (Chen *et al*. 1998) and has been shown to be present in all tissues but is most highly expressed in the brain (Rosenthal *et al*. 1999). Loss of epsin function results in defects in actin dynamics through recruitment of Hip1R (Brady *et al*. 2010), and reduction in the maturation of CCPs (Mettlen *et al*. 2009).

Due to its high level of expression in the brain and its role in endocytosis and vesicle formation epsin has been implicated in synaptic development and plasticity (Vanlandingham *et al*. 2013). Epsin has also been implicated in the trafficking of the influenza virus as KD of this protein reduces uptake of the virus and directs the virus to enter through a clathrin independent pathway (Chen and Zhuang 2008). Interactions with cargo specific systems have also been noted such as the requirement of epsin for the internalisation of the VEGFR2 receptor which is crucial for angiogenesis (Rahman *et al*. 2016). However, blocking epsin interaction with the same receptor is important in down regulating VEGFR2 signalling and angiogenesis and therefore reducing growth of metastatic cells in cancer (Pasula *et al*. 2012; Dong *et al*. 2015). And like other adaptor proteins epsin is involved in mitosis (Smith and Chircop 2012) where it is involved in membrane remodelling through its ENTH domain which helps promote spindle assembly (Liu and Zheng 2009). Finally, the strong promotion of clathrin assembly by epsin has been used to create functionalised clathrin lattices on surfaces that could potentially be used as functional bio-surfaces for various industrial purposes (Dannhauser *et al*. 2015b).

1.8.2 Epsin adaptor structure

Epsin, like many adaptors such as CALM and AP180, has a globular membrane binding domain at the N-terminus of the protein followed by a long unstructured region that contains numerous binding motifs that are required for interacting with clathrin, cargo and other adaptor proteins (Chen *et al*. 1998; Drake *et al*. 2000; Drake and Traub 2001; Kalthoff *et al*. 2002; Dafforn and Smith 2004) (see Figure 1.05). The ENTH domain, or epsin N-terminal homology domain, binds to PI(4,5)P2 as is found in many adaptor proteins (Ford *et al*. 2002; Hom *et al*. 2007). This domain also

facilitates interactions with other adaptors such as Hip1R (Brady *et al*. 2010) and is crucial for the recruitment of this adaptor to the plasma membrane during CME (Messa *et al*. 2014). The ENTH domain contains an amphipathic helix (termed helix 0) that forms from an unstructured region on binding to PI(4,5)P2 which the inserts into the membrane (Ford *et al*. 2002; Lai *et al*. 2012). The insertion of the helix into the membrane increases the fluidity of the membrane and allows the ENTH to induce membrane curvature and bind preferentially to these highly covered surfaces (Lai *et al*. 2012; Holkar *et al*. 2015).

After the ENTH domain the first of many interaction motifs is presented: the ubiquitin interacting motif (UIM). Epsin contains 3 UIMs which interact which are able to interact with polyubiquitin (Polo *et al*. 2002; Oldham *et al*. 2002; Hawryluk *et al*. 2006) and are important for internalisation of various receptors such VEGFR2 (Dong *et al*. 2015) and EGFR (Fortian *et al*. 2015).

The region following the UIM is an unstructured region termed the clathrin/adaptor domain (Kalthoff *et al*. 2002). This region contains two clathrin boxes at residues 257 and 480 that bind to the clathrin terminal domain (Drake *et al*. 2000) and in between a series of DPW motifs. This DPW region containing 8 such motifs is able to interact primarily with α adaptin ear domain (Brett *et al*. 2002) although it also provides low affinity binding to β2 ear (Edeling *et al*. 2006a) and to clathrin (Drake *et al*. 2000). Interestingly, this unstructured region has also been shown to facilitate membrane curvature (Busch *et al*. 2015) and also links to the ability of the full length protein to initiate membrane fission (Brooks *et al*. 2015) and clathrin assembly (Dannhauser and Ungewickell 2012; Holkar *et al*. 2015). Finally, the N-terminal region contains 3 NPF motifs that interact with epsin hand (EH) domains on adaptors such as eps15 (Chen *et al*. 1998).

1.8.3 Functions of epsin in endocytosis

Epsin has a wide range of functions in endocytosis. One of the most important studies that highlights many of these functions was conducted by Messa *et al*. (2014). The authors knocked out all 3 epsin isoforms in the brains of mice individually and together to test the differing functions and phenotypes of these proteins (Messa *et al*. 2014). All mice were dead after 4 weeks and, consummate with epsin's role in mitosis (Smith and Chircop 2012), cells exhibit abnormal large and clustered nuclei. In live cells

transferrin uptake was severely limited and EM of cells showed shallow and U-shaped pits with an absence of late stage proteins such as endophilin and dynamin as well as abnormal actin organisation. The abnormal actin organisation was attributable to the loss of Hip1R recruitment to the membrane, confirming the need of epsin for Hip1R recruitment. In addition, epsin was shown to interact with actin directly and hence epsin has a key role in regulating actin assembly.

Like many adaptors epsin is important in regulating the size of vesicles. Disrupting epsin interactions via antibody binding results in an increase in the the number of flat clathrin coated structures as well as larger CCPs in synapses (Jakobsson *et al*. 2008). Epsin has also been shown to promote the formation of small cage structures *in* vitro in a similar manner to AP180 (Kalthoff *et al*. 2002). It is likely that this regulation of size is mediated by the clathrin box motifs that are predicted to bind to different locations on the clathrin terminal domain (Drake *et al*. 2000; Drake and Traub 2001; Miele *et al*. 2004; Dafforn and Smith 2004). Given the distances that the unstructured region could potentially stretch (Kalthoff *et al*. 2002) it is possible that these motifs co-operatively assemble clathrin by linking together multiple triskelia. Support for this hypothesis has recently been made by Holkar *et. al.* 2015 who looked at the ability of epsin to bind and assemble clathrin on membranes. In addition to showing that epsin preferentially assembles clathrin on curved surfaces they showed that mutating either of the clathrin boxes in epsin substantially inhibited its ability to assemble clathrin (Holkar *et al*. 2015). The requirement for both clathrin boxes to assemble clathrin on membranes indicates that epsin promotion of assembly is mediated by interactions with multiple triskelia.

The precise fate of epsin during endocytosis is still up for debate. *In vivo* trafficking studies have shown that epsin is recruited early and shows similar dynamics to clathrin (Mettlen *et al*. 2009; Saffarian *et al*. 2009; Taylor *et al*. 2011) with disruption of epsin halting endocytosis at the early and mid-stages (Jakobsson *et al*. 2008; Mettlen *et al*. 2009). Given its role in membrane remodelling and fission and role in actin polymerisation in conjunction with Hip1R it is likely that epsin preferentially migrates to the neck to perform this role at late stages of endocytosis (Saffarian *et al*. 2009). These observations, combined with competitive binding between AP2/clathrin and epsin (Edeling *et al*. 2006a; Schmid *et al*. 2006) have led to the suggestion that epsin is excluded from the coat and pushed to the edges of the growing coat (Praefcke *et al*.

2004; Saffarian *et al*. 2009) along with the adaptor protein eps15 (Chen *et al*. 1998; Wang *et al*. 2006; Edeling *et al*. 2006a) and hence is not present in the budded CCV. Evidence for the presence of epsin in CCVs is mixed and therefore the precise location of epsin during cage disassembly is not known (Chen *et al*. 1998; Rappoport *et al*. 2006; Edeling *et al*. 2006a; Hawryluk *et al*. 2006; Saffarian *et al*. 2009; Henne *et al*. 2010).

1.9.1 Disassembling the clathrin coat: Hsc70 & auxilin/GAK

Once a coated vesicle has been formed the question then arises as to how this process is reversed. The disassembly of the clathrin-adaptor coat complex must occur in order that the adaptor and clathrin components can be recycled to engage in successive rounds of endocytosis and so that the vesicle can go on to fuse with its target compartment. Early work implicated an 'uncoating ATPase' Hsc70 in the function of disassembly (Schlossman *et al*. 1984) but could not disassemble clathrin on its own (Tsai and Wang 1994). Like many chaperones Hsc70 requires a co-chaperone to recruit it to its substrate with the protein auxilin initially identified as fulfilling this role in neurones (Ahle and Ungewickell 1990; Ungewickell *et al*. 1995) with the subsequent discovery of GAK fulfilling this function in non-neuronal cells (Greener *et al*. 2000; Umeda *et al*. 2000). Recruitment of Hsc70 to the region under the hub domain of the clathrin triskelion by auxilin/GAK followed by hydrolysis of ATP facilitates disassembly of the clathrin cage (see Figure 1.08).

1.9.2 Roles of auxilin and GAK

Auxilin is a 100 kDa protein that is a member of the J-domain family of proteins that are important regulators of chaperone function (Cheetham *et al*. 1996). GAK shares the same structure with 50% homology to auxilin (Eisenberg and Greene 2007) but with the addition of the N-terminal kinase domain which is able to phosphorylate the μ2 subunits of AP2 and AP1 and therefore promote their association and binding with cargo and the plasma membrane (Umeda *et al*. 2000; Korolchuk and Banting 2003). Loss of auxilin/GAK through KD or knock out (KO) experiments results in defects in CME in yeast (Pishvaee *et al*. 2000), *C.elegans* (Greener *et al*. 2001) and human cell lines with characteristic loss of receptor uptake (Zhang *et al*. 2005) and depletion of clathrin and adaptors at the plasma membrane combined with the formation of clathrin

structures devoid of cargo and lipids (Lee *et al*. 2005). KO of auxilin or GAK is lethal in mice (Yim *et al*. 2010; Park *et al*. 2015) with both auxilin and GAK proposed to play a role in the development of such as through the Notch signalling pathway in Drosophila (Eun *et al*. 2008; Banks *et al*. 2011).

1.9.3 Structure and function of auxilin/GAK

The N-terminal PTEN (phosphatase and tensin homology) domain of auxilin/GAK binds preferentially to mono-phosphorylated (PI4P) PIPs in contrast to other membrane binding adaptor proteins that preferentially bind to bi-phosphate PIPs (PI(4,5)P2 or PI(3,4)P2) (Lee *et al*. 2006; Massol *et al*. 2006; Guan *et al*. 2010) (see Figure 1.05). It has been suggested that this preference for PI4P drives recruitment of auxilin after formation of the CCV and is driven by dephosphorylation of PI(4,5)P2 by synaptojanin (Cousin *et al*. 2001; Stefan *et al*. 2002; Stefan *et al*. 2005), which is recruited shortly prior to scission of the CCV (Taylor *et al*. 2011). The increase in PI4P in the membrane of the CCV would therefore ensure that disassembly is only triggered after scission. However, disassembly *in vitro* does not require the PTEN domain with constructs consisting of residues 547-910 being sufficient to disassemble clathrin cages (Ungewickell *et al*. 1995). In addition, studies of GAK mutants in both cell and mouse models show that KO of both auxilin and GAK can be rescued by the a clathrin binding domain-J-domain GAK construct (Lee *et al*. 2008; Park *et al*. 2015). Therefore, whether this domain plays a role in the temporal recruitment of auxilin/GAK is debatable.

The central region of auxilin facilitates binding to clathrin and AP2 through various motifs (Scheele *et al*. 2001; Scheele *et al*. 2003; Smith *et al.* 2004) (see Figure 1.05). The C-terminal end of this region contains a clathrin box motif that interacts with the clathrin terminal domain (Smith *et al*. 2004) with multiple DLL motifs that provide affinity to the CHC and DPF motifs that bind to both the CHC and the ear domain of α adaptin (Scheele *et al*. 2001). Whilst the clathrin box motif provides affinity for the terminal domain the other binding motifs seem to be able to target both the terminal domain and the distal region of the CHC suggesting that auxilin can interact with multiple regions on the leg of the triskelion. This is supported by the fact that clathrin lacking the terminal domain can be disassembled *in vitro* (Ungewickell *et al*. 1995) suggesting that binding to the terminal domain is not crucial to disassembly.

Binding of auxilin to clathrin has been shown to saturate at 1 auxilin molecule for every CHC (Holstein *et al*. 1996; Ma *et al*. 2002) however only one auxilin molecule per triskelion (i.e. 1 per 3 CHC) is required for the optimal stimulation of Hsc70 ATP hydrolysis and for the maximal rate of cage disassembly (Holstein *et al*. 1996; Barouch *et al*. 1997; Rothnie *et al*. 2011). This has lead to the hypothesis that auxilin acts catalytically to stimulate cage disassembly and the clathrin-adaptor binding region is important in this. Disassembly of clathrin cages with a fusion protein consisting of the clathrin binding region of AP180 and the J-domain of auxilin is able to disassemble cages with Hsc70 but requires stoichiometric binding (Ma *et al*. 2002). This is because the AP180 binding region does not dissociate from triskelia after disassembly where as auxilin does, hence the clathrin-adaptor region is important for allowing auxilin to dissociate from triskelia and re-bind to cages to initiate further rounds of disassembly. The CLC has also been implicated in the ability of auxilin to act catalytically as removal of the CLC inhibits disassembly and requires larger concentrations of auxilin to promote complete disassembly (Young *et al*. 2013). This suggests that some form of conformational change driven by the CLC induces dissociation of auxilin and allows it to recycle.

Finally, the C-terminus contains the J-domain that recruits Hsc70 and stimulates its ATP hydrolysis activity (Ungewickell *et al*. 1995; Holstein *et al*. 1996; Barouch *et al*. 1997) but is not able to interact with clathrin (Scheele *et al*. 2001). The J-domain and adjacent clathrin binding region consists of 6 α helices with the DLL and HPD motifs integral to clathrin and Hsc70 binding respectively present at the ends of these helices (Jiang *et al*. 2003; Gruschus *et al*. 2004). Mutations to the HPD motif in auxilin inhibit disassembly *in vitro* and in synapses suggesting that this interaction is crucial to the function of auxilin (Morgan *et al*. 2001).

Whilst auxilin cannot disassemble clathrin without Hsc70, auxilin binding to the terminal domain/distal leg induces a conformational change whereby the terminal domains twist at the ankle and also induce an expansion of the overall lattice (Smith *et al*. 2004; Fotin *et al*. 2004a; Xing *et al*. 2010). Therefore, whilst auxilin alone is not sufficient to initiate disassembly these structural changes are thought to assist in destabilising the cage structure and assist Hsc70 induced uncoating. However other work indicates that auxilin can promote clathrin assembly *in vitro* and so the

interactions of auxilin with the clathrin are not actively inimical to cage interactions (Holstein *et al*. 1996; Scheele *et al*. 2003).

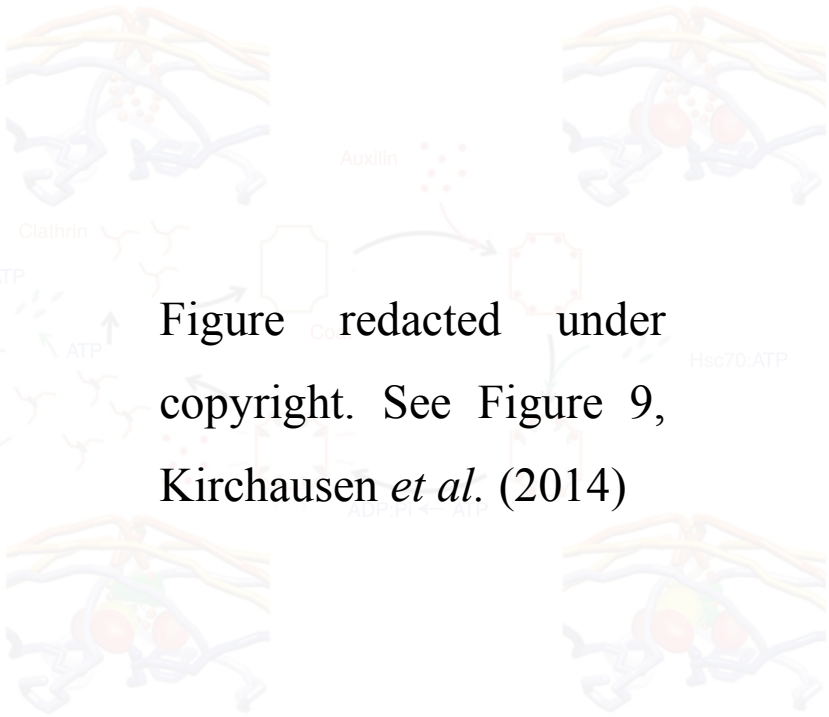

Figure redacted under copyright. See Figure 9, Kirchausen *et al*. (2014)

Figure 1.08 Recruitment of auxilin and Hsc70 to the clathrin trimerisation domain induces cage disassembly. *Clockwise from top left: The hub of the triskelion contains the unstructured C-terminal tails containing the Hsc70 binding motif (orange). The knee domains of neighbouring triskelia are shown with the distal and terminal domain regions of further triskelia shown in blue. Recruitment of auxilin (red) through terminal domain-leg interactions under the hub allows recruitment of Hsc70 (green/yellow). Subsequent ATP hydrolysis stimulated by the J-domain facilitates ATP hydrolysis and tight binding of Hsc70 to clathrin and dissociation of triskelia from the clathrin cage. Auxilin is able to recycle from clathrin where as Hsc70 remains bound to clathrin until ADP+Pi is exchanged for ATP. Reproduced from (Kirchhausen et al. 2014)*

1.9.4 Hsc70 structure and function in clathrin mediated endocytosis

Hsc70 or heat shock cognate protein 70, is a member of the protein chaperone family involved in a diverse range of functions including protein folding, aggregation, degradation, translocation and import mechanisms to name a few (Liu *et al*. 2012; Stricher *et al*. 2013). As such Hsc70 can contribute up to 1% of the protein content of the cell. Given the wide range of roles of Hsc70 an impact one one area of function can impact on Hsc70 in CME. For example, aggregates of proteins found in neurodegenerative diseases such as huntingtin can sequester Hsc70 and therefore inhibit endocytosis suggesting a role in the physiology of these diseases (Yu *et al*. 2014). The role of Hsc70 in CME *in vivo* has been confirmed through the use of ATPase deficient mutants of Hsc70 that inhibit the uptake of transferrin and stall both

uncoating of CCVs and an apparent loss of cargo clustering, suggesting that Hsc70 is involved in the assembly of clathrin at the membrane (Newmyer and Schmid 2001; Yim *et al.* 2005). In addition, the mutants lead to the formation of empty clathrin structures that form in the cytosol in the absence of lipid which suggests that Hsc70 may remain in complex with clathrin to prevent it from assembling in the cytosol.

This family of proteins broadly contains two major component parts, with an N-terminal nucleotide binding domain (NBD) (De Luca-Flaherty *et al*. 1990) and a C-terminal substrate binding domain (SBD) (Zhu *et al*. 1996). The SBD contains a 15 kDa β sandwich that binds peptides and 10 kDa lid R-helix that hides the peptide binding pocket (Qi *et al*. 2013). Conformational changes in the NBD are transferred to the SBD on nucleotide binding and hydrolysis through a linker region (Jiang *et al*. 2005). In the absence of nucleotides, the binding pocket is closed, preventing substrate binding (Jiang *et al*. 2005) where as in the presence of ATP the SBD opens to allow access to the binding pocket (Liu and Hendrickson 2007) but not tight binding. Stimulation of ATP hydrolysis by J-domains is believed to occur through an interaction with a cleft on the NBD (Jiang *et al*. 2007). On ATP hydrolysis a conformational change in the NBD (Sousa and McKay 1998; Jiang *et al*. 2007) results in a further conformational change that closes the lid over substrate, therefore facilitating tight binding to the substrate (Bertelsen *et al*. 2009). Dissociation of ADP is slow and hence modulation of binding to substrate can be modulated through nucleotide exchange factors such as Bag1 and Hsp110 that stimulate the exchange of ADP to ATP, therefore opening the structure and allowing substrate release (Hohfeld and Jentsch 1997; Jinag et al. 2007; Schuermann *et al*. 2008).

In CME Hsc70 interacts with the QLMLT motif on the unstructured C-terminal domain of the CHC (Rapoport *et al*. 2008) which positions Hsc70 below the trimerisation hub (Xing *et al*. 2010) (see Figure 1.08). Despite this knowledge questions remained as to how Hsc70 binding induces disassembly. Structural and functional studies suggested that Hsc70 binding trapped transient fluctuations in the triskelia that were not favourable for cage competent interactions (Xing *et al*. 2010; Boecking *et al*. 2011). The other topic of debate was over the number of Hsc70 molecules required to induce disassembly. Hsc70 has been shown to saturate at binding at 3 molecules of Hsc70 per triskelion (Prasad *et al*. 1994) which is supported by the availability of 3 QLMLT motifs per triskelion with one per CHC (Rapoport *et*

al. 2008). However, there has been debate over whether saturation of binding to clathrin is required with different studies suggesting that a minimum of 2 Hsc70 molecules are required to release a triskelion (Boecking *et al*. 2011) or full occupation of all sites with 3 Hsc70 molecules (Rothnie *et al*. 2011).

A recent investigation by Sousa *et al.* (2016) helped answer both of these questions through confirming that a minimum of 2 Hsc70 molecules are required to initiate the dissociation of a triskelion and has leant support to a model where by Hsc70 recruitment acts to dissociate triskelia through a 'collision pressure' mechanism (Sousa *et al*. 2016). This mechanism suggests that, rather than a 'pulling' or 'trapping' mechanism as suggested in Brownian ratchet or power stroke models of Hsp activity, that the presence of a large molecule inserted under the trimerisation domain effectively pushes apart the triskelia through collisions. The key results from this study showed that by extending the distance between the QLMLT motif and the underside of the triskelia the authors were able to show that the rate of disassembly is reduced and by replacing the Hsc70 binding motif with a FLAG affinity tag they were able to induce disassembly through the addition of anti-FLAG Fab (Sousa *et al*. 2016). Therefore, the addition of mass to these regions of the clathrin cages is the primary driving force of disassembly.

1.9.5 The role of Hsc70 and auxilin in active remodelling of the maturing clathrin coat

The transition from a planar lattice structure to ones of increased curvature requires the re-organisation of the clathrin coat to accommodate the structural switch from pentagons to hexagons in order to accommodate the new curvature (Jin and Nossal, 2000). Recent *in vivo* studies have confirmed that curvature of the CCP is continually remodelled to accommodate the increasing curvature of the membrane (Avinoam *et al*. 2015). It has been suggested that auxilin/GAK and Hsc70 may function to actively facilitate this maturation of the CCP.

This has been supported by the fact that disassembly of CCVs is slowed through the formation of clathrin/auxilin/Hsc70/AP180 complexes *in vitro* which are able to re-bind to a membrane (Jiang *et al*. 2000). Subsequent *in vivo* fluorescent work has shown that clathrin rapidly transitions between the cytosol and the plasma membrane which is dependent on Hsc70 (Wu *et al*. 2001; Yim *et al*. 2005). Further implications for the

role of Hsc70 in this process is that Hsc70 has been hypothesised to remain in complex with clathrin to form a pool of triskelia that can readily be incorporated into the growing pit (Schlossman *et al*. 1984; Black *et al*. 1991). Cell based studies with Hsc70 ATPase mutants (Newmyer and Schmid 2001) and RNAi of auxilin and GAK (Lee *et al*. 2005; Hirst *et al*. 2008) have been implicated in the formation of empty clathrin structures that appear to form in the cytosol in the absence of lipid. Finally, recent work by Holkar *et al.* (2015) has shown that clathrin and epsin assemble onto membranes faster in the presence of Hsc70 and auxilin (Holkar *et al*. 2015). In addition to the interactions with clathrin and adaptors auxilin has been shown to interact with dynamin and may play a role in helping to drive constriction of the CCP, possibly by recruiting Hsc70 (Newmyer *et al*. 2003; Sever *et al*. 2006). Taken together these observations point towards Hsc70 and auxilin playing a direct role in the regulation of assembly and re-modelling of clathrin, adaptors and the cytoskeleton in CME.

1.10.1 Other roles of endocytic proteins

In addition to roles in CME clathrin and adaptor proteins function in many other related cellular functions ranging from other components of the trafficking pathway to mitosis.

1.10.2 Clathrin in other trafficking pathways

As well as its role CME at the plasma membrane clathrin also plays a role in trafficking between the Trans-Golgi Network (TGN) and endosomes (Mogelsvang *et al*. 2004; Johannes and Popoff 2008; Daboussi *et al*. 2012). For example, clathrin is involved in interacting with the ESCRT0 protein to facilitate degradation of ubquitinated cargo in late endosomes (Henne *et al*. 2011; Shields and Piper 2011). Another specific role in TGN trafficking is fulfilled by the second clathrin isoform CHC22 named for its location on chromosome 22 (Brodsky 2012). CHC17 forms the well characterised functions of CME and as has been described so far in this introduction. CCHC22 is less well characterised but plays a more specific role in trafficking at the TGN (Esk *et al*. 2010). In particular, CHC22 is implicated in transport of the Glut4 receptor (Vassilopoulos *et al*. 2009) which plays an important role in skeletal muscle regeneration (Hoshino *et al*. 2013) and defects in this pathway are implicated in type 2 diabetes (Brodsky 2012). Another major difference between CHC17 and CHC22 is

that CHC22 does not bind to CLC (Liu *et al*. 2001) and does not appear to interact with CHC17, suggesting a fundamentally different role in the cell.

1.10.3 The role of endocytic proteins in mitosis

One of the most diverse roles for CME related proteins is the role they play in mitosis with many adaptors and clathrin forming integral interactions with the mitotic machinery (Royle 2013). Endocytosis is shut down during mitosis due to increased membrane tension (Kaur *et al*. 2014) and subsequent recruitment of endocytic proteins to other roles in mitosis. Clathrin interacts with the microtubule stabilising proteins TACC3 and ch-TOG which allows clathrin to cross link microtubule bundles in kinetochore fibres, thus stabilising this interaction with the centrosome during mitosis (Royle *et al*. 2005; Foraker *et al*. 2012; Royle 2013). KO of other proteins such as GAK also leads to duplication of the centrosome and subsequent arrest of the cell cycle during mitosis (Olszewski *et al*. 2014). Phosphorylation of adaptors such as epsin reduces their affinity for other adaptors (AP2) and is believed to direct endocytic proteins to roles in mitosis (Kariya *et al*. 2000; Smith and Chircop 2012) with the ENTH domain of epsin has been implicated in promote spindle assembly during mitosis (Liu and Zheng 2009).

1.11.1 Aims and thesis structure

The field of endocytosis has made great advances in our understanding of clathrin mediated endocytosis. Structural studies such as those revealing the interactions between clathrin and adaptors (Smith *et al*. 2004; Fotin *et al*. 2004b; Xing *et al*. 2010) and *in vivo* studies such as those by Taylor *et al.* (2011) which have revealed the temporal recruitment of proteins during endocytosis (Saffarian *et al*. 2009; Taylor *et al*. 2011). However, questions relating to the specific mechanisms of protein-protein interactions still remain to be answered although recent advances such as those relating to Hsc70 induced cage disassembly have been made (Sousa *et al*. 2016). This project therefore set out to address some of these unanswered interaction questions by using *in vitro* assays to study these systems:

- Can we measure quantitatively the effect of adaptor binding on cage size during polymerisation?

How adaptor proteins promote clathrin polymerisation and regulate the size of these structures has been largely deduced from indirect and qualitative measurements. By applying a novel analytical technique utilising dynamic light scattering to this system I aimed to build on previous observations of adaptor promotion of clathrin cage assembly to provide a greater insight into the mechanisms of adaptor promotion of cage assembly.

- Investigate how auxilin and Hsc70 induce clathrin cage disassembly by investigating how functional mutants of these proteins alter disassembly kinetics.

  Hsc70 and auxilin facilitate the disassembly of clathrin cages *in vivo* but questions remain as to the precise mechanisms behind this process. By building on previous work using Hsc70 and auxilin mutants I aimed to monitor the effects of these mutants on the disassembly of clathrin cages in real-time using a perpendicular light scattering assay and gain a greater insight into these mutants and their effects on disassembly.

- Do adaptor interactions with clathrin cages that promote the formation of these structures alter the ability of Hsc70 and auxilin to disassemble clathrin cage structures?

  The effect of adaptor proteins on disassembly has not been investigated in detail. By disassembling adaptor-clathrin cage complexes I aimed to determine if these proteins have any effect on the ability of auxilin and Hsc70 to disassemble clathrin cages in order to gain a greater understanding of clathrin-adaptor interactions and gain a greater insight into potential interactions between adaptors and auxilin-Hsc70.

In order to answer these questions, I detail the procedures used to obtain purified protein from animal material and through recombinant expression as well as the analytical techniques used. These methods are detailed in Chapter 2 and in Chapter 3 I detail the purification and quantification of the proteins used. Chapters 4,5 and 6 relate to the 3 aims detailed above and address adaptor assembly, Hsc70 and auxilin disassembly and adaptor effects on disassembly respectively. Subsequent questions that arose from observations of the effect of epsin on assembly and disassembly are

addressed in Chapters 7 and 8. Finally in Chapter 9 I summarise and contextualise the results obtained.

Chapter 2: Materials and Methods

'...I have always found that plans are useless but planning is indispensable' Dwight D. Eisenhower

2.1.1 Overview

This chapter provides and overview of the material and methods used during my thesis. Variations to the protocols described here will be found in the relevant chapter.

2.2.1 Materials and Reagents

Abcam (UK): Prism Ultra Protein Ladder (10-245 kDa), Rat monoclonal anti-Hsc70 antibody (Ab 19136), Rabbit polyclonal anti-Rat IgG antibody (Ab 102172)

Affymetrix (USA): 2-(N-morpholino)ethanesulfonic acid monohydrate (MES)

Agar Scientific (UK): Formvar/Carbon 300 mesh copper grids

AGTC Bioproducts Ltd. (UK): Agarose

Alfa Aesar (USA): Manganese chloride

Alta Bioscience (UK): Synthetic peptides (see Chapter 8)

AMRESCO (USA): Hepes free acid

Applichem Lifesciences (USA): Dithiothreitol (DTT), sodium dodecyl sulphate (SDS)

Bio-Rad (USA): Hydroxyapatite Bio-Gel HG Ge

Expedeon (UK): Instant Blue

First Link Ltd. (UK): Porcine brain

Fisher Scientific (UK): Ammonium sulphate, glycerol, magnesium acetate tetrahydrate, nitric acid, potassium acetate, potassium chloride, potassium phosphate dihydrate, potassium phosphate monohydrate, sodium chloride, sodium hydroxide

GE Healthcare (USA): ECL Western Blotting Detection Reagents, Ficoll™ PM70, Glutathione sepharose 4B, GSTrap™FF column, HiLoad 26/600 Superdex 200 pg column, HiPrep 26/60 Sephacryl S-500 HR, HWM-SDS Marker Kit, Hybond TM-P PVDF membrane, LWM-SDS Marker Kit, PreScission Protease, TALON ® Superflow™ resin, XK 16/100 Superdex 75 column

Fuji (Japan): SuperRX film

Life Technologies (USA): NuPAGE Transfer Buffer (x20)

Lonza (Switzerland): Insect Xpress Protein Free Media

Macherey-Nagel (Germany): NucleoBond BAC 100 purification kit

Melford Laboratories Ltd. (UK): Isopropyl β-D-1-thiogalactopyranoside (IPTG)

Medicell Membranes Ltd. (UK): visking dialysis tubing 19 mm 12-14 kDa cut-off

National Diagnostics (USA): 30% w/v Accugel acrylamide (acrylamide 29:1 bis-acrylamide)

New England Bioscience (USA): 10x NEB buffer, Bsu36I, Gel loading dye (6x)

PanReac AppliChem (USA): 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)

Premier International Foods (UK): Marvel Milk Powder

Promega (USA): FugeneHD

Qiagen (Germany): Nucleospin Gel and PCR Clean-up Kit, QIAprep Spin Miniprep Kit

Roche Diagnostics Ltd. (Switzerland): c0mplete HisTrap (5 mL), c0mplete Protease Inhibitor Tablets

Sartorius Stedim (Germany): Vivaspin 20 centrifugal concentrators

Sigma-Aldrich (UK): Ammonium per sulphate (APS), ampicilin, ATP-agarose cyanogen bromide activated, ATP monosodium, beta-2-mercaptoethanol (βME), bovine serum albumin (BSA), chloramphenicol, dimethyl sulphoxide (DMSO), ethidium bromide, ethylene-diamine-tetraacetic acid (EDTA), ethylene-glycol-bis(β aminoethyl ether)-tetraacetic acid (EGTA), glutathione (GSH), glycine, hydrochloric acid, imidazole, kanamycin, magnesium chloride hexahydrate, rubidium chloride,

sucrose, triethylamine (TEA), tetra-methyl-ethylene-diamine (TEMED), thrombin, Tris-HCl

SPI-Supplies (USA): Uranyl acetate

Thermo Scientific: Pierce BCA Protein Assay Reagents, Slide-A-Lyzer MINI Dialysis Units 7000 MWCO

VWR Chemicals (UK): 3-*N*-morpholinopropanesulfonic acid (MOPS), bromophenol blue, ethanol, methanol

Whatmann (UK): 3 mm filter paper 0.2 μm, blotting paper

Kind Gift: Activated Charcoal

2.1.2 Buffer and gel components

1M Tris pH 7.2

50 mM Tris base, 950 mM Tris HCl, 1 mM EDTA, 1 mM DTT

6.25% Ficoll / 6.25% sucrose

0.0625 g/ml Ficoll PM70, 0.0625 g/ml sucrose, 25 mM Hepes, 125 mM Potassium acetate, 5 mM Magnesium acetate

3% SDS PAGE stacking mix

150 mM Tris, 4% acrylamide - 29% acylamide, 1%bis-acrylamide, 0.12% SDS

12% SDS PAGE resolving mix

250 mM Tris HCl pH 8.8, 12% acylamide - 29% acylamide, 1%bis-acrylamide, 0.12% SDS

Buffer A pH 7.2

20 mM Hepes, 200 mM NaCl

Buffer C pH 7.0

20 mM Hepes, 25 mM KCl, 3 mM MgCl2

Depolymerisation buffer (DEPOL) pH 8.0

20 mM TEA, 1 mM EDTA, 1 mM DTT or 0.1% βME

GSH elution buffer pH 7.0

20 mM Hepes, 200 mM NaCl, 10 mM GSH

HKM pH 7.2

25 mM Hepes, 125 mM Potassium acetate, 5 mM Magnesium acetate

Hsc70 Dialysis buffer pH 7.0

20 mM Hepes, 25 mM KCl, 10 mM EDTA

Imidazole elution buffer pH 7.9

25 mM Tris, 500 mM NaCl, 200 mM imidazole

Loading dye (x2) pH 6.8

250 mM Tris-HCl, 5 % w/v SDS, 25 % w/v glycerol, 50 mM DTT, 4 % w/v β mercaptoethanol, 0.05 % w/v Bromophenol blue

McKay buffer pH 7.0

40mM Hepes, 75mM KCl, 4.5mM Magnesium acetate

Peptide buffer pH 7.8

200 mM ammonium sulphate, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4

Phosphate buffered saline pH 7.4

137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4

Polymerisation buffer (POL) pH 6.2-6.5

100 mM MES, 1.5 mM $MgCl_2$, 200 μM EGTA

Saturated ammonium sulphate buffer pH 7.0

770 g/L ammonium sulphate, 10 mM Tris, 100 nM EDTA

TFB1 pH 5.8

30 mM potassium acetate, 10 mM calcium chloride, 50 mM manganese (II) chloride, 100 mM rubidium chloride, 15 % w/v glycerol

TFB2

10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15 % w/v glycerol

Tris/Glycine buffer pH 8.3

25 mM Tris, 185 mM glycine, 3.5 mM SDS

Tris/Salt buffer pH 7.9

25 mM Tris, 500 mM NaCl

2.1.3 Expression constructs

Construct	**Properties**	**Reference**	**Source**
BAC10:KO_{1629}	BAC10 with knock out of ORF1629	Zhao *et al*. 2003	Ian Jones (University of Reading)
pET32c-epsin	pET32c + thioredoxin-His_6-epsin 1_{1-575}	Kalthoff *et al*. 2002	Ernst Ungewickell (Hannover Medical School)
pQE32-ΔENTH-epsin1	pQE32 + His_6-$epsin1_{144-575}$	Kalthoff *et al*. 2002	Ernst Ungewickell (Hannover Medical School)
pETDuet-1-Hip1cc	pETDuet-1 + His_6- $Hip1_{361-637}$	Wilbur *et al*. 2008	Frances Brodsky (UCL)
pETDuet-1-mHip1Rcc	pETDuet-1 + His_6- $mHip1_{346-655}$	Wilbur *et al*. 2008	Frances Brodsky (UCL)
pGEX4T2-aux401-910	pGEX4T2 GST-$auxilin_{401-910}$	Smith *et al*. 2004	Helen Kent (MRC LMB)

pGEX4T2-β2-616-937	pGEX4T2 + GST- β2 adaptin$_{616-937}$	Owen *et al*. 2000	David Owen (CIMR)
pGEX4T2-α695-893	pGEX4T2 + GST- α adaptin$_{695-893}$	Owen *et al*. 1999	David Owen (CIMR)
pGEX4T2-CHC-1-361	pGEX4T2-GST-CHC$_{1-361}$	NA	David Owen (CIMR)
pOPINE-Hsc70	pOPINEΔHis$_6$-Hsc70	Rothnie *et al*. 2011	Jo Nettleship (OPPF)
pQE31-ap180	pQE31 + His$_6$-AP180$_{1-907}$	Kalthoff *et al*. 2002	Ernst Ungewickell (Hannover Medical School)
pVL1393-hsc70	pVL1393 + Hsc70	Höfeld *et al*. 1997	Jörg Höfeld (Friedrich-Wilhelms-University Bonn)
pVL1393-hsc70-baculovirus	pVL1393-hsc70 in recombinant baculovirus	Rothnie *et al*. 2011	Yvonne Vallis and Harvey McMahon (MRC LMB)

Table 2.01: Details of DNA constructs used for the expression of recombinant proteins in E.coli and insect cells *including the original reference where they were first used and the source of the construct. Mutations generated from these original constructs for use in this project are addressed in the relevant chapters in section 2.1.4.*

2.1.4 Generation of auxilin, Hsc70 and epsin mutants

Design of the mutations to auxilin clathrin binding and AP2 binding motifs was conducted with reference to Scheele *et al*. 2003. The following mutants were designed and generated from base pGEX4T2-aux$_{401\text{-}910}$ construct with numbers indicating the first residue of each motif in auxilin:

^{579} DPF->APA + ^{674} DPF->APA (hereafter referred to as auxilin APAx2)

^{781} DLL->ALA + ^{591} DLL->ALA (hereafter referred to as auxilin ALAx2)

^{781} DLL->DAA + ^{591} DLL->DAA (hereafter referred to as auxilin DAAx2)

Generation of epsin clathrin box binding motifs was conducted in line with reference to Drake *et. al*. 2000. The following mutants were generated from the base pET32c-epsin construct with numbers indicating the first residue of each motif in epsin:

^{257} LMDLA -> AAAAA (hereafter referred to epsin Δ257)

^{480} LVDLD -> AAAAA (hereafter referred to as epsin Δ480)

^{257} LMDLA -> AAAAA and ^{480} LVDLD -> AAAAA (hereafter referred to epsin DKO)

Mutagenesis of all these constructs was conducted by GenScript (NJ, USA).

2.2.1 Cell lines

The cell lines used for molecular biology and protein expression work are detailed here.

2.2.2 *E.coli* cell lines

Strain	**Properties**
BL21(DE3)	huA2 [lon], ompT gal, (λ DE3) [dcm], ΔhsdS λ DE3 = λ sBamHIo, ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1), i21 Δnin5
DH5α	supE44, ΔlacU169 (80lacZΔM15), hsdR17, recA1, endA1, gyrA996, thi-1, relA1

Table 2.02: E.coli strains used in this work with the genetic properties of each strain detailed.

2.2.3 Generation of chemically competent *E.coli cells*

Chemically competent DH5α and BL21 (DE3) cells were generated from a single colony streaked on an LB-agar plate incubated overnight at 37°C. The single colony was used to inoculate 5 mL of LB (10 g/L NaCl, 10 g/L bactotryptone and 5 g/L yeast extract produced in house) which was in turn grown overnight at 37°C. The 5 mL overnight culture was used to inoculate 250 mL of LB supplemented with 20 mM $MgSO_4$ and grown whilst shaking at 180 rpm at 37°C until OD_{600} reached 0.4-0.6. Cells were pelleted at 4500 x g for 5 minutes at 4°C. The cell pellet was re-suspended in 100 mL of TFB1 buffer chilled on ice with a further incubation of the cell suspension on ice for 5 minutes. Cells were then pelleted again as described previously. The cell pellet was re-suspended in 10 mL of TFB2 buffer before incubation on ice for 60 minutes. Cells were dispensed into 100 µL aliquots and snap frozen in dry ice/ liquid nitrogen. Aliquots were stored at -80°C.

2.2.4 Transformation of competent *E.coli* cells

100 μL of competent *E.coli* cells were defrosted on ice before the addition of 100 ng of plasmid DNA with subsequent incubation on ice for 20 minutes. Cells underwent heat shock in a 42°C water bath for 30 seconds before incubation on ice for a further 5 minutes. The 100 μL of cells were added to 400 μL of LB and incubated at 37°C for 30 minutes. 100 μL and 25 μL of cell suspension were streaked onto LB-agar plates inoculated with an antibiotic appropriate to the strain and plasmid. Plates were incubated overnight.

2.2.5 Purification of plasmid DNA from *E.coli*

Purification of plasmid DNA from *E.coli* was conducted from 5 mL of overnight culture using the QIAprep Spin Miniprep Kit (Qiagen) as per the manufacturers instructions.

2.2.6 Insect cell line

The insect cell line used for amplification of Hsc70 baculovirus and expression of Hsc70 was the *Spodoptera frugiperda* 9 or Sf9 cell line. The cells were a kind gift from the Napier group at the University of Warwick.

2.2.7 Sf9 standard culture

A static culture of Sf9 cells was passaged every 3-4 days at a starting concentration of $4x10^5$ cells/mL in Insect Xpress media at a temperature of 28°C. Insect cells were maintained in static culture in Insect Xpress Media at 28°C up to passage 40. Stocks of cells were frozen at at a low passage number (<10) at $3x10^6$ cells/mL in Insect Xpress media with 10% DMSO at -80°C. Cells were thawed from storage by defrosting at room temperature before dilution to approximately $5x10^5$ cells/mL and allowed to settle for 1 hour at 28°C. Media was then exchanged to remove remaining DMSO.

2.2.8 Amplification and purification of bacmid DNA

E.coli cells containing the $BAC10KO_{1629}$ plasmid were plated from glycerol stocks on LB agar impregnated with 0.35 mg/mL of chloramphenicol. Single colonies were picked and used to inoculate a 10 mL of LB 0.35 mg/mL-chloramphenicol and grown for 6 hours at 37°C. The culture was then used to inoculate 400 mL of LB 0.35 mg/mL-

chloramphenicol and grown overnight at 37°C. DNA was extracted using the NucleoBond BAC100 kit as per the manufacturers instructions. DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (DE USA). Bacmid DNA was digested using the Bsu36I enzyme in NEB buffer for 2 hours at 37°C. Heat inactivation was conducted by incubation at 72°C for 20 minutes. Digestion was confirmed by resolving using agarose gel electrophoresis. 1 μg of digested DNA was diluted in 6x loading buffer (NEB) and water before running on a 0.8% agarose gel in TAE buffer at 110V/100 mA for 1 hour. The gel was stained with ethidium bromide (0.5 μg/mL) and imaged using a Syngene G:Box. Cut bacmid was stored at -20°C.

2.2.9 Transfection of Sf9 cells with pOPINE-Hsc70 & BAC10KO$_{1629}$

Sf9 cells were seeded on a 6 well cell culture plate at $5x10^5$ cells/mL in 2 mL and allowed to sediment for 1 hour at 28°C. A transfection mix was created consisting of 4 ng/μL linearized bacmid, 4 ng/μL pOPINE-hsc70 and 0.02% FugeneHD in Insect Xpress media and incubated at room temperature for 30 minutes at room temperature. 200 μL of transfection mix was added to each well and gently mixed by swirling. Cells were incubated for 6 days at 28°C. Supernatant containing recombinant virus was removed and stored at 4°C.

2.3.1 Protein expression and purification

2.3.2 SDS poly acrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE was used to resolve proteins during protein expression and purification and during pull-down experiments. Gels were cast using a 12 % resolving gel and a 4 % stacking gel for use in a Mini-PROTEAN® system (Bio-Rad). Samples were diluted 1:1 with gel loading dye (x2) and incubated at 37°C for 10 minutes before loading onto the gel. Gels were run at 160 V (~50 mA) for 1 hour or until the dye front reached the bottom of the gel. Gels were stained using Instant Blue for a minimum of 30 minutes prior to washing of excess stain in water and imaged.

2.3.3 Clathrin purification

Clathrin was purified as described in Rothnie *et al*. 2011. All dialysis, incubation and centrifugation steps were conducted on ice or 4 °C. 8 pig brains (approximately 600

g) snap frozen in liquid N_2 were homogenised in HKM buffer supplemented with protease inhibitor. Centrifugation at 12,000 x g for 30 minutes was conducted to clarify the homogenate, before isolation of coated vesicles from the supernatant by centrifugation at 140,000 x g for 45 minutes. Pellets were homogenised before centrifugation in the presence of 6.25% Ficoll 6.25% Sucrose buffer at 45,000 x g for 20 minutes. The supernatant containing the CCVs was removed and diluted before centrifugation at 140,000 x g for 1 hour. The supernatant was removed and pellets stored over night at 4°C. Pellets were re-suspended in HKM buffer before homogenisation. Centrifugation at 13,000 rpm in a microfuge was conducted to remove cytoskeletal contaminants. Lipids were stripped from the protein content by adding an equal volume of x 2 concentration of 1 M Tris Buffer and incubating at 4°C for 1 hour. The majority of lipids were then isolated by centrifugation at 135,000 x g for 30 minutes. The supernatant was loaded onto a sephacyl 500 HR column (GE) connected to an ÄKTA FPLC system (GE Healthcare). The column separates clathrin from the remaining lipids and the majority of adaptor proteins. Fractions collected from the ÄKTA system were assayed for purity by SDS-PAGE and fractions containing the purest clathrin were pooled. An equal volume of saturated ammonium sulphate buffer was added to precipitate out the protein content before storage over night. The precipitated protein was isolated by centrifugation at 48,000 x g and re-suspended in a low volume of 1 M Tris buffer before dialysis into depolymerisation buffer for a minimum of 2 hours. Clathrin that was unable to depolymerise was removed by centrifugation at 130,000 x g. The supernatant was the loaded onto a Superdex 200 HR column with isolated fractions pooled and again precipitated with ammonium sulphate before over night storage. Precipitated protein was then re-suspended as described previously and dialysed into depolymerisation buffer for a minimum of 2 hours. The dialysis buffer was then switched to polymerisation buffer (pH 6.4) and left to dialyse over night with at least one buffer change. Polymerised clathrin was isolated after centrifugation at 140,000 x g and re-suspension in polymerisation buffer.

2.3.4 Recombinant Hsc70 expression and purification

Hsc70 was expressed using the baculovirus expression system in Sf9 insect cells essentially as described in Rothnie *et al*. 2011 using either pVL1393-hsc70 virus

stocks or BAC10KO$_{1629}$-pOPINE-hsc70 recombinant virus. Viral titre and expression of Hsc70 was confirmed using SDS-PAGE and Western blotting. The recombinant baculovirus was amplified at a multiplicity of infection (MOI) of 0.2 for 6 days. Expression of Hsc70 was conducted in Sf9 cells in suspension seeded at $1x10^6$ cells/mL in 300 mL with a MOI of 3 for 4 days. Cells were pelleted at 2000 x g for 5 minutes and stored at -20°C. Purification of Hsc70 was conducted by re-suspension of an Sf9 cell pellet in buffer C in the presence of protease inhibitor. The cells were lysed by sonication before removal of cell debris by centrifugation at 45,000 x g at 4 °C for 20 minutes. The supernatant was then loaded onto a hydroxyapatite column and washed with buffer C + 20 mM K_2PO_4 to remove non-specific interactions. Elution of bound proteins was achieved through the use of buffer C + 200 mM K_2PO_4. Eluted fractions were assayed by SDS-PAGE and the fractions containing the purest Hsc70 were pooled and EDTA added to a final concentration of 10 mM EDTA. The pooled protein was then dialysed against Hsc70 dialysis buffer with activated charcoal at 4 °C over night. Post-dialysis, 4 mM $MgCl_2$ was added back to pooled Hsc70, which was then loaded onto an ATP-agarose column. Non-specific binding interactions were removed by washing with Buffer C + 1M KCl. Elution of ATP-binding proteins was conducted using buffer C + 3 mM ATP. Pooled eluted protein was concentrated by centrifugation and loaded onto a Superdex 75 16/60 size exclusion column to remove final protein contaminants and excess ATP. Fractions containing the most pure Hsc70 were determined by SDS-PAGE before pooling, concentration and storage at -80 °C.

2.3.5 Western blotting

To determine the level of expression of Hsc70 and to optimise viral titre for said expression cell samples were prepared for gel electrophoresis as described in section 2.3.2 with an added sonication step to lyse cells and break down genomic DNA. After resolving by SDS-PAGE as described protein was transferred to a methanol activated PVDF membrane using a Mini Trans-Blot® system (Bio-Rad). Transfer was conducted in NuPAGE buffer for a period of 1 hour at 200 mA.

After transfer the membrane was blocked using a 10 % w/v milk powder PBS-tween solution overnight at 4°C. The membrane was then rinsed with 3 successive 10 minute PBS-tween washes before incubation with rat anit-Hsc70 monoclonal antibody in 5 % w/v milk powder PBS-tween at a dilution of 1:10,000 for a period of 1 hour. The

membrane was again washed with PBS-tween before incubation with the rabbit anti-rat IgG HRP conjugate secondary antibody at a dilution of 1:10,000 in 5% w/v milk powder PBS-tween for 1 hour. After a final round of washing the membrane was incubated with ECL reagents as per manufacturers instructions. X-ray films were developed and imaged.

2.3.6 GST-tagged protein expression and purification

All GST-fusion proteins used in this study were purified as follows with GST-auxilin$_{401-910}$ used as the example. GST-auxilin$_{401-910}$ was expressed in BL21 (DE3) *E.coli* and purified through glutathione (GSH) affinity chromatography as described in Rothnie *et al*. 2011. Chemically competent BL21 (DE3) *E.*coli cells were transformed with pGEX4T2-aux$_{401-910}$ plasmid by heat shock and streaked onto an LB-ampicillin (0.1 mg/ml) plate before incubation overnight at 37 °C. Single colonies were picked into 5 mL of LB-ampicillin and grown overnight at 37 °C . 5 mL of overnight culture was added to 400 mL of LB-ampicillin and grown at 37 °C until OD_{600} reached 0.6-0.8. IPTG was then added to the culture at a final concentration to 0.5 mM and grown for overnight at 25 °C. Cells were isolated from culture by centrifugation at 9000 x g for 10 minutes and storage at - 20 °C. Purification from cell pellets was conducted by re-suspension in buffer A with protease inhibitors and lysis by sonication. Cell debris was removed by centrifugation at 45,000 x g for 20 minutes and the supernatant loaded onto a GSTrap FF column. GST-Aux$_{401-910}$ was eluted with GSH elution buffer. Excess GSH was removed by dialysis against buffer A. In the case of un-cleaved auxilin the protein was concentrated by centrifugation and stored at – 80 °C. In the case of cleaved auxilin the GST-tag was removed using digestion with thrombin over night at 4 °C at a ratio of 1 Unit/10 µg protein and the cleaved protein and tag were again separated by GST affinity chromatography.

GST-β2 adaptin$_{616-951}$ was expressed and purified as per GST-auxilin$_{401-910}$ with the use of the PreScission enzyme to cleave the GST tag. GST-α adaptin$_{695-983}$ was expressed in DH5α cells and purified essentially as for GST-β2 adaptin$_{616-951}$. GST-α adaptin$_{695-983}$ was further purified from degraded protein contaminants using superdex 75 size exclusion chromatography.

2.3.7 Poly-histidine tagged protein expression and purification

The His_6 tagged fusion proteins $epsin1_{1\text{-}575}$, $epsin1_{144\text{-}575}$, AP180 and Hip1/1R CC proteins were expressed in BL21 DE3 *E.coli* as previously described. Cell pellets were re-suspended in buffer with a proteinase inhibitor tablet. The proteins were then purified after centrifugation of cell lysate at 50,000 x g to remove lipid contaminants. Initially proteins were purified from lysed supernatant after incubation with TALON Co^{2+} affinity purification for a minimum of 2 hours at 4 °C and purified by gravity flow. The protocol was then subsequently changed for purification with a c0mplete His-trap column (Roche) for use with an ÄKTA FPLC system. In either case the proteins were allowed to bind in 50 mM Tris 500 mM NaCl buffer. Washing of the medium was conducted using 20 mM imidazole and elution of the His-tagged protein was conducted using 200 mM imidazole. Fractions containing pure protein were pooled and concentrated using a centrifugal concentration before storage at -80°C.

2.3.8 Protein quantification

Protein quantification was conducted using a NanoDrop ND-1000 Spectrophotometer (DE USA). A280 readings used to quantify proteins based on their extinction coefficient as calculated from their amino acid sequence using the online tool ProtParam (ExPASy). Clathrin concentration was assayed by titration in 1M Tris-HCl buffer to disassemble cages into triskelia before assaying absorbance at 280 nm using a Cary 100 UV-Vis Spectrophotometer. Hip1/1R CC concentrations were assayed using the Pierce BCA Protein Assay (Thermo Scientific) as per the manufacturers instructions with BSA used to generate a standard curve.

2.4.1 Analytical Techniques

2.4.2 Circular Dichorism

Conformation of Hip1/1R CC α-helical structure was conducted using circular dichroism. Both proteins were dialysed into 20 mM NaH_2PO_4 buffer. Hip1 CC and Hip1R CC were added to a 1 mm cuvette at a concentration of 0.035 mg/mL and 0.038 mg/mL respectively over a spectrum of 350 nm to 180 nm with 6 replicates. Data where the HT[V]>600 was discarded. The CD signal was converted to mean residue

elipticity using the peptide sequence of both proteins with the following equations: The concentration of protein (C) in molar is multiplied by the number of amino acids (N) in the protein to provide the mean residue concentration (C_{MR}):

$$C_{MR} = C \times N$$

$$\Delta\varepsilon_{MR} = \Delta A/(C_{MR} \times l$$

Where ΔA is the absorbance change as detected and I is the path length.

2.4.3 Clathrin cage formation in the presence of adaptor proteins

Cage competent clathrin was disassembled at concentrations between 15 and 4 μM though dilution in 1 M Tris buffer and dialysis into depolymerisation buffer for a minimum of 4 hours with a buffer change. Depolymerisation was confirmed through dynamic light scattering (DLS) (see section 2.3.4). Polymerisation was then induced via dialysis back into polymerisation buffer (pH 6.4) in the presence or absence of adaptor proteins at various concentrations of clathrin and adaptors for a minimum period of 12 hours in a volume of 100 μL. All dialysis steps were conducted with at least on buffer change with a minimum sample to dialysis buffer volume ratio of 1:200. Polymerisation was once again confirmed by DLS and ultracentrifugation/SDS-PAGE.

2.4.4 Dynamic Light Scattering

Dynamic Light Scattering (DLS) was conducted using a Zetasizer Nano S (Malvern) to determine clathrin particle sizing during polymerisation and depolymerisation in the presence or absence of adaptor proteins. Measurements were taken at 10 °C with data collected over 30 seconds with 30 replicates made for each sample. Size distribution data was analysed using a modified fitting algorithm using a custom script in the software package R courtesy of Joseph Jones (Jones 2016).

2.4.5 Electron microscopy and particle measurement

Copper Formvar/carbon grids were prepared for sample loading by glow discharging using an EMtech K100x unit at 10 mA for 30 seconds. Clathrin-adaptor samples were added to the surface of the grid at a concentration of 1 μM of clathrin cages unless otherwise stated in a volume of 5 μL. After incubation for 1 minute grids were blotted

using filter paper before the addition of 5 μL of 2 % uranyl acetate, which was again incubated and blotted as previously. Grids were imaged variously using JOEL 2011, JEOL 2010F and a JEOL 2200 FS transmission electron microscopes. The 2011 and 2010F were equipped with a Gatan Ultrascan 4000 CCD camera and the JEOL 2200 FS with a Gatan 2k x 2k Ultrascan CCD camera. Images of cages were taken at x 25,000 magnification unless otherwise stated.

Clathrin cage particles diameters were measured in ImageJ software (Version 1.49u (100)) using the measure tool as calibrated to the scale bar appropriate to the image. For a given polymerisation condition a minimum of 150 particles were measured and the diameters values halved to obtain a radius. Particle radii were binned in class sizes of 5 nm between 0 and 100 nm to produce a histogram of the size distribution.

2.4.6 Perpendicular light scattering assay

Perpendicular light scattering was conducted using a LSB50 fluorimeter (Perkin Elmer) as described previously (Rothnie *et al*. 2011) using an excitation wavelength of 390 nm and a detection wavelength of 395 nm. Slit width for excitation and emission was set at 3 nm. Clathrin/clathrin-adaptor cage complexes were diluted to a final concentration of 250 nM in McKay buffer. GST-auxilin$_{401\text{-}910}$ or auxilin$_{401\text{-}910}$ was added at varying concentrations (10 nM to 100 nM) with an excess of ATP (500 μM). Disassembly was then initiated though the addition of Hsc70 (100 nM to 1 μM) and data collected over a period of 500 seconds with data collected every 0.25 s. A minimum of 3 replications were made for each experiment and intensity normalised to initial scattering signal of the cage samples added. Two parameters were assessed to determine changes to disassembly: the end scatter intensity and the half life ($t_{1/2}$). Average end scatter intensity was calculated by averaging the intensity over the final 30 seconds of disassembly. The half life ($t_{1/2}$) of the signal decay was calculated by using the end scatter intensity to determine the half-intensity signal. The time point at which this value was reached is given as the $t_{1/2}$.

2.4.7 GST-Pull downs

GST-auxilin$_{401\text{-}910}$ fusion protein or GST-TD was immobilized at 4 °C with packed GSH-Sepharose 4B in a spin column and incubated with epsin at different concentrations for 1 hour in HKM buffer. The GSH-sepharose beads were centrifuged

at 13000 rpm for 30 seconds at 4 °C before washing 3 times with HKM + 500mM NaCl 1mM DTT and 1% Triton X-100 and 3 times with HKM. Finally, SDS-PAGE loading buffer was added to the GSH-sepharose beads for 10 minutes at 37°C before a final spin to elute bound protein. Samples for SDS-PAGE analysis were taken at various stages to determine protein interactions.

2.5.8 General software

Disassembly data was prepared from LSB50 proprietary software and analysed using Microsoft Excel for Mac (2016) and graphs were generated using GraphPad Prism 7.0a. Particle size measurements from EM data was conducted using Image J (version 1.49u (100)). Searching of motifs in adaptor proteins was conducted using Jalview (version 2.9.0b2). Manipulation of protein structural data and images was conducted using USCF Chimera (version 1.11). Word processing was conducted using Microsoft Word for Mac (2016). Figures were prepared using Microsoft PowerPoint for Mac (2016).

Chapter 3: Protein Expression and Purification

'Ph.D. is a misunderstood acronym in protein biochemistry. It actually stands for "Protein has Degraded" Anonymous

3.1.1 Introduction

This chapter details the purification of recombinant adaptor proteins expressed in *E.coli* and Sf9 insect cells and the purification of clathrin from porcine brain using the methods as described in Chapter 2. Data includes assessment of purification from chromatographic data and SDS-PAGE. Further biophysical characterisation of purified Hip1/1R CC proteins by circular dichroism (CD) are also detailed here. The functional and binding activity of these proteins is addressed in later chapters.

3.2.1 Clathrin purification

3.2.2 Isolation of clathrin coated vesicles (CCVs)

Initially pig brain was homogenised by blending in the presence of HKM buffer and the homogenate clarified by centrifugation. The supernatant containing soluble lipid and protein was then centrifuged at 140,000 x g to pellet out the lipid faction containing CCVs. Uncoated lipids and CCVs were separated via a Ficoll-sucrose centrifugation step that removed the majority of the larger lipid contaminants leaving the smaller CCVs and other lipid components in solution. After the removal of the Ficoll-sucrose solution by dilution and centrifugation, the homogenised pellets were centrifuged at low speed to remove cytoskeletal contaminants and other protein aggregates, leaving the CCVs in solution. The protein coat on the CCVs was separated from the lipid surface through the addition of a 1 M Tris buffer. This buffer has a high ionic strength which disrupts the interactions of the lipid with the adaptor proteins as well as the interactions between triskelia in the coat complex, effectively depolymerising the structure. The majority of lipid material was removed by centrifugation at 135,000 x g, leaving any depolymerised clathrin and adaptor proteins in solution.

Protein was separated from the lipid content through the use of high ionic strength buffer prior to further separation of lipids, clathrin and adaptors by size exclusion chromatography. 'Cage competent' clathrin was selected through dialysis into depolymerisation and polymerisation buffers with insoluble aggregates or un-polymerised triskelia removed by centrifugation at the appropriate steps.

3.2.3 Clathrin purification by size exclusion chromatography and buffer dialysis

The first chromatographic separation step using Sephacryl 500 HR media (300 mL volume) is presented in Figure 3.01. This step separates the remaining lipids from the protein content, appearing at an elution volume between ~ 140 and 225 mL (Figure 3.01 A). The following peak from ~230 to 320 mL is the clathrin peak, consisting of depolymerized clathrin triskelia along with remaining adaptor proteins which form an 'adaptor tail' which merges with the clathrin peak, eventually dropping in absorbance. Because of the incomplete resolution of clathrin and adaptors, fractions from the clathrin peak were collected and assayed for purity of clathrin by SDS-PAGE (Figure 3.01 B). Fractions were picked and pooled for ammonium sulphate precipitation based on the abundance of clathrin relative to adaptor proteins. Note that this step does not remove all contaminant proteins.

After removal of saturated ammonium sulphate and re-constitution of precipitated protein in 1 M Tris buffer and dialysis into depolymerisation buffer, the protein solution underwent centrifugation at 130,000 x g. As described earlier the 1 M Tris buffer's ionic strength disrupts triskelia-triskelia interactions and dialysis into the high pH depolymerisation buffer is sufficient to prevent the formation of larger clathrin complexes. Centrifugation at 135,000 x g therefore removes large polymerised or aggregated protein from solution, leaving triskelia in the supernatant.

The supernatant from this step was then further purified by a further size exclusion step using Superdex 200. This step removes low molecular weight contaminants with clathrin eluting as a single broad peak (Figure 3.01 C). Analysis of fractions by SDS-PAGE confirms the presence of heavy chain and light chain in the peak with the absence of large amounts of contaminating protein.

After a repeat of the ammonium sulphate precipitation step the purified clathrin was assessed for 'cage competency' through dialysis from depolymerisation buffer to

polymerisation buffer. Polymerised clathrin was pelleted via centrifugation at 135,000 x g and the pell*et al*lowed to re-suspend in a smaller volume of polymerisation buffer. This step serves to concentrate clathrin which increases its stability and makes it more conducive to long term storage.

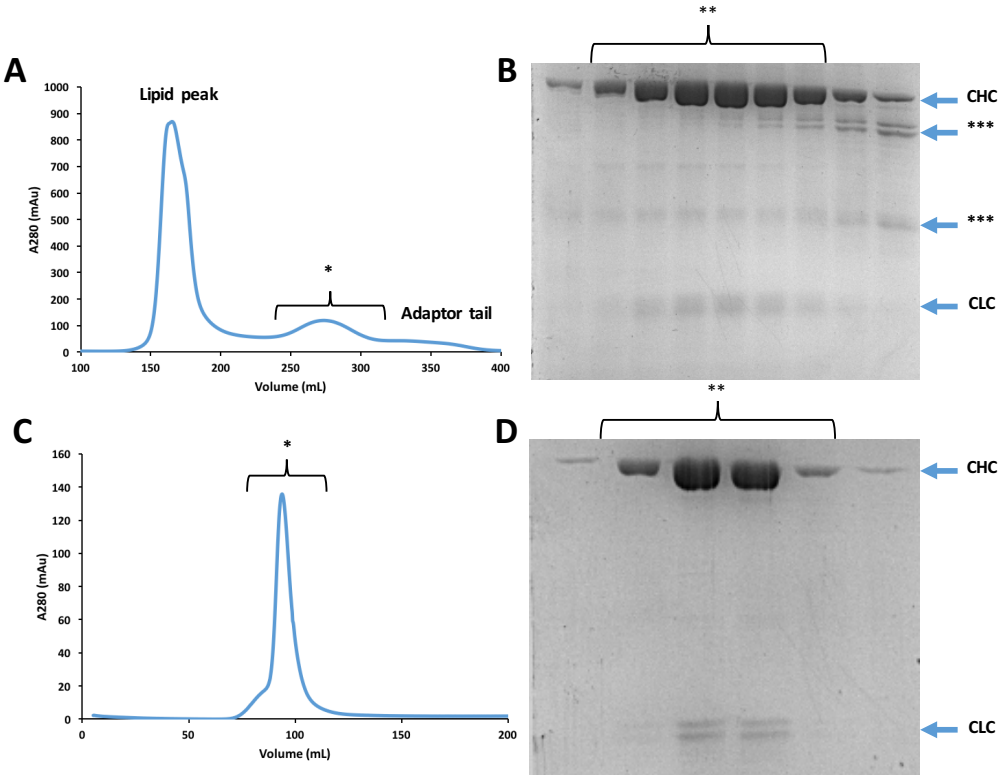

Figure 3.01: Purification of clathrin by size exclusion chromatography and assessment of purity as determined by A280 absorbance and SDS-PAGE analysis. A) *Separation of lipid, clathrin and adaptor proteins using a sephacryl 500 HR column was monitored by A280 absorbance with elution volume. The peak at ~140-230 mL corresponds to lipids eluting from the column as indicated. The peak between ~240 to 320 mL contain clathrin and adaptor proteins and factions (*) were selected for SDS-PAGE analysis.* ***B)*** *SDS-PAGE allowed visual selection of fractions containing the purest clathrin (**) with other fractions being discarded. Bands corresponding to CHC and CLC are indicated along with bands corresponding to contaminating adaptors (***).* ***C)*** *A280 absorbance plotted against elution volume from a Superdex 200 pg column with fractions containing clathrin selected for SDS-PAGE analysis (*).* ***D)*** *Fractions containing the purest clathrin were selected after visual conformation by SDS-PAGE (**).*

3.2.4 Quantification of purified clathrin by titration

To quantify purified clathrin a titration curve was produced by dilution of clathrin stock solution in 1 M Tris buffer. Dilution into this buffer serves to reduce the protein concentration to allow accurate absorbance at 280 nm but also serves to depolymerise clathrin though the action of the ionic strength, high pH and slight denaturing effect of the buffer and through low concentration. Triskelia free in solution give a more accurate measure of protein content than polymerised cages where the protein interactions in the cage block absorbance. A standard curve was generated and the

concentration determined through the use of Beer's Law. The extinction co-efficient used assumed a 1:1 molar ratio of CHC to CLCb.

3.2.5 Considerations on the estimation of clathrin concentration

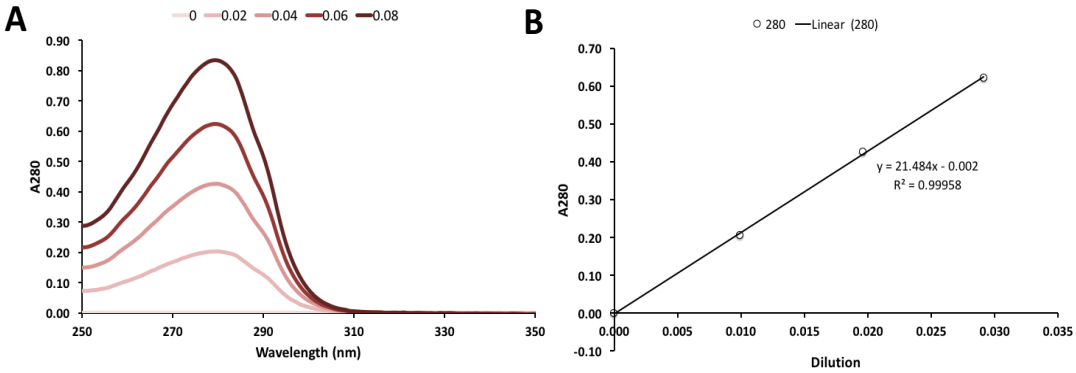

Figure 3.02 Quantification of clathrin via titration and absorbance at 280 nm. ***A)*** *Increasing volumes of clathrin were diluted in 1 M Tris buffer and scanned for an absorbance spectrum between 250 and 350 nm using a Cary 100 UV-Vis spectrophotometer with the legend indicating the dilution factor.* ***B)*** *The peak absorbance at 280 nm was plotted against the dilution factor to generate a standard curve. The concentration of the stock clathrin was determined by substituting the gradient into Beer's Law using the extinction coefficient* $\varepsilon = 222780\ M^{-1}\ cm^{-1}$ *for CHC:CLCb. In this example the concentration was calculated as 97.3 μM.*

The reasons for the approximation using CHC:CLCb are two fold. Previously calculations of protein content with clathrin have assumed that the entire protein absorbance is contributable to CHC. Making this assumption results in the overestimation of CHC/protein content due to the smaller extinction coefficient used (201810 M^{-1} cm^{-1} for CHC compared to 222780 M^{-1} cm^{-1} for CHC:CLCb). Secondly, whilst the presence of both CLCa and CLCb forms of light chain can be seen by SDS-PAGE analysis it is not clear what the relative abundance of these proteins is. Although mass spectrometric analysis could be used to determine the ratio of CHC:CLCa:CLCb this ratio would likely vary between each protein preparation and it would not be feasible or cost effective to do this with each purification. The use of CHC:CLCb as opposed to CHC:CLCa was due to the combined extinction coefficient having a value mid way between the CHC only and CHC:CLCa value ((201810 M^{-1} cm^{-1} CHC < 222780 M^{-1} cm^{-1} CHC:CLCb < 229770 M^{-1} cm^{-1} CHC:CLCa). In addition, although evidence suggests that almost all heavy chains are saturated with light chains (Girard *et al*. 2005) it is not possible to accurately determine this with mass spectrometry. It was therefore felt that the use of 1:1 CHC:CLCb was a sufficient compromise between discounting light chains altogether and the issues surrounding the precise ratios of CHC:CLCa:CLCb. Given the range of the extinction coefficients

we can calculate their standard deviation and from that the error inherent in this assumptions it approximately 6.5%.

3.3.1 Hsc70: expression and purification of WT and ATP hydrolysis mutants

3.3.2 Transfection and amplification of Hsc70 baculovirus

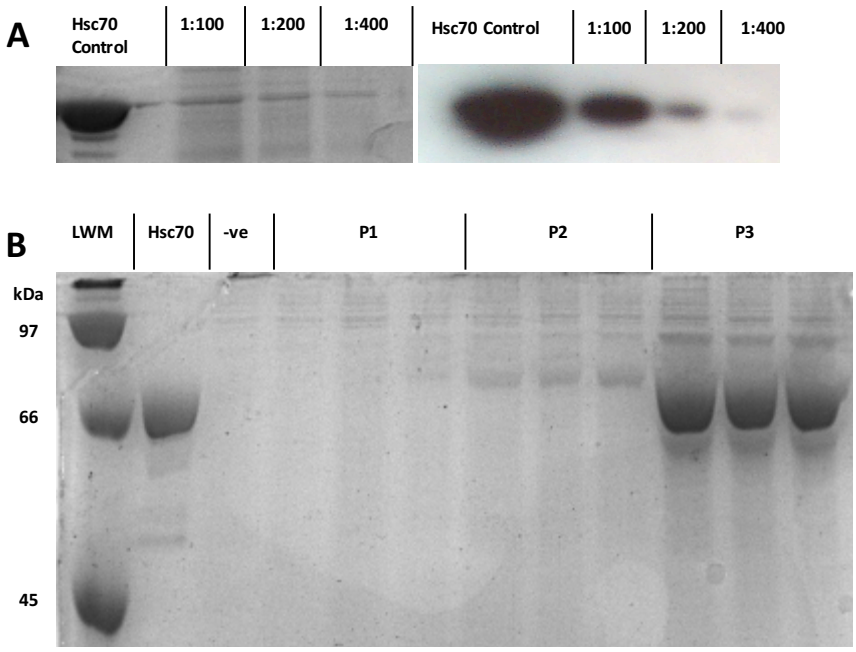

Figure 3.03 Amplification of Hsc70 expressing baculovirus. A) *SDS-PAGE and Western Blot of Sf9 cell material infected with P0 Hsc70 baculovirus at dilution in 1:100, 1:200 and 1:400 in 5 mL media after 5 days of infection. Higher concentrations of P0 virus resulted in increased expression of Hsc70.* ***B)*** *SDS-PAGE of Sf9 cell material infected with Hsc70 baculovirus (1:100 virus to media in 5 mL) for 5 days after infection at increasing passage. Infections were conducted in triplicate. A band consistent with a purified Hsc70 control increases in intensity with increased passage as expected. This band is not present in the non-infected control (-ve).*

The expression of Hsc70 was carried out using the baculovirus system in Sf9 insect cells. This was conducted using both previously generated stocks of baculovirus stored at 4°C (pVL1393-hsc70-baculovirus) and virus generated from transfection of insect cells with pOPINEΔHis_6-Hsc70 (modified to removed the C-terminal His_6-tag) and the BAC10:KO_{1629} to generate recombinant baculovirus. For both constructs the presence of Hsc70 expression was confirmed through SDS-PAGE analysis and Western blotting (Figure 3.03 A). This in turn determined optimal infection ratios of virus to media volume and hence optimised amplification and expression of Hsc70. In order to produce sufficient quantities of virus for large scale expression, both in volume and in viral titre, multiple rounds of infection were conducted using supernatant from a previous infection to amplify the viral titre. Figure 3.03 B shows

an example of an increase in the expression of Hsc70 by SDS-PAGE as viral passage number is increased. Once the titre was deemed to be sufficiently high (usually passage 3-5) a large scale suspension culture of Sf9 cells (300 mL at 1 x10^6 cells/mL) was infected and incubated for 4 days before harvesting of cells by centrifugation and storing at -20°C before use.

3.3.3 Purification of Hsc70

The purification of WT Hsc70 was conducted from Sf9 cell pellets as expressed at the Oxford Protein Production Facility (OPPF, Didcot, UK) and produced in house. Supernatant from lysed Sf9 cells infected with Hsc70 baculovirus was first purified through the use of hydroxyapatite affinity/ion exchange chromatography as detailed in figure 3.04 A and B. The binding to hydroxyapatite is determined by the calcium and phosphate binding sites that provide positively charged and negatively charged sites respectively. Hsc70 itself binds through affinity for phosphate ions and as such can be eluted through the use of potassium phosphate.

Separation of Hsc70 from other contaminating proteins was then conducted through the use of its affinity for ATP. Prior to loading the protein material onto an ATP agarose column ATP was removed through dialysis against activated charcoal. The activated charcoal in the dialysis buffer acts to sequester ATP and thus actively maintains a concentration gradient, ensuring as much of the ATP is removed as possible. Once loaded onto the ATP-agarose column non-ATP binding proteins and non-specific binding proteins are lost in the flow through and through the use of a salt wash (Figure 3.04 C). Elution by the addition of excess ATP results in a high A280 reading due to the ATP itself and SDS-PAGE was needed to confirm the presence of protein hidden by the nucleotide absorbance (Figure 3.04 D).

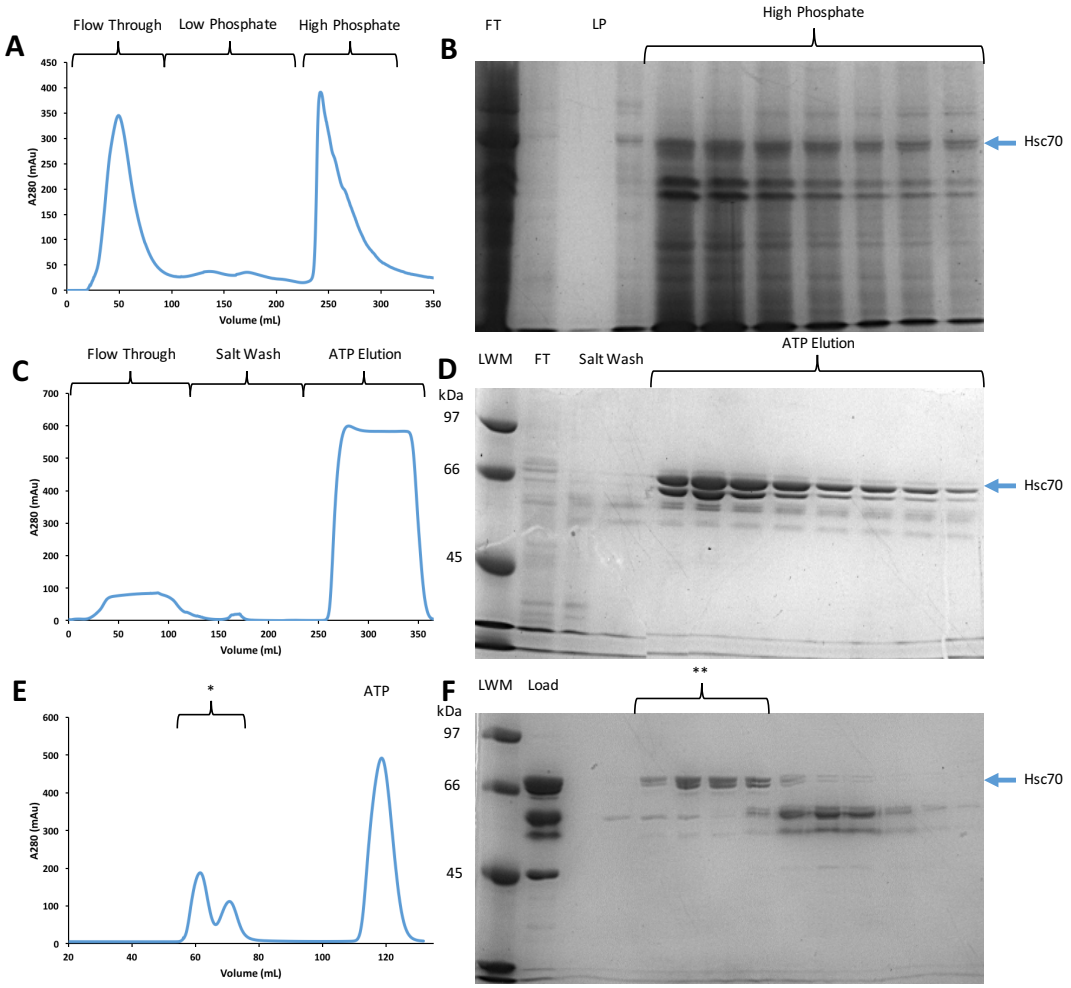

Figure 3.04 Purification of Hsc70 by ion exchange, affinity and size exclusion chromatography as monitored by A280 absorbance (A,C,E) and by SDS-PAGE (B,D,F). A) *Supernatant from lysed Sf9 cells expressing Hsc70 was loaded onto a hydroxyapatite column with non-binding material eluting from the column as the flow through. Protein material was eluted using phosphate buffers with a low phosphate wash (20 mM sodium phosphate) to remove low affinity binding proteins followed by a high phosphate elution (200 mM sodium phosphate).* ***B)*** *Samples taken from each stage were run on an SDS-PAGE gel with FT and LP corresponding to Flow Through and Low Phosphate in A). This confirms Hsc70 along with other components elutes from the column in the high phosphate buffer.* ***C)*** *The Hsc70 containing solution was then purified by affinity to an ATP-agarose column.* ***D)*** *SDS-PAGE corresponds to the A280 trace with Hsc70 eluting from the column with an excess of ATP with the flow through and salt wash (1M KCl) removing other contaminants.* ***E)*** *The final purification by size exclusion using Superdex 75 separates Hsc70 from other ATP-binding contaminants (labelled: *) and remaining ATP.* ***F)*** *SDS-PAGE confirmed the separation of Hsc70 from other proteins and samples pooled for concentration (**).*

Finally, Hsc70 itself was separated from the remaining ATP and the majority of other contaminants through the use of superdex 75 size exclusion. Figure 3.04 E and F show samples from fractions taken across the double peak eluting between ~55 and 80 mL (*). Fractions indicated by ** contain the purest Hsc70 which were pooled, concentrated and stored at -80°C for long term storage.

3.4.1 Expression and purification of GST-tagged proteins

Three different proteins: auxilin$_{401-910}$ (auxilin), α adaptin$_{695-893}$ (α HA) and β2 adaptin$_{616-937}$ (β2 HA) containing GST affinity tags were expressed and purified on the pGEX4T-2 plasmid using *E. coli*. All proteins were initially purified using GST trap affinity columns with subsequent cleavage of the GST-tag for β2 HA and auxilin with size exclusion chromatography used to separate GST-α HA from degradation products.

3.4.2 GST-auxilin$_{401-910}$

Expression of WT and mutant auxilin (DLL->ALAx2, DLL->DAAx2 and DPF->APAx2) was conducted in BL21 DE3 *E. coli* cells. Cells were induced with 0.5 mM IPTG at OD 600 of 0.6-0.8 and incubated overnight at 25°C. Purification from lysed cell supernatant was loaded onto a GSTtrap column and eluted through the addition of 10 mM GSH (Figure 3.05 A and B). Initially this eluted protein was concentrated and stored. Previous work by Alice Rothnie had shown that the presence of the tag had no effect on the activity of the protein (Rothnie *et al*. 2011), however due to concerns about variations in activity between protein batches it was decided that further purification by cleavage of the GST tag would result in increased purity so that protein concentration would more accurately reflect the amount of active protein. After cleavage of the tag by incubation with thrombin the protein material was again loaded onto the GST affinity column. Cleaved auxilin passed through the column and was collected, concentrated and stored (Figure 3.05 C and D).

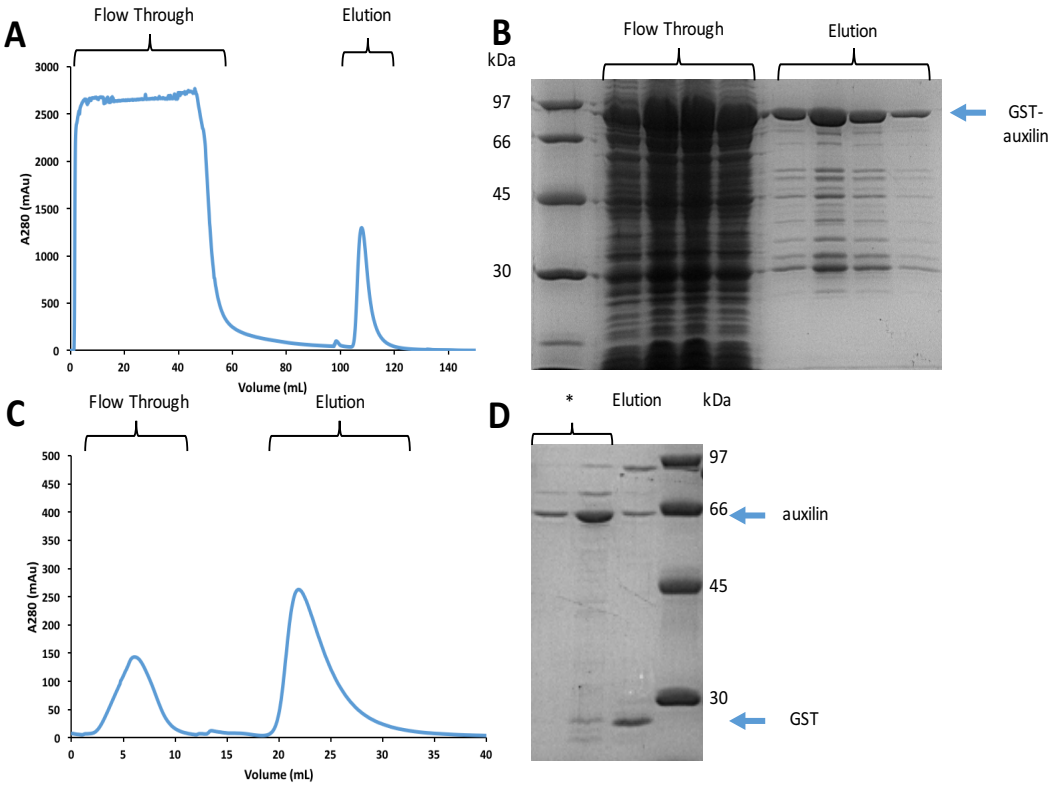

Figure 3.05 Purification of GST-auxilin$_{401-910}$ by affinity chromatography as monitored by A280 absorbance (A, C) and by SDS-PAGE (B, D)). ***A)*** *Supernatant from E. coli lysed cells was loaded onto a GST affinity column with non-binding protein passing through the column in the flow through. GSH buffer (10 mM GSH) was used to elute the glutathione bound protein.* ***B)*** *SDS-PAGE confirms the separation of contaminating E. coli proteins in the flow through (with the loss of some GST-auxilin) with GST-auxilin eluting with GSH.* ***C)*** *Where the cleavage of the GST tag by thrombin digestion was conducted the protein material was re-loaded onto the GSTrap column with now cleaved auxilin passing through the column with non-cleaved GST-auxilin and remaining GST-tag eluting with GSH buffer.* ***D)*** *Fractions from the flow through were confirmed to contain cleaved auxilin by SDS-PAGE and these fractions were pooled, concentrated and stored (*).*

3.4.3 GST-α-adaptin$_{695-893}$

Expression of GST-α adaptin (GST-α HA) was conducted in DH5α *E coli* cells. Cells were induced with 0.5 mM IPTG at OD 600 of 0.6-0.8 and incubated overnight at 25°C. Purification from lysed cell supernatant was loaded onto the GSTtrap column and eluted through the addition of 10 mM GSH (Figure 3.06 A and C). In contrast to Owen *et al*. 1999 the eluted protein contained a large amount of degradation products and so an additional purification step was included through the use of superdex 75 size exclusion chromatography (Owen *et al*. 1999). The elution peak seen in Figure 3.06 B was analysed be SDS-PAGE in part C (**) where the first fraction contains primarily pure GST-α adaptin. This fraction was selected for concentration and storage. The GST tag was retained for use in pull downs via the affinity tag.

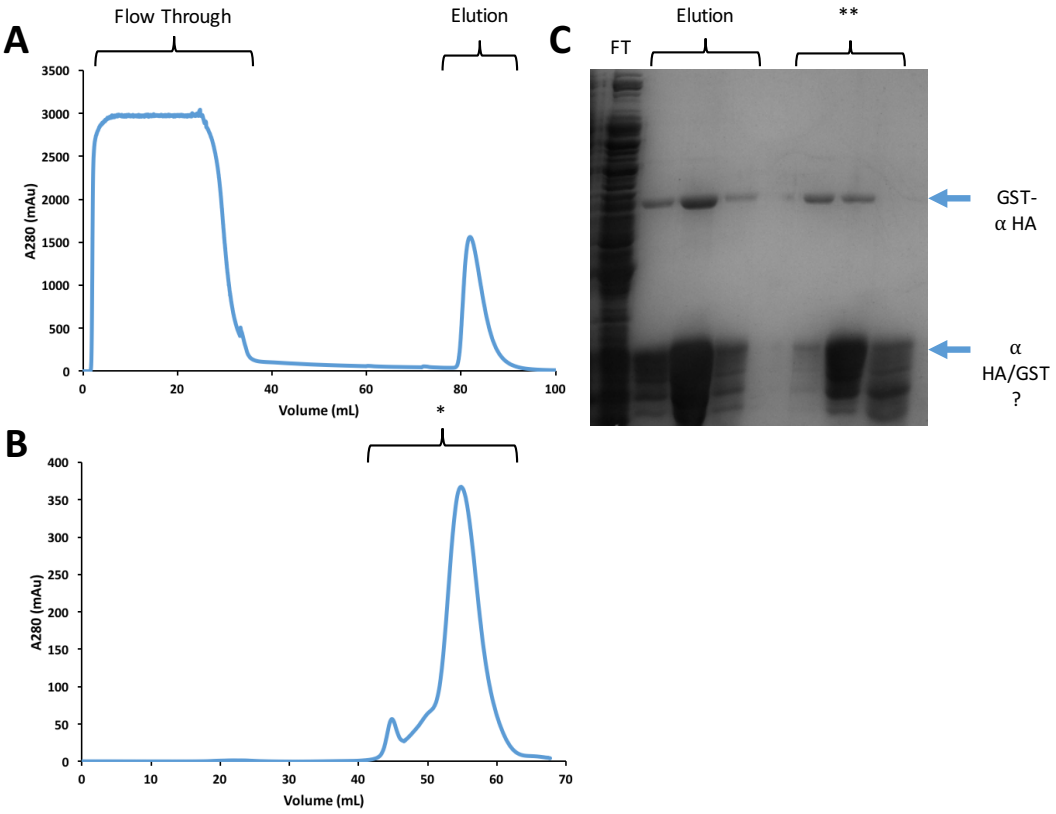

Figure 3.06 Purification of GST-α adaptin$_{695-893}$ by affinity chromatography as monitored by A280 absorbance and by SDS-PAGE. ***A)*** *Supernatant from E. coli lysed cells was loaded onto a GST affinity column with non-binding protein passing through the column in the flow through. GSH buffer (10 mM GSH) was used to elute the glutathione bound protein.* ***B)*** *GST-α adaptin was separated from GST/degradation products through superdex 75 size exclusion and samples from the elution peak (*) were analysed for purity by SDS-PAGE.* ***C)*** *SDS-PAGE of samples from GST affinity and superdex 75 chromatography. Elution corresponds to the elution of GST-α adaptin from the first round of purification as in A which was taken through to further purification by size exclusion. Fraction analysed from B (**) indicate separation of low molecular weight contaminants from the full length protein with the first fraction containing the purest GST-α adaptin and hence this fraction was concentrated and stored for future use.*

3.4.3 GST-β2-adaptin$_{616-937}$

Expression of β2-adaptin$_{616-937}$ (β2 HA) was conducted in BL21 DE3 *E. coli* cells. Cells were induced with 0.5 mM IPTG at OD 600 of 0.6-0.8 and incubated overnight at 25°C. Purification from lysed cell supernatant was loaded onto the GSTtrap column and eluted through the addition of 10 mM GSH (Figure 3.07 A and C). Cleavage of the GST tag was conducted through incubation with the PreScission protease and separation of the cleavage product and tag conducted again through loading back onto the GST affinity column. The flow through containing cleaved β2 HA was pooled and concentrated and the GST tag eluted (Figure 3.07 C).

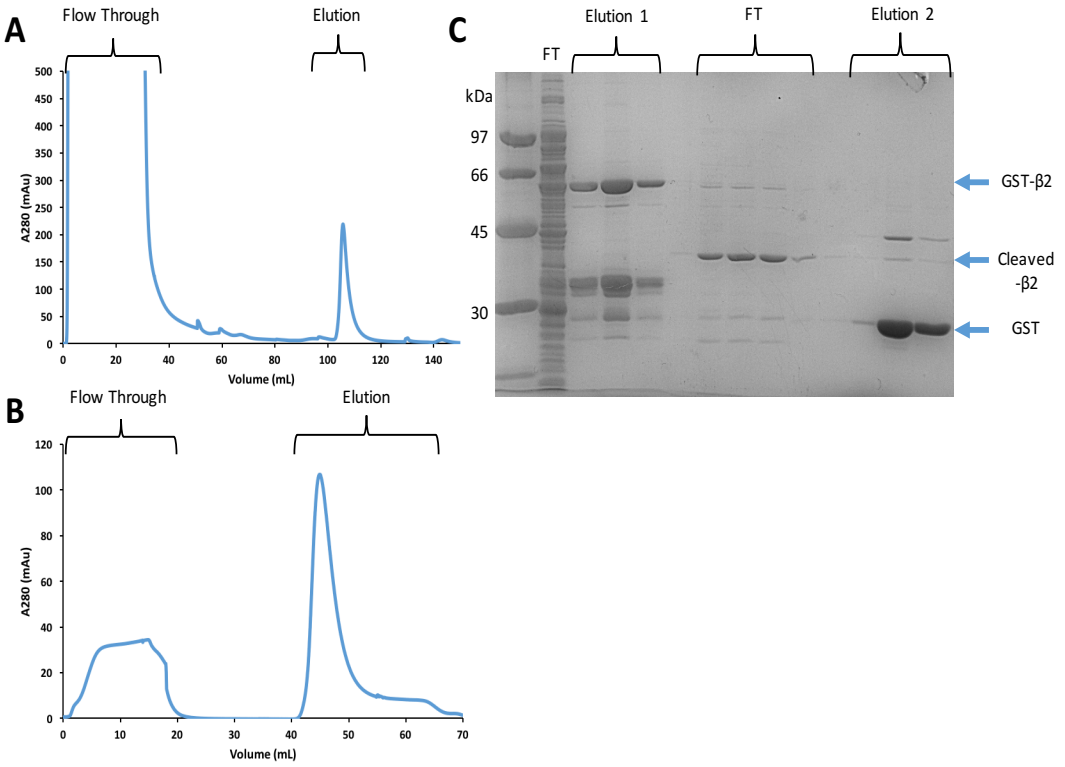

Figure 3.07 Purification of GST-β2 adaptin$_{616-937}$ by affinity chromatography as monitored by A280 absorbance and by SDS-PAGE. A) *Supernatant from E. coli lysed cells was loaded onto a GST affinity column with non-binding protein passing through the column in the flow through. GSH buffer (10 mM GSH) was used to elute the glutathione bound protein.* ***B)*** *Cleavage of the GST tag by PreScission digestion was conducted and the protein material was re-loaded onto the GSTrap column with now cleaved β2 HA passing through the column with non-cleaved GST-β2 HA and remaining GST-tag eluting with GSH buffer.* ***C)*** *SDS-PAGE both pre and post PreScission cleavage. Elution 1 corresponds to the elution of GST-β2 from the first round of purification as in A. FT (Flow through) and Elution 2 correspond to post-cleavage purification as in B. Fractions from the FT containing cleaved β2 HA were pooled and concentrated for storage.*

3.4.4 GST-CHC$_{1-375}$

The terminal domain (TD) of the CHC was expressed and purified by Mary Halebian. BL21 DE3 cells were induced with 0.1 mM IPTG at an OD600 of 0.6 and grown overnight at 25°C. Purification from lysed cell supernatant was loaded onto the GSTtrap column and eluted through the addition of 10 mM GSH (Figure 3.08 A and B). The GST tag was retained for use in pull downs via the affinity tag.

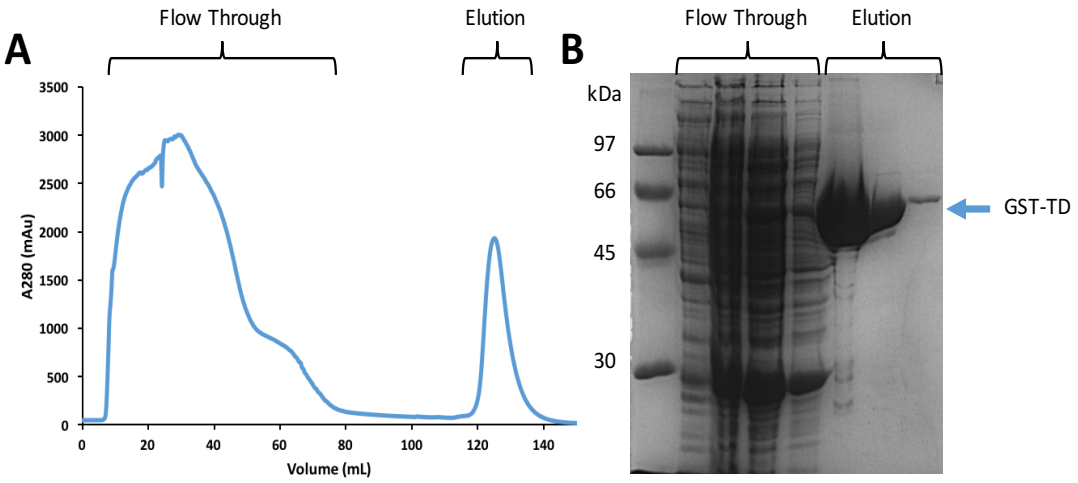

Figure 3.08 Purification of GST-CHC$_{1-375}$ (GST-TD) by affinity chromatography as monitored by A280 absorbance and by SDS-PAGE. Conducted by Mary Halebian. A) *Supernatant from E. coli lysed cells was loaded onto a GSTtrap affinity column with non-binding protein passing through the column in the flow through. GSH buffer (10 mM GSH) was used to elute the glutathione bound protein.* ***B)*** *SDS-PAGE confirms the separation of contaminating E. coli proteins in the flow through with GST-TD eluting with GSH. Eluted protein was pooled, concentrated and stored for future use.*

3.5.1 Purification of His$_6$ tagged proteins

Three different proteins: His$_6$ epsin$_{1-575}$ (epsin), His$_6$Hip1$_{361-637}$ (Hip1CC), His$_6$Hip1R$_{346-655}$ (Hip1RCC) were expressed in *E. coli.* Initially proteins were purified through incubation with Co^{2+} resin and elution by gravity flow. However, after initial purification via this method the protocol was switched to the use of a His trap affinity column and all data here is from protein purified by this method. The reason for this change in protocol was due to the apparent loss of protein during the binding phase where as switching to an Ni^{2+} based affinity column increased yields (data not shown). An additional size exclusion step using superdex 200 was used to purify degradation products from epsin.

3.5.2 Epsin 1

Expression of WT and mutant epsin (clathrin box mutants 257, 480 and DKO) was conducted using BL21 (DE3) *E. coli* cells. Cells were induced with 1 mM IPTG at OD 600 of 0.6-0.8 and incubated overnight at 25°C. Purification from lysed cell supernatant was loaded onto the His trap column and eluted through the addition of 200 mM imidazole (Figure 3.09 A and B). Size exclusion by use of a superdex 200 column was employed to separate the large number of low molecular weight contaminant proteins (Figure 3.09 C). SDS-PAGE analysis of fractions was used to

determine which fractions contained the purest epsin (** in Figure 3.09 D). In most cases the tag was not cleaved and fractions were pooled, concentrated and stored at this stage. In the case of cleavage of the His_6-thioredoxin-tag this was conducted through incubation with the thrombin protease and separation of the cleavage product and tag conducted again through loading back onto the His_6-affinity column. The flow through containing cleaved epsin was pooled and concentrated and the un-cleaved protein eluted (Figure 3.09 E).

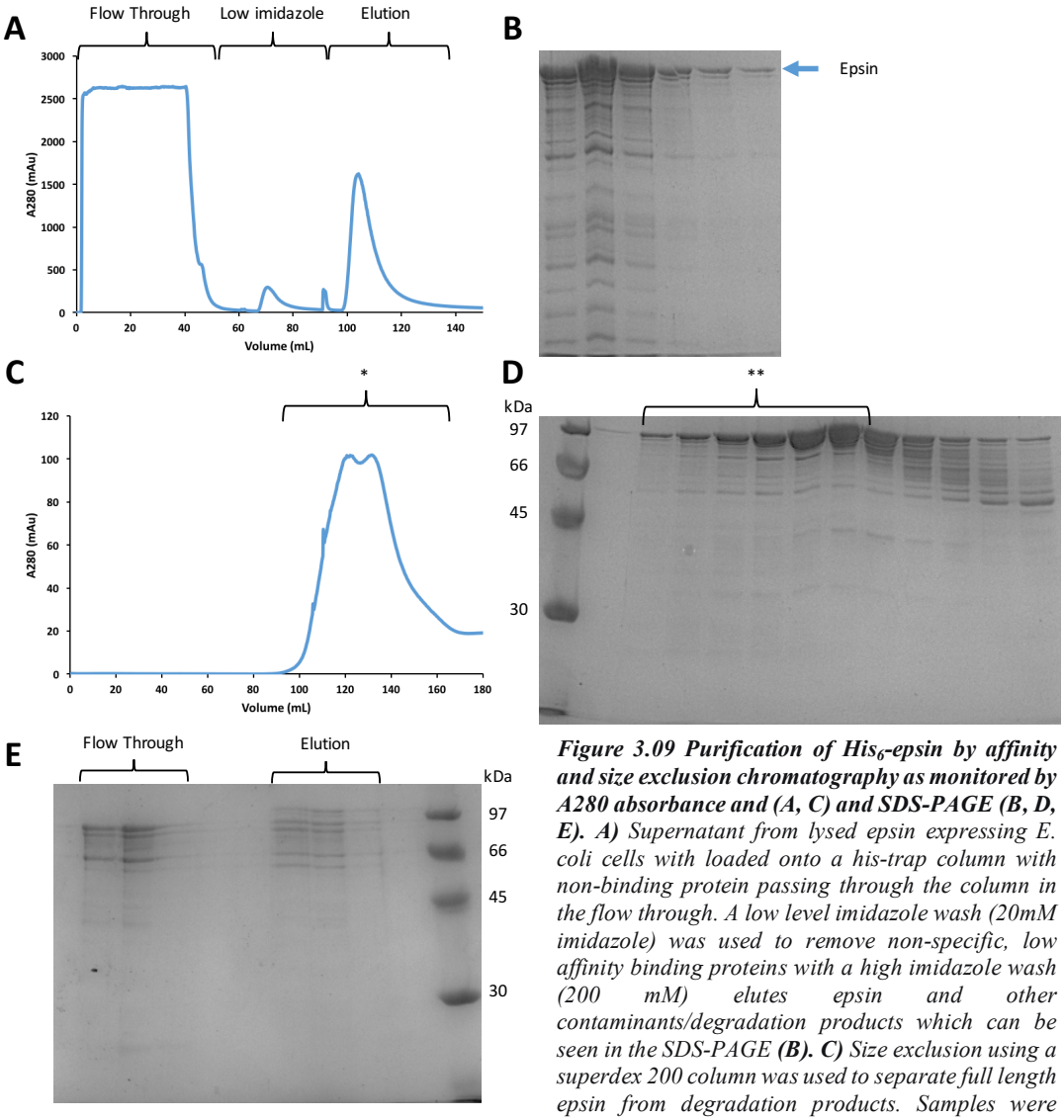

Figure 3.09 Purification of His_6-epsin by affinity and size exclusion chromatography as monitored by A280 absorbance and (A, C) and SDS-PAGE (B, D, E). A) *Supernatant from lysed epsin expressing E. coli cells with loaded onto a his-trap column with non-binding protein passing through the column in the flow through. A low level imidazole wash (20mM imidazole) was used to remove non-specific, low affinity binding proteins with a high imidazole wash (200 mM) elutes epsin and other contaminants/degradation products which can be seen in the SDS-PAGE* ***(B). C)*** *Size exclusion using a superdex 200 column was used to separate full length epsin from degradation products. Samples were taken from elution fractions (*) and analysed by SDS-PAGE* ***(D).*** *Separation was confirmed by SDS-PAGE and fractions containing the purest epsin were selected (**) and pooled for concentration and storage.* ***E)*** *Where the His_6-thiredoxin tag was cleaved from epsin by thrombin digestion the material was passed back through the his-trap column with the flow through of cleaved protein collected and confirmed by SDS-PAGE.*

3.5.3 Hip1CC and Hip1RCC

Expression of Hip1CC and Hip1RCC was conducted using BL21 (DE3) *E. coli* cells. Cells were induced with 1 mM IPTG at OD 600 of 0.6-0.8 and incubated for 3 hours at 37°C. Purification from lysed cell supernatant was loaded onto the His trap column and eluted through the addition of 200 mM imidazole (Figure 3.10 for Hip1CC and Figure 3.11 for Hip1RCC). In the case of Hip1CC all the eluted protein was pooled and stored but in Hip1R CC fractions indicated by the * in Figure 3.11 B were used as these fractions contain fewer contaminants.

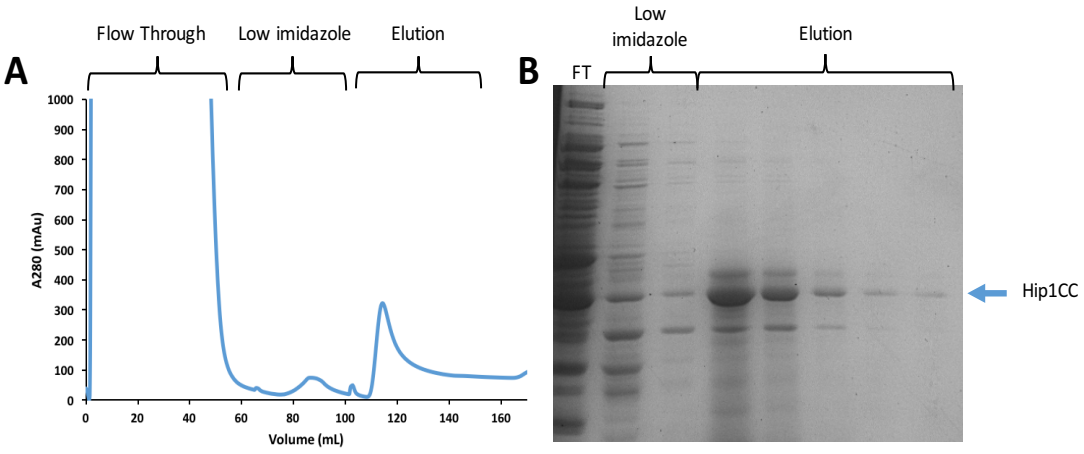

Figure 3.10 Purification of His_6-Hip1CC by affinity chromatography as determined by A280 absorbance and SDS-PAGE. A) *Supernatant from lysed Hip1CC expressing E. coli cells with loaded onto a his-trap column with non-binding protein passing through the column in the flow through. A low level imidazole wash (20mM imidazole) was used to remove non-specific, low affinity binding proteins with a high imidazole wash (200 mM) elutes HipCC which can be seen in the SDS-PAGE* ***(B).*** *All elution fractions were pooled, concentrated and stored for future use.*

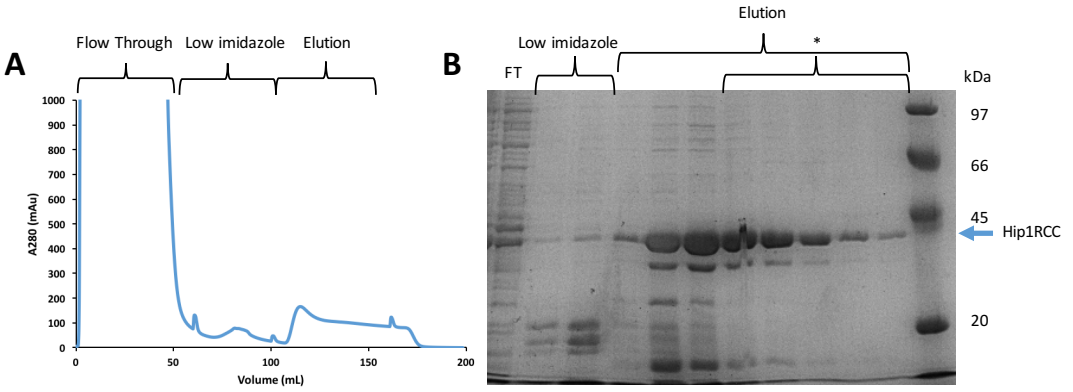

Figure 3.11 Purification of His_6-Hip1RCC by affinity chromatography as determined by A280 absorbance and SDS-PAGE. A) *Supernatant from lysed Hip1RCC expressing E. coli cells with loaded onto a his-trap column with non-binding protein passing through the column in the flow through. A low level imidazole wash (20 mM imidazole) was used to remove non-specific, low affinity binding proteins with a high imidazole wash (200 mM) elutes Hip1RCC which can be seen in the SDS-PAGE* ***(B).*** *Elution fractions indicated by * were pooled, concentrated and stored for future use as these contained the fewest contaminants.*

3.5.4 Characterisation of Hip1 CC and Hip1R CC secondary structure by CD

As both the Hip1CC and Hip1R CC are by nature α-helical in structure the use of CD was used to determine if there was no significant miss folding. The alpha helix forms a characteristic double minimum at 222 nm and 208 nm and this is exhibited in both protein constructs (Figure 3.12). Both proteins were diluted to 0.035 mg/mL and measured between 280 nm and 180 nm with data only retained for an HT[V]<600. Heating to 100 at 1°C/min and then measuring reveals loss of signal as the protein is denatured before cooling back to 20°C where the signal is mainly restored-suggesting re-folding. Spectra are consistent with previously published work (Wilbur *et al.* 2008).

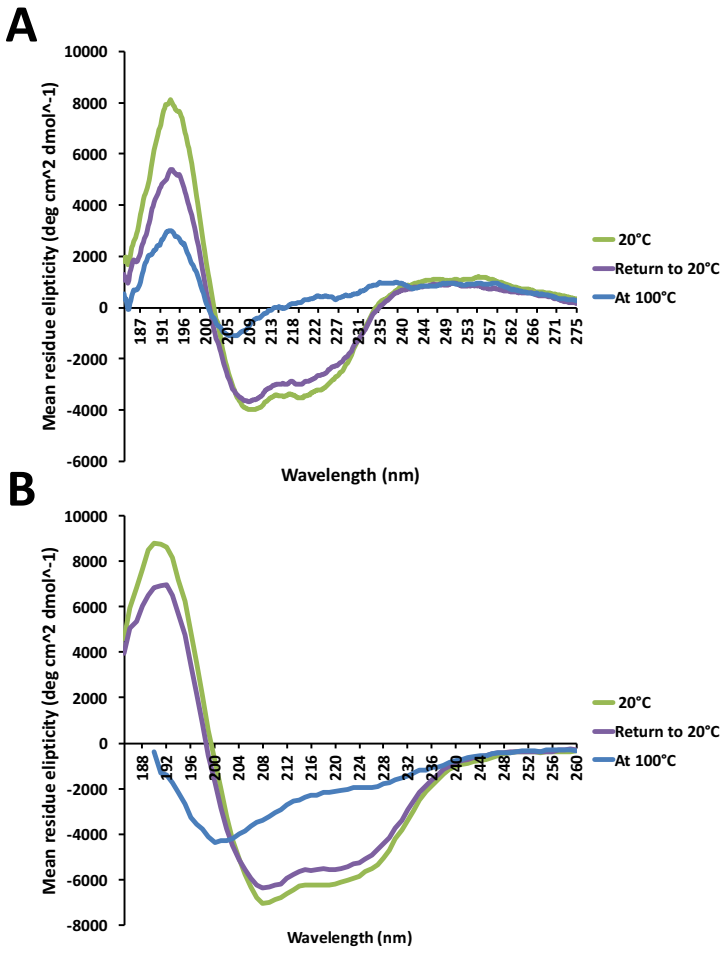

Figure 3.12 Assessment of protein folding of Hip1/1R CC by CD. *Near UV CD spectrum of Hip1 (A) and Hip1R (B) CC domains at 20°C, at 100°C and return after cooling back to 20°C at a concentration of 0.035 mg/mL for both Hip1CC and Hip1RCC. Heating to 100°C was conducted at 1°C/min. Both proteins show expected α-helical signature and an element of structural stability as the protein returns to a similar signature. Data are only shown with HT[V]<600.*

3.6.1 Protein quantification

Quantification of proteins other than clathrin was conducted either using a A280 absorbance or through use of the Pierce ® BCA protein assay. In the case of A280 absorbance values were determined through the use of a Nanodrop ND-1000 spectrophotometer. Protein concentration was derived using Beer's Law with extinction co-efficient derived from the protein sequence and calculated using the online tool ProtParam (http://web.expasy.org/protparam/). Extinction co-efficients for each protein are detailed in Table 3.01.

Protein	**Extinction co-efficient ($M^{-1}cm^{-1}$)**
Clathrin (CHC:CLCb)	222780
Thioredoxin-His_6-epsin 1_{1-575}	97400
epsin 1_{1-575}	83420
His_6- epsin$1_{144-575}$	50990
His_6- Hip$1_{361-637}$ (Hip1CC)	9970
His_6-mHip1$R_{346-655}$ (Hip1RCC)	2980
GST-auxilin$_{401-910}$	101300
auxilin$_{401-910}$	58440
β2 adaptin$_{616-937}$ (β2 HA)	72770
GST-α adaptin$_{695-893}$ (α adaptin)	52830
Hsc70	33350

Table 3.01 Protein constructs purified as described in Chapter 3 *with the extinction co-efficient used to calculate their concentration once purified. Extinction co-efficient were calculated from the protein sequence using the ProtParam online tool (http://web.expasy.org/protparam/).*

3.7.1 Chapter conclusion

In this chapter I have discussed how the proteins that were used in this study were acquired, either directly from tissue or from recombinant expression, and purified from source. These proteins are listed in Table 3.02

Protein	**Variants and Mutants**
Clathrin (CHC:CLCb)	WT
Epsin1	WT, Δ257, Δ480, DKO
Hip1 CC	WT
Hip1R CC	WT
GST-auxilin$_{401-910}$	WT, ALAx2, DAA x2, APA x2
auxilin$_{401-910}$	WT, ALAx2, APA x2
β2 adaptin$_{616-937}$ (β2 HA)	WT
GST-α adaptin$_{695-893}$ (α HA)	WT
Hsc70	WT

Table 3.02 Proteins purified in this chapter and used in subsequent investigations*. Mutant variants of each protein are listed.*

Chapter 4: Monitoring the assembly of clathrin cages in the presence or absence of adaptor proteins

'Errors using inadequate data are much less than those using no data at all' Charles Babbage

4.1.1 Introduction

4.1.2 Overview

The polymerisation of clathrin cages and the effects of adaptor proteins on assembly *in vitro* has been studied previously but much of the work has been qualitative in nature. In this chapter I use a newly devised method and analysis developed at the University of Warwick that uses dynamic light scattering (DLS) to monitor the shift in particle radius during polymerisation and depolymerisation of clathrin. I use this technique to determine the effect adaptor proteins have on the size distribution of clathrin cages when polymerised in their presence. I compare the DLS data obtained to measurements of cage size made from negative stain EM micrographs and use these preliminary observations to make insights into the promotion of cage assembly by adaptors.

4.1.3 Analysing clathrin assembly

The ability of clathrin to self assemble into cages/coats in the presence or absence of membrane has been studied both *in vitro* and *in vivo*. Clathrin polymerisation and depolymerisation *in vitro* can be driven through varying pH and the level of the divalent cations Ca^{2+} and Mg^{2+} in buffers (Liu *et al*. 1995). Below pH 6.5 clathrin will self assemble, driven in part by the protonation of histidines at key interface sites between neighbouring legs in the triskelia (Ybe *et al*. 1998; Bocking *et al*. 2014). The

divalent cations, Mg^{2+} and Ca^{2+} have been shown to interact with a specific binding site on the clathrin CLC (Liu *et al*. 1995) and thereby cause a conformational change in the CHC that is conducive to cage formation (Wilbur *et al*. 2010).

4.1.4 Adaptor proteins and assembly

The adaptor proteins as a whole have been shown to be integral to the promotion of clathrin coat formation both *in vivo* and *in vitro*. Observations of adaptor-clathrin complexes formed *in vitro* point to adaptors promoting the narrowing of the distribution of cage size, forming cages of smaller diameter than with clathrin alone and promote cage assembly under high pH conditions were clathrin does not readily assemble (Ahel and Ungewickell 1990; Greene *et al*. 2000; Morgan *et al*. 2000; Engqvsit-Goldstein *et al*. 2001; Kalthoff *et al*. 2002; Legendre-Guillemin *et al*. 2005; Chen *et al*. 2005). Although mechanisms have been proposed for how these proteins function to bring together clathrin cages there is still more to learn and confirm about these mechanisms.

The Hip1 and Hip1R proteins are unusual in that they interact with the CLC (Engqvist-Goldstein *et al*. 2001; Legendre-Guillemin *et al*. 2002; Legendre-Guillemin *et al*. 2005; Chen *et al*. 2005) as opposed to the terminal domain, which is the primary binding site for most clathrin adaptors (Lemmon and Traub 2012). Both proteins interact with clathrin via their coiled-coil domains (CC) and are proposed to regulate assembly through these interactions. The CLCs have been implicated as important regulators of assembly (Wilbur *et al*. 2010) and the CC domains of Hip1/R are proposed to regulate assembly by interacting with and neutralising the acidic EED residues on the CLC that negatively regulate assembly (Ybe *et al*. 1998; Legendre-Guillemin *et al*. 2005; Ybe *et al*. 2007a; Ybe *et al*. 2010). Previous *in vitro* studies for Hip1 have used ultracentrifugation-SDS-PAGE analysis to demonstrate that Hip1CC binding to clathrin results in clathrin polymerisation (Chen *et al*. 2005; Legendre-Guillemin *et al*. 2005) whereas Hip1R has been shown to actively promote the formation of cages to produce a distribution similar to other adaptors by measurement from EM (Engqvist-Goldstein *et al*. 2001).

Given the differing method of cage promotion to other adaptors and the lack of information on cage distribution of cage sizes when polymerised by Hip1,

investigating the effect of binding by the CC domains of these proteins on clathrin assembly would give us a greater insight into the proposed mechanism.

AP2 as the primary and essential adaptor has long been studied for its ability to assemble clathrin cages. The addition of AP2 to form clathrin cages has been integral to efforts to determine the structure by cryo-EM as the adaptor increases the prevalence of smaller cage complexes such as the D6 barrel which has a high level of symmetry suitable for single particle averaging (Vigers *et al*. 1986; Smith *et al*. 1998; Fotin *et al*. 2004b).

The β2 hinge appendage region (β2 HA) of AP2 contains the clathrin box implicated as the primary interaction site for AP2 with clathrin terminal domain (Owen *et al*. 2000). In addition the β2 appendage is also able to contact the ankle domain of clathrin through a second site and therefore been hypothesised to make contacts with neighbouring TD/ankle domains of triskelia to promote assembly of clathrin (Greene *et al*. 2000; Edeling *et al*. 2006a; Kneul *et al*. 2006). One study on the specific effect of the β2 HA on clathrin polymerisation by Greene *et al.* (2000) showed that this region is able to promote assembly and noted that the cages formed were of a similar size to those formed by AP2 (Greene *et al*. 2000). However, no quantitative measurements of the effect were made. Therefore, exploring the apparent effect by using a quantitative measure would improve our understanding of the mechanism of AP2 promotion of assembly.

Epsin has been studied extensively for its ability to remodel membranes and assemble clathrin on surfaces (Dannhauser and Ungewickell 2012; Dannhauser *et al.* 2015a; Holkar *et al*. 2015). Epsin has been shown to interact with clathrin through its unstructured region containing two clathrin box motifs that bind to clathrin TD and multiple DPW motifs that also provide affinity to clathrin (Drake *et al*. 2000). It has been proposed that the multiple binding sites in this unstructured region, combined with the potential distance that this structure could reach would facilitate interactions between multiple triskelia and thus promte cage formation (Drake *et al*. 2000; Drake and Traub 2001; Kalthoff *et al*. 2002; Dafforn and Smith 2004). Polymerisation of clathrin *in vivo* in the presence of epsin has been shown to facilitate the formation of small cages as seen with other adaptors although quantitative measurements were not made (Kalthoff *et al*. 2002). In addition, disruption of epsin binding in cells results in

the formation of larger coated pit structures suggesting that the control of vesicle size is a physiological role of epsin (Jakobsson *et al*. 2008). Therefore, investigating the effect of epsin on assembly in a quantitative fashion would reveal more about its mechanisms of assembly.

4.1.5 Measuring clathrin cage and vesicle size

In vitro the measurement of clathrin cages has been primarily through the use of negative stain or cryo-transmission electron microscopy (EM). Being a large complex clathrin lends itself well to EM and clathrin cages are routinely studied using this technique. Negative-stain EM fixes the protein to the surface of a copper-carbon grid using a heavy metal stain such as uranyl-acetate that provides contrast and fixes the sample. Measurement of particle size by negative stain EM has the major drawback that the protein is dehydrated on a surface and fixed with a stain and hence the dimensions of the protein under observation can be distorted. This is particularly true of clathrin cages in the absence of vesicles where the absence of support in the centre of the cage can cause the particles to broaden on the surface, giving a larger apparent diameter than is expected. Another option is to use cryo-EM where particles in vitreous ice are equivalent to their native solvated form and hence the dimensions are more accurate. This approach can also be used to pick and average particles to obtain classes of cage topologies as well as structural information on structures with high levels of symmetry. As such this approach has been the primary means for obtaining high resolution structural information on clathrin cages. However, this approach is technically challenging: in particular, with particle classification and averaging of large cage structures that are inherently more flexible and less symmetrical means that the approach is not feasible for determining size distribution.

4.1.6 Using dynamic light scattering to determine clathrin cage particle sizes in solution

A novel approach to measure clathrin particle sizes has been to apply the technique of dynamic light scattering (DLS). DLS uses a single beam of incident light that passes through a solution containing particles. The particles generate a scattering signal that fluctuates with time due to the Brownian motion of these particles in solution. The scattered light in turn, undergoes constructive and destructive interference with other particles in solution. This change in signal fluctuation is dependent on the motion of

the particle in solution, which in turn is related to the particle size and shape. Therefore, a size distribution of particles in solution can be estimated. This is usually given as the hydrodynamic radius (Rh) of the particle.

Joseph Jones of the Smith Group at the University of Warwick has pioneered the use of this technique to analyse the polymerisation and depolymerisation of clathrin cages (Joseph Jones 2016, thesis manuscript in preparation). It was initially noted that whilst the technique was able to differentiate between depolymerised samples and polymerised clathrin the proprietary analysis software did not accurately reflect the size distributions (see Figure 4.01). This is in part because calculations of hydrodynamic radius assume a solid sphere. This is clearly not the case with triskelia or with higher order oligomers of clathrin, nor the cages themselves (which are hollow in these experiments). Therefore, a novel analytical approach to address the physical properties of clathrin was needed.

M_i (triskelia)	R_i = Rh (nm)
1	15.6
2	18.9
3	21.3
5	25.5
28	30.9
36	35.2
60	44.9
108	58.9
180	77.7

Table 4.01 A table indicating the particle classes used in the modified fitting algorithm for determining clathrin cage size from DLS data. *Classes are given by the median number (M_i) of triskelia corresponding to their theoretical hydrodynamic radius (R_i = Rh) as calculated using the modelling software HYDRO.*

The novel analysis takes into account the relative scattering signal of different classes of particles containing different numbers of triskelia. By building on previous work

on the modelling of triskelia in solution (Ferguson *et al.* 2006) and the preferential topologies of clathrin cage assemblies (Schein and Sands-Kidner 2008) a theoretical hydrodynamic radius for various classes of monomers and oligomers could be calculated. (see Table 4.01). The technique has been shown to more accurately reflect the theoretical expected Rh of the triskelia as determined by Ferguson *et al.* (2006) and, as shown in this chapter, more closely matches the size distribution as determined by negative stain EM.

Here I present the use of this novel technique to study how clathrin assembly is effected by the presence of adaptor proteins. This preliminary work was conducted in collaboration with sample preparation and data collection conducted by the author and data analysis conducted by Joseph Jones. I compare the DLS data to cage radii as measured from negative stain EM micrographs. By comparing the proprietary data analysis to the modified fitting algorithm for the clathrin only control, I show that the modified fitting algorithm better matches the size distribution of measured cages compared to the proprietary analysis. Finally, I show that different adaptors have different effects on the size distribution of cages and relate this to previous work and to possible implications for *in vivo* function and assembly mechanisms. Note that all experimental results shown here are as the result of single experiment and hence any conclusions associated with results should be viewed with this in mind. As such, all data errors from the fitting analysis have been removed for clarity.

4.2.1 Depolymerisation and re-polymerisation of clathrin as analysed by EM and DLS

The polymerisation and depolymerisation of clathrin was driven in each of these experiments through the change in buffer conditions from the pH 8.0 depolymerisation buffer to the pH 6.4 polymerisation buffer. Clathrin cages purified as described (see section 2.3.3) were diluted from stock solution in 1 M Tris Buffer to 3.5 μM to initiate disassembly and then dialysed into depolymerisation buffer. The high ionic strength (and partial denaturing effect) of the 1 M Tris buffer works to disrupt interactions between clathrin triskelia and thereby cause depolymerisation. Dialysis into depolymerisation buffer is conducted to ensure clathrin is retained as triskelia via high pH (pH 8.0) but allows for a direct transition to low pH polymerisation conditions when the protein is dialysed into polymerisation buffer (pH 6.4).

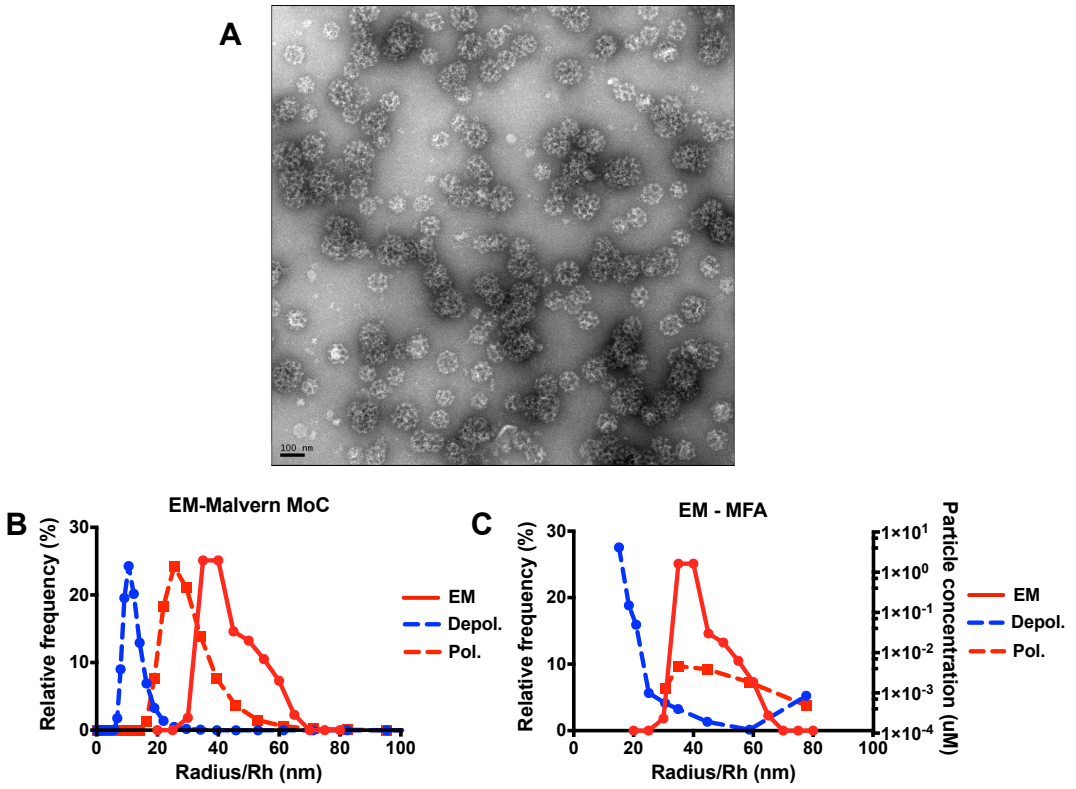

Figure 4.01 Polymerisation and depolymerisation of clathrin cages as determined by EM and DLS. *A) Polymerised clathrin (diluted from 3.5 μM during polymerisation to 1 μM) imaged by negative stain (2% uranyl-acetate) EM shows clathrin cages which vary in size. B) A histogram of particle radii as measured in ImageJ (n=219) using negative stain EM images as shown in A (EM solid red) is compared to the size distribution of the data of polymerised clathrin (Clathrin dashed red) and depolymerised clathrin (Depol dashed blue) from the Malvern Method of Cumulants (Malvern MoC). The EM distribution shows a peak radius of 40 nm with a shoulder extending to larger radii up to 70 nm. With the Malvern MoC data the difference between depolymerised clathrin as triskelia and polymerised clathrin can be seen. However, the DLS data for polymerised clathrin shows a much smaller hydrodynamic radius with a peak at around 30 nm. C) Using the modified fitting algorithm (MFA) and comparing to the EM distribution we can see a much greater concordance in terms of size distribution for the polymerised sample suggesting that this output gives a more accurate picture of the particle size in solution.*

An example of polymerised clathrin imaged by negative stain EM can be seen in Figure 4.01 A and the size distribution as measured from these images can be seen in Figure 4.01 B (solid red line). As seen previously clathrin forms a wide distribution of cage sizes. In this distribution the cages show a peak at a radius of around 35-45 nm with larger cage radii forming a shoulder. This same graph also shows the DLS size distribution of 3.5 μM of clathrin in depolymerisation buffer (dashed blue, Depol.) and polymerisation buffer (dashed red, Pol.) as determined by the proprietary Malvern software (Malvern-MoC). Both depolymerised clathrin and polymerised clathrin clearly show a difference in distribution, consistent with the presence of triskelia and low order oligomers in depolymerisation buffer and cage particles in polymerisation buffer. However, the peak of the size distribution as seen here is at a lower Rh (~30 nm) compared to the EM distribution radius (~40 nm). Analysing the same data using the modified algorithm reveals a shift to a distribution that is much more consistent

with the EM data (see Figure 4.01 C). This would suggest that the modified algorithm produces a Rh distribution that is much more consistent with the physical radius as observed through EM.

4.3.1 Hip1 CC & Hip1R CC assembly

4.3.2 Hip1 CC and Hip1R CC bind to clathrin cages

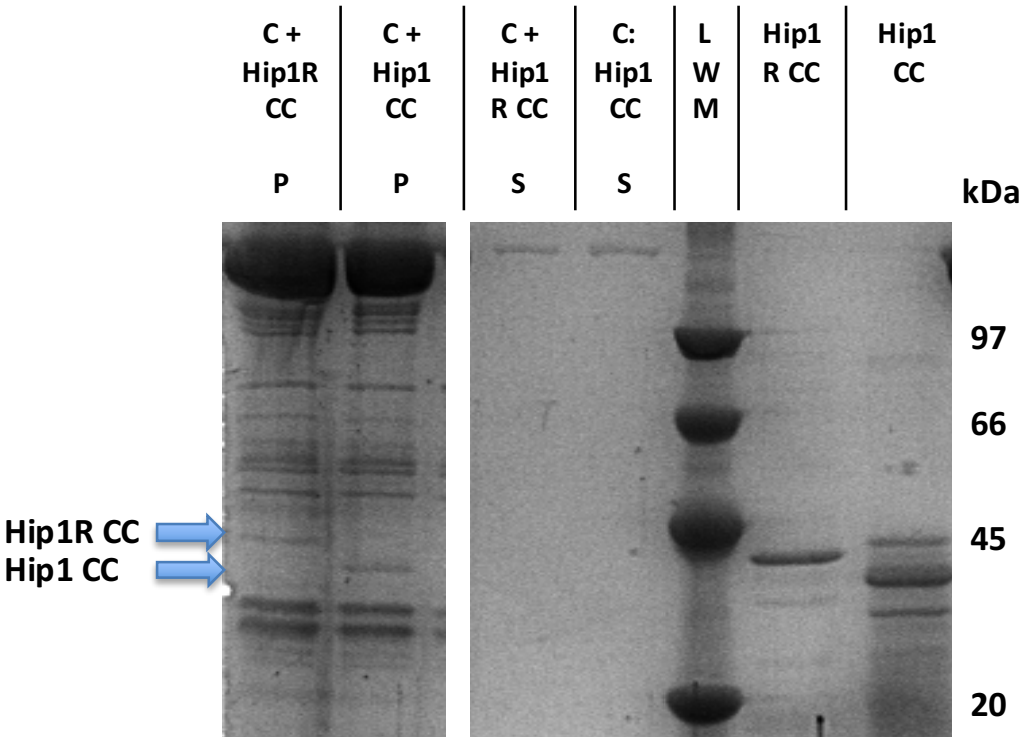

Figure 4.02 SDS-PAGE analysis of binding of Hip1/1R CC to clathrin cages. *Clathrin cages incubated with 1:1 molar ratio of Hip1 CC or Hip1R CC were pelleted at 140,000 x g and pellet and supernatant fractions separated. Bands consistent with Hip1 CC and Hip1R CC (H1R CC and H1 CC) are present in the pellet fraction (C:H1R P and C:H1 P) and not in the supernatant fractions, suggesting the proteins have bound to clathrin as expected.*

To confirm the binding of Hip1 CC and Hip1R CC to clathrin cages pull downs of clathrin cages incubated with Hip1 CC and Hip1R CC were conducted (see Figure 4.02). As expected, bands corresponding to Hip1R CC and Hip1 CC were detected in pellet fraction on an SDS-PAGE gel with both clathrin CHC and CLC, suggesting that the proteins are able to bind to clathrin as expected.

4.3.3 Polymerisation with Hip1 CC

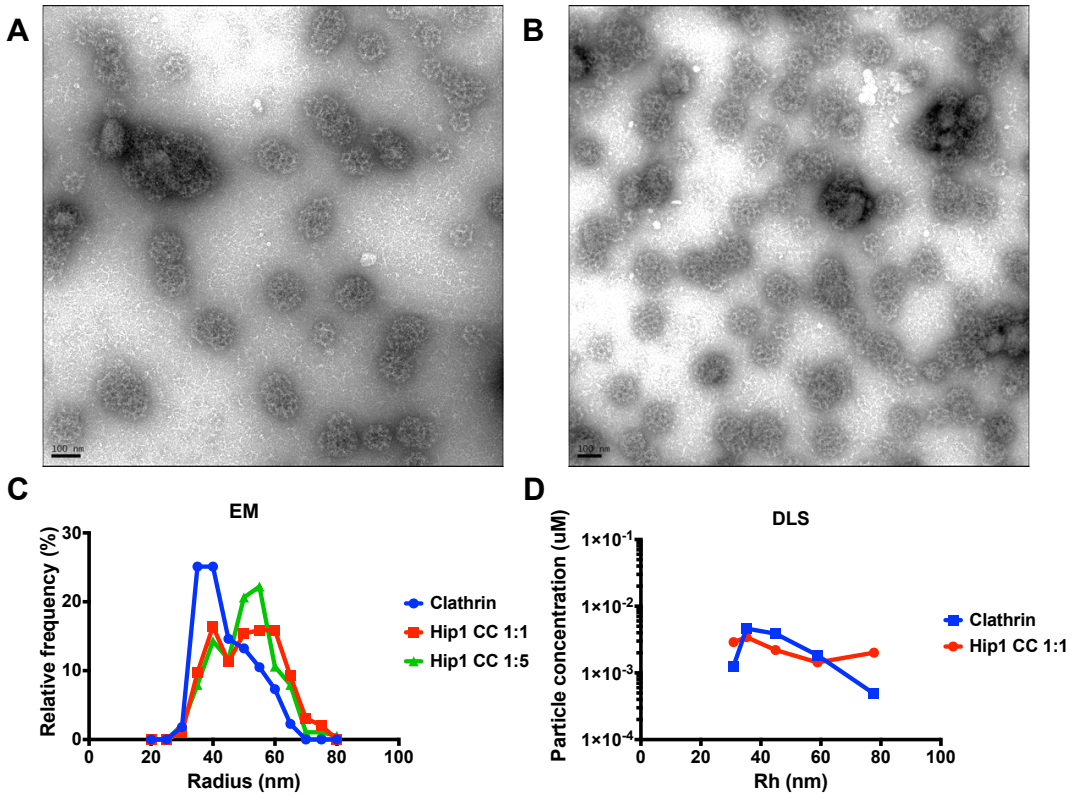

Figure 4.03 Polymerisation of clathrin cages in the presence of Hip1 CC as determined by DLS and EM. *Clathrin at 3.5 μM was polymerised at either a 1:1 or 1:5 molar excess of Hip1 CC with example EM images at these concentrations shown in A and B respectively. C) The size distribution of cage radii as measured from EM images in ImageJ (n=195 and n = 189 for 1:1 and 1:5 respectively) indicates a shift to larger radii with increased concentrations of Hip1 CC with the possible formation of two distinct peaks at 40 nm and ~55 nm. D) When analysing the same sample using DLS a similar increase in Rh can be seen at the 1:1 ratio of Clathrin:Hip1 CC with an increase in particles at the 77.7 nm class and a similar change in the distribution to form two separate peaks. However, the 1:5 concentration is not shown due to the sample having apparently aggregated with the cage sizes being apparently larger than the range used by the model.*

Polymerisation in the presence of Hip1 CC was conducted as with the clathrin control with the addition of Hip1 CC at 1:1 and 1:5 molar excess of clathrin (3.5 μM). Note that all adaptors were dialysed into depolymerisation buffer over the same time period as clathrin in order to negate any effects of residual purification buffer on assembly. The effects on cage size distribution as determined by EM and DLS are described in Figure 4.03. EM images indicate the presence of predominantly larger cage particles than in the control, which is confirmed in the histogram in Figure 4.03 C. A relative drop in the number of cages at around 40 nm in radius is observed with a commensurate increase in cages with a radius of 55-65 nm which seems to be at least partially dependent on the concentration of Hip1 CC.

When looking at the DLS data a similar effect of increased cage size relative to the Hip1 CC concentration is noted for the 1:1 sample. However, at a 5-fold excess of

Hip1 CC (1:5) almost all of the particles were classified as 77.7 nm or greater suggesting sample aggregation and hence this data is excluded from the graph for clarity. Aggregated particles and large clumps of cages were noted at this concentration when imaged. These particles were not measured as part of the EM distribution but were noted in the images. One possible explanation for the slight discrepancy between the DLS data and the EM data is that the cage samples were diluted from 3.5 μM to 1 μM prior to imaging to prevent over saturation of cages on the surface. The act of diluting the cages may have reduced the number of cages that were clumped together at 3.5 μM in the DLS and hence allowed the measurement of more particles.

4.3.4 Polymerisation with Hip1R CC

Polymerisation in the presence of Hip1R CC was conducted as with Hip1 CC experiment with Hip1R incubated at 1:1 and 1:5 molar excess of clathrin (3.5 μM) and the results summarised in Figure 4.04. EM data seems to indicate a largely similar distribution to the clathrin only control on first observation, however at high concentrations of Hip1R CC there appears to be a slight increase in the population of larger cages. When looking at the histogram of the cage particles radii shown in Figure 4.04 C at the 1:1 molar ratio a shift towards larger cage particles is observed as with Hip1 CC. At the higher concentration this trend seems to change slightly with the emergence of two populations of cages, one with a peak at around 35 nm and the other with a peak around 55 nm.

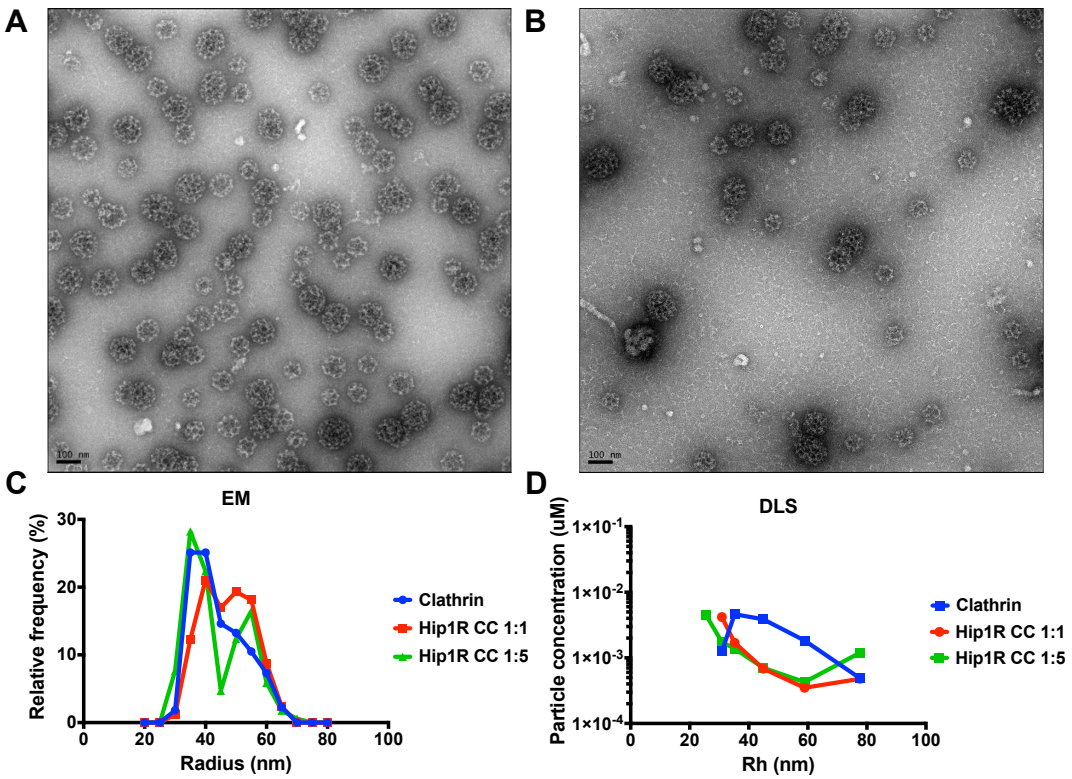

Figure 4.04 Polymerisation of clathrin cages in the presence of Hip1R CC as determined by DLS and EM. *Clathrin at 3.5 μM was polymerised at either a 1:1 or 1:5 molar excess of Hip1R CC with example EM images at these concentrations shown in A and B respectively. C) The size distribution of cage radii as measured from EM images in ImageJ (n=171 and n = 170 for 1:1 and 1:5 respectively) indicates a shift to larger radii with increased concentrations of Hip1R CC with the possible formation of two distinct peaks at 40 nm and ~55 nm as seen with Hip1 CC (see Figure 4.03). D) Analysis of the sample using DLS seems to show a similar split in distributions with the loss of mid sized cage particles (~45 nm) and an increase in particles at the size extremes that increases with higher concentrations of Hip1R CC.*

When these observations are compared to the DLS results we see an apparent loss of cages with diameters in the 40 – 60 nm range and an increase in smaller and larger particles. Again this seems to be consistent with the formation of two separate populations of cages but the DLS results suggest both smaller and larger particles than those observed under EM. As discussed in the last section the dilution of the clathrin-Hip1R CC sample prior to staining and imaging may have altered the dynamics of cage interactions and hence larger clumps of cages become individual particles rather than larger complexes. However, this does not easily explain the apparent prevalence of smaller particles seen, although these particles may correspond to the triskelia and smaller oligomers that seem to be evident in the EM images (see Figure 4.04 B). The size of these particles is not taken into consideration in the EM histogram. Again this observation highlights how differences in concentration may alter the apparent distribution of cage particles as well as the differences between the analytical techniques (see discussion 4.6.2).

4.4.1 β2 HA assembly

4.4.2 β2 HA binds to clathrin cages

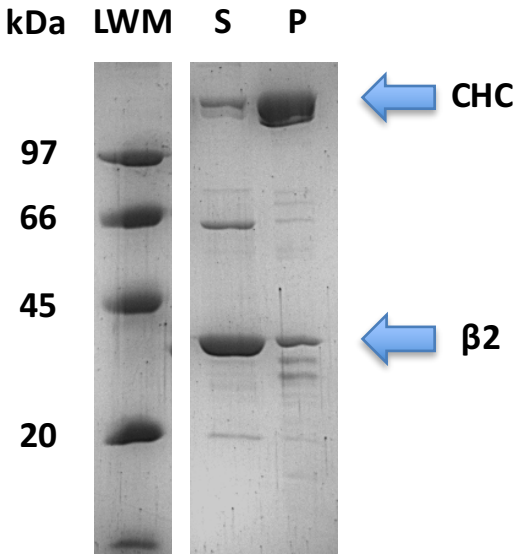

Figure 4.05 SDS-PAGE analysis of binding of β2 HA to clathrin cages. *Clathrin cages incubated with 1:5 molar ratio of β2 HA were pelleted at 140,000 x g and pellet and supernatant fractions separated. Bands consistent with β2 HA are present in pellet fraction (P) suggesting the protein has bound to clathrin as expected.*

As with Hip1 CC and Hip1R CC the binding of β2 HA was assessed through incubation with clathrin cages and pelleting by ultracentrifugation. In Figure 4.05 we can see that β2 HA is present in the pellet fraction on the gel along with the CHC, confirming that β2 HA binds as expected.

4.4.3 Polymerisation with β2 HA

Polymerisation with β2 HA was conducted as with previous experiments with β2 HA incubated at 1:1 and 1:5 molar excess of clathrin (3.5 μM) and the results summarised in Figure 4.06. Note that β2 HA samples were imaged with a JEOL 2200 FS with a Gatan 2k x 2k Ultrascan CCD camera and hence the difference in magnification used. When comparing the histogram of the size distribution of β2 HA polymerised cages we can see that the distribution is the same as the control in the absence of β2 HA. This same pattern is noted in the DLS although with a general increase in particle concentration across all size classes, suggesting a possible increase in the conversion of triskelia to cages, although variations in concentrations cannot be discounted.

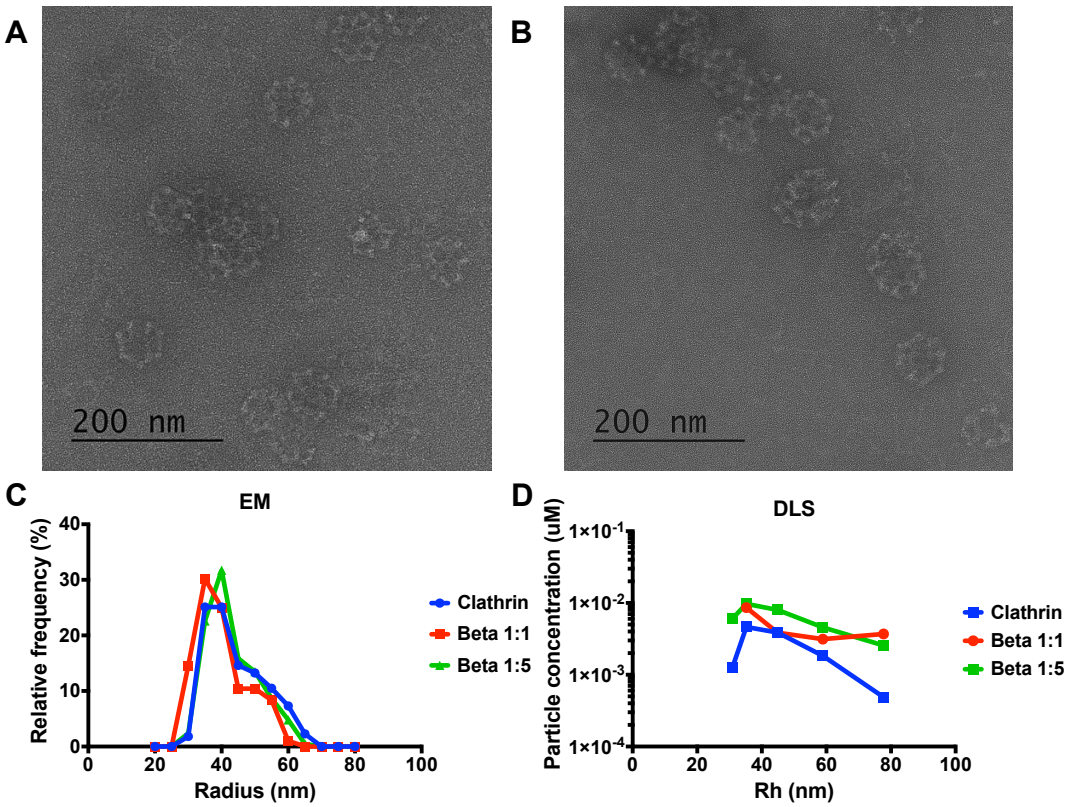

Figure 4.06 Polymerisation of clathrin cages in the presence of β2 HA as determined by DLS and EM. *Clathrin at 3.5 μM was polymerised at either a 1:1 or 1:5 molar excess of β2 HA (Beta) with example EM images at these concentrations shown in A and B respectively. C) The size distribution of cage radii as measured from EM images in ImageJ (n=200 and n = 208 for 1:1 and 1:5 respectively) indicates a similar distribution to the clathrin control. D) Analysis of the sample using DLS shows a similar pattern of distribution in the presence of β2 HA with an overall increase in particle concentration in the presence of β2 HA.*

4.5.1 Epsin assembly

4.5.2 Epsin binds to clathrin cages

Epsin was incubated with clathrin cages at increasing conentrations as with the other adaptors and cages pelleted by centrifugation and the pellet and supernatant fractions analysed by SDS-PAGE (see Figure 4.07). Epsin binds to clathrin cages as expected and does not appear in the pellet fraction in the absence of clathirn. Interestingly the clathrin control contains clathirn both in the pellet and supernatant, suggesting an element of depolymerisation. However in the presence of epsin there is little or no clathrin evident in the supernatant fraction, suggesting that epsin may be having a stabilising effect on clathrin cages.

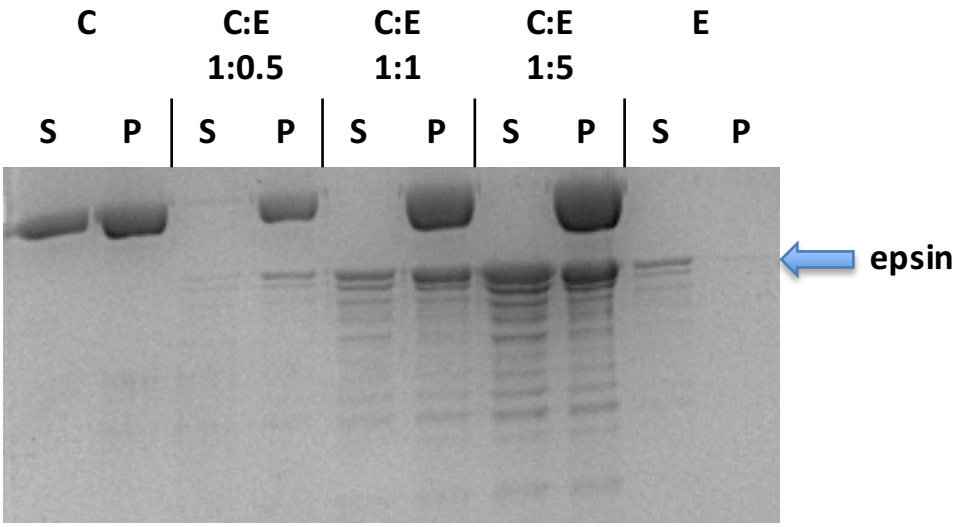

Figure 4.07 SDS-PAGE analysis of binding of epsin to clathrin cages. *3 μM Clathrin cages incubated with 1:0.5, 1:1 and 1:5 molar ratio clathrin:epsin were pelleted at 140,000 x g with pellet (P) and supernatant (S) fractions separated. Increasing the relative concentration of epsin results in an increasing concentration of epsin in the pellet fraction with clathrin. Epsin itself does not pellet on its own, suggesting that the protein is binding to clathrin as expected.*

4.5.3 Polymerisation with epsin

The final adaptor assessed for its assembly effect in this chapter is epsin. Epsin was incubated with depolymerised clathrin (3.5 μM) at 1:1 and 1:5 molar ratios and assembled through dialysis into polymerisation buffer as previously described. Initial observations from EM images revealed a dramatic change in cage size distribution at both 1:1 and 1:5 epsin ratios (see Figure 4.08 A and B) with a clear increase in uniformity of cage size. Measuring cage radii from these images indicates that the cage sizes seen here are almost exclusively in the range of 30-45 nm with an almost total lack of cages in the 50-70 nm range (see Figure 4.08 C). In the DLS analysis a similar shift is noted with a relative increase in apparent particle concentration in the presence of epsin with a particular increase in particle concentration around 35 nm. Interestingly with the 1:5 fold excess the presence of particles at 25 nm and a similar concentration of larger particles compared to the control is seen. The increase in large particles may be due to an increase in the number of small cages directly interacting or bumping into each other as these structures are not seen in the EM images. As described previously the act of diluting the sample for imaging is likely to have disrupted these interactions. The reason for the appearance of the small particles is not known but could reflect smaller cage intermediates present in solution.

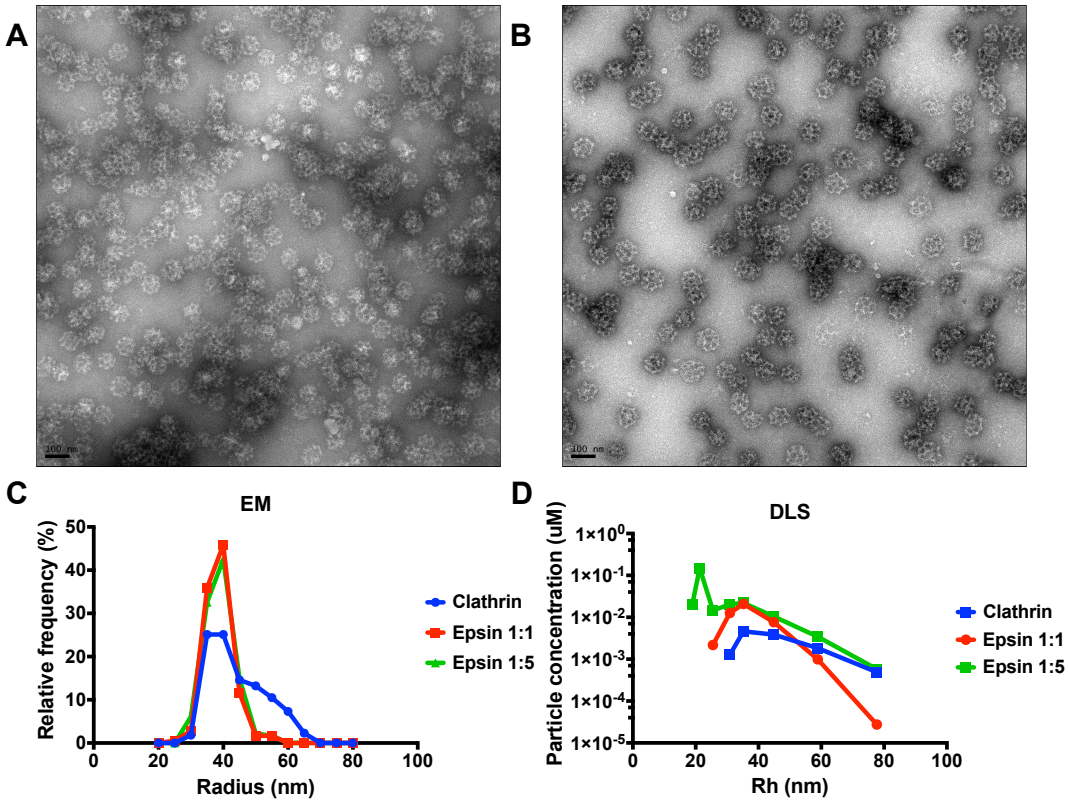

Figure 4.08 Polymerisation of clathrin cages in the presence of epsin as determined by DLS and EM. *Clathrin at 3.5 μM was polymerised at either a 1:1 or 1:5 molar excess of epsin with example EM images at these concentrations shown in A and B respectively. C) The size distribution of cage radii as measured from EM images in ImageJ (n=181 and n = 181 for 1:1 and 1:5 respectively) indicates a tightening in size distribution with the loss of larger particles and a relative increase in particles around 35 to 45 nm in radius. D) Analysis of the sample using DLS shows a similar pattern of distribution in the presence of epsin with an overall increase in particle concentration in the presence of epsin with the additional appearance of particles at 25.5 nm with the 1:5 concentration.*

4.6.1 Discussion

In this chapter I applied the technique of DLS, for monitoring the polymerisation and depolymerisation of clathrin cages and shown how adaptor proteins alter the assembly of these complexes. Whilst these results are preliminary they none the less provide important insights into adaptor assembly effects with the potential biological relevance of the effects discussed here.

4.6.2 Experimental Design

The use of DLS to monitor clathrin cage assembly and disassembly has significant advantages over previous assays. The main advantage of the technique is the ability to determine that clathrin has been fully depolymerised, and that no significant aggregation has occurred, before the addition of adaptors and re-polymerising. This

reduces the chance that variations in the extent of clathrin depolymerisation or aggregation would alter the effect of adaptor binding on re-polymerisation.

It has also been shown that the fitting algorithm, on the whole, accurately reflects the size distribution of clathrin particles measured from EM images and therefore suggests that the technique is applicable for studying this process. The algorithm appears to associate a hydrodynamic radius with cages and oligomers that is applicable to their physical size due to the similarity between the distributions seen from EM measurements and DLS measurements.

There are however differences observed between the two sets of analyses, in particular the apparent increase in the concentration of larger cage particles/aggregates as well as smaller particles in the DLS data compared to EM measurements. The first point to note is that aggregate particles were not directly measured under EM due to the difficulty in accurately measuring the diameter of an amorphous particle. For the same reason smaller particles which were not 'cage like' were ignored and hence this technique is biased towards particles that are easily measured. The second observation is the difference in concentration between the EM images and the data collected using DLS. In order to accurately count cages the samples were diluted from 3.5 μM to 1 μM. Although dilution was conducted shortly prior to blotting and staining on grids this may have reduced the number of larger particles formed from cages reversibly interacting with one another. In the DLS these particles are not differentiated and hence the balance between concentrations conducive to assembly and concentrations where reversible interactions occur must be taken into account. It would therefore be important to vary concentrations of clathrin and adaptors in future experiments.

One significant difference should be noted when comparing the results obtained here and the previous work conducted on the effect of adaptor proteins on cage polymerisation. The vast majority of *in vitro* assembly experiments have been conducted with buffers that are not conducive to clathrin assembly on their own, typically MES based buffers as pH 6.7-6.8 (Goodman *et al*. 1996; Greene *et al*. 2000; Morgan *et al*. 2000; Engvist-Goldstein *et al*. 2001; Legendre-Guillemin *et al*. 2002; Chen *et al*. 2005), or pH 7.1 HKM buffers (Scheele *et al*. 2001; Kalthoff *et al*. 2002). Polymerisation buffer at pH 6.4 was used as the assay had primarily been developed with the use of these buffers to drive clathrin assembly in the absence of adaptors and hence this buffer was used. Given that adaptors can promote assembly at higher pH it

would be important for any future investigations to look into the effect of varying these conditions on adaptor polymerisation.

4.6.3 Adaptor protein assembly effects

4.6.4 Hip1 CC and Hip1R CC polymerisation shows novel and contradictory results

Hip1 CC and Hip1R CC interact with the CLC to initiate assembly which is in contrast to most other adaptors which initiate assembly via interaction with the CHC. The effect of Hip1 binding on cage polymerisation has been investigated previously but the effect on cage size itself has not been investigated. Both Chen *et al.* (2005) and Legendre-Guillemin *et al*. (2002) showed that Hip1 CC or longer constructs can promote clathrin pelleting at pH 6.7/6.8 where clathrin does not naturally polymerise (Legendre-Guillemin *et al*. 2002; Chen *et al*. 2005). Measurements of polymerisation were made from the movement of heavy chain from supernatant to pellet fractions from high speed centrifugation. Therefore, the change in size distribution to one of larger cages has not been previously observed. Interestingly an *in vivo* study noted that over-expressing the central region of Hip1 including the CC domain in various human cell lines resulted in the formation of large vesicular structures (Waelter *et al*. 2001). It is therefore possible that the effect seen here related to the function of Hip1 *in vivo*.

In contrast to Hip1 CC where the promotion effect on assembly has been analysed by centrifugation and SDS-PAGE, work has been conducted to measure the size distribution of Hip1R-polymerised cages. Engqvist-Goldstein *et al*. (2001) measured the size distribution of cages formed in the presence of full length Hip1R at pH 6.8 where clathrin does not naturally assemble (Engqvist-Goldstein *et al*. 2001). They found a particle range of around 50-90 nm in diameter corresponding to 25-45 nm in radius which is significantly smaller than the data observed here (30-80 nm). There are significant differences between this study and the previously conducted one which may explain the differences in the observations made. The first is that the concentrations of protein, in particular clathrin, are significantly higher in this study (200 nM to 3.5 μM). Observations made by Joseph Jones in optimising the DLS approach have shown how lowering the concentration of clathrin to <1 μM shifts the distribution to smaller cage particles (Joseph Jones 2016). It is therefore possible that the cages seen in this study preferentially adopt a small size distribution due to clathrin

concentration rather than the presence of Hip1R. Secondly, the higher pH used by Engqvist-Goldstein *et al*. (2001) may have resulted in a preference for smaller cage sizes. Since the experimental conditions for the DLS measurements had been optimised for clathrin only samples which do not routinely assemble above pH 6.5 the effect of high pH on cage size distribution in this instance has not been determined. Finally, the Engqvist-Goldstein study used the full length Hip1R protein as opposed to the CC domain as used in this study. Although the CC domain is necessary and sufficient for its clathrin interaction it is possible that the full length protein alters the assembly properties of the protein (although Hip1R has no other reported binding regions for clathrin).

In summary the experiments presented here, both the Hip1 and Hip1R CC proteins, in contrast to previous work, seemed to have a negative effect on cage assembly according to the DLS results. However, there are a number of differences in approach between the work presented here and the published study, which include concentration, pH and whether full length or truncated domains were used, which could account for the discrepancy. It is nonetheless interesting to note that the cages formed by both of these proteins tended to be of larger size than those in the absence of either. This may simply be due to changes in apparent concentrations of free clathrin through aggregation. But, given that both of these proteins promote cage formation through interactions with the CLC it is tempting to conclude that this atypical mechanism results in increased cage size.

4.6.5 β2 HA results are also inconsistent with previous observations

The results seen here with β2 HA again seem to contradict previous work indicating that AP2 and β2 HA itself promote the formation of small cages (Greene *et al*. 2000). As described earlier AP2 was the first adaptor shown to induce the preferential formation of small cages (Goodman *et al*. 1996) and β2 HA was shown to have similar activity (Greene *et al*. 2000). In contrast, the results here show a similar size distribution to controls conducted in the absence of clathrin although with the possible increase in overall conversion to cages as determine by DLS. The reasons for this discrepancy are unknown but as noted with previous Hip1/1R CC results it is likely that differences in pH between the studies may have an important role to play in the

differences seen. In addition, Greene *et al.* (2000) used a molar ratio of β2 HA: clathrin of < 1. It may be the case that β2 HA promotes smaller cages at sub stoichiometric concentrations which would become apparent with a wider range of protein concentrations.

4.6.6 Epsin promotes the formation of smaller cages as seen with other adaptors

Direct measurement of the effect of epsin on cage polymerisation has not been investigated before but here we show that epsin is indeed able to promote cage formation with the size distribution created being similar to that seen previously with other adaptors (Ahle and Ungewickell 1986; Goodman *et al*. 1997; Greene *et al*. 2000; Morgan *et al*. 2000; Engvist-Goldstein *et al*. 2001; Kalthoff *et al*. 2002). Although work by Kalthoff *et al*. (2002) inferred that epsin does not produce cages as small and as uniform as with AP180 the results shown here compare more closely to those collected with AP180 at a lower pH than was used in the Kalthoff study (Kalthoff *et al*. 2002). An *in vitro* study by Jakobsson *et al*. (2008) showed that injecting antibodies against either the ENTH domain or the clathrin/adaptor binding region into Lamprey synapses resulted in various changes in synaptic vesicle formation (Jakobsson *et al*. 2008). The crucial observation here is that targeting the clathrin/adaptor region resulted in the the increased size of coated pits (although not diameter) suggesting that epsin may have a role in controlling vesicle size. Similar effects have been noted for AP180 *in vivo* (Nonet *et al*. 1998; Zhang *et al*. 1998; Morgan *et al*. 2000) and *in vitro* (Kalthoff *et al.* 2002) so this lends support that interaction of these adaptors with clathrin is important for regulating vesicle size.

It is interesting to draw comparisons between AP180 and epsin as, although they have a similar structure with a membrane binding domain and unstructured clathrin/adaptor interacting domain, both proteins interact with clathrin through different motifs. AP180 interacts with clathrin primarily though DLL motifs (although some sequences may potentially act as more traditional clathrin boxes) (Kalthoff *et al*. 2002), which bind to the terminal domain (Zhou *et al.* 2015) but also potentially non-specifically to the CHC, whereas epsin primarily interacts with clathrin via its clathrin box motifs that are proposed to be specific for the TD (Drake *et al*. 2000; Drake and Traub 2001). As with observations with other adaptors it is interesting to note that these different

binding modes might result in the same physical outcome – that of a smaller uniform clathrin cages.

4.6.7 Future work

As stated throughout this chapter the work conducted here is preliminary and serves to illustrate that DLS, with the appropriate analysis, can be used to effectively monitor the changes in clathrin cages polymerisation in the presence of adaptor proteins. The technique is highly sensitive to changes in concentration and so large data sets with multiple concentrations of adaptors and clathrin and buffer conditions must be conducted.

In Chapter 5 I introduce a construct of the protein auxilin containing clathrin/adaptor binding mutants to determine their effect on physiological disassembly of clathrin cages through the actions of auxilin and Hsc70. Although auxilin has typically been shown to be recruited to CCVs after scission from the membrane (Saffarian *et al*. 2009; Taylor *et al*. 2011) auxilin has been shown to promote the formation of clathrin cages (Ahle and Ungewickell 1990; Scheele *et al*. 2003). Using similar mutations to the ones introduced in the next chapter Scheele *et al.* showed that mutations to clathrin/adaptor binding motifs reduce binding, reducing the effectiveness of auxilin as a promoter of assembly, although only through the use of pelleting assays (Scheele *et al*. 2003). It would be interesting therefore to employ the use of DLS and EM to study the effect of the mutants in greater detail than in previous studies.

The technique could also be extended to the study of other adaptor proteins. AP180 would be an ideal candidate due to the remarkably similar effects on size distribution to epsin with the effect observed *in vitro* (Morgan *et al*. 2000; Kalthoff *et al*. 2002) and *in vivo* (Nonet *et al*. 1998; Zhang *et al*. 1998; Meyerholz *et al*. 2005; Pretralia *et al*. 2013). Another adaptor protein of interest would be β arrestin. β arrestin interacts with the clathrin terminal domain via a clathrin box motif, however it has not been shown to promote clathrin cage formation (Goodman *et al*. 1996; Kim *et al*. 2002). Quantifying this intriguing observation using this assay would help better understand the mechanisms behind cage assembly.

In addition to the variation of clathrin and adaptor concentrations as well as pH in future experiments the other avenue to explore would be through the use of multiple adaptors in concert. The clathrin coat is a complex interaction between multiple

adaptor proteins, some of which can bind co-operatively whilst others compete for interaction with clathrin. It would therefore be of interest to see how these adaptors interact during clathrin assembly and what the cumulative effects of these interactions may be on the extent or size distribution of the cages assembled. In addition, the technique could be used to investigate the effects of inhibitors of assembly such as the presence of free clathrin terminal domain which has been shown to inhibit assembly (Greene *et al*. 2000). Other inhibitors CME such as the PITSTOP chemicals could be employed (von Kleist *et al.* 2011). These inhibitors are proposed to bind to the terminal domain although their precise binding and mechanism of action have been called into question (Lemmon and Traub 2012). Assembly with adaptors in the presence of these chemicals would be a useful tool for probing binding and assembly mechanisms.

4.6.8 Conclusion

In this chapter I have shown how the DLS technique, combined with a novel analytical algorithm, can be used to monitor the assembly of clathrin cages and the differences induced by the presence of adaptor proteins. I have shown that the data collected by this technique records a similar size distribution to that determined by EM with the advantages and disadvantages of each technique compared. I have shown that different adaptors appear to have different effects on the size distribution of cages formed. It now remains to confirm these preliminary results by repeating the experiments to determine the consistency of the data.

Chapter 5: Disassembly of clathrin cages: investigating the function of Hsc70 and auxilin

"If you thought that science was certain - well, that is just an error on your part" Richard Feynman

5.1.1 Introduction

5.1.2 Overview

In the previous chapter I investigated the effect of adaptor proteins on the assembly of clathrin cages and how they alter the size distribution of the cages formed. The other major aspect of clathrin function in CME that I have investigated during this project is how the clathrin cage is disassembled. This process plays an important role in the cell by allowing the recycling of protein coat components and allowing efficient trafficking of vesicles formed during CME. In the following 3 chapters I investigate the kinetics of disassembly of clathrin cages with the effect of adaptor proteins addressed in Chapter 6 and finally a detailed investigation into the epsin inhibitory effect in Chapters 7 and 8.

In this chapter I specifically address the role of the clathrin cage disassembly proteins Hsc70 and auxilin through the use of functional mutants of auxilin. Here I show how mutations to auxilin clathrin/adaptor binding motifs alters the ability of auxilin to effectively disassemble clathrin.

5.1.3 Summary of the role of Hsc70 and Auxilin in clathrin cage disassembly

The mechanism of clathrin cage disassembly is briefly summarised again here, a more detailed appraisal of the process and mechanisms can be found in section 1.9.1. The disassembly of the clathrin coat from the budded vesicle is driven *in vivo* by Hsc70

and auxilin/GAK. Whilst the precise mechanism hasn't been fully elucidated we know that the process is driven by recruitment of Hsc70 to the clathrin structure through the J-domain of auxilin or GAK (Ungewickell *et al*. 1995; Greener *et al*. 2000; Umeda *et al*. 2000). Auxilin/GAK associates with clathrin through various clathrin binding motifs that bind to the CHC (Sheele *et al*. 2001; Sheele *et al*. 2003) which allow auxilin to interact with the clathrin terminal domain and distal leg under the hub of the triskelion (Smith *et al*. 2004; Fotin *et al*. 2004a; Xing *et al*. 2010). The J-domain of these proteins recruits Hsc70 to the structure (Holstein *et al*. 1996) where Hsc70 binds to a specific QLMLT motif on the unstructured C-terminal of the CHC, positioned under the hub of the clathrin triskelion (Rapoport *et al*. 2008; Boecking *et al*. 2011; Sousa *et al*. 2016). The J-domain stimulates ATP hydrolysis by Hsc70 which in turn promotes tight binding of Hsc70 to clathrin (Holstein *et al*. 1996; Barouch *et al*. 1997). Recent evidence gathered by Sousa *et al*. suggests that recruitment of Hsc70 induces disassembly by a collision induction mechanism, with the mass of Hsc70 placed under the trimerisation domain causing disruption of the clathrin cage structure (Sousa *et al*. 2016). Evidence from various studies suggests that the binding and ATP hydrolysis at a stoichiometry of at least 2 Hsc70 molecules per triskelion is required to cause dissociation (Rothnie *et al*. 2011; Boecking *et al*. 2011) although up to 3 Hsc70 molecules can bind per triskelion (Schlossman *et al*. 1984; Prasad *et al*. 1994). Again, although up to 3 auxilin molecules may bind to a single triskelion the maximum rate of disassembly is reached at a ratio of 1 auxilin molecule per triskelion (Holstein *et al*. 1996; Ma *et al*. 2002; Rothnie *et al.* 2011). Auxilin/GAK is able to dissociate from the free triskelia and recycle to recruit further Hsc70 proteins to the clathrin structure (Barouch *et al*. 1997; Ma *et al*. 2002; Rothnie *et al.* 2011) where as Hsc70 is believed to remain associated with triskelia, possibly to prevent re-formation of the clathrin coat and to provide a pool of free triskelia ready to form new clathrin structures (Black *et al*. 1991; Schlossman *et al*. 1984).

5.1.4 Studying clathrin cage disassembly

The mechanism of disassembly has been studied *in vitro* using a variety of methods. Much work has focused on incubating CCVs or cages in the presence of Hsc70, ATP and auxilin which is then analysed by ultracentrifugation/SDS-PAGE pelleting (e.g. Ungewickell *et al*. 1997) or electron microscopy (e.g. Dannhauser and Ungewickell 2012). These methods have provided us with a lot of information on the disassembly

of clathrin structures but lack the ability to monitor the process in real time. Another approach has been to use fluorescently labelled clathrin and Hsc70 by Böcking *et al.* (2011) to correlate in real time the recruitment of Hsc70 to clathrin and this has indicated that recruitment of 2 Hsc70 for every 3 available binding sites (i.e. 2 Hsc70 per triskelion) is required for disassembly. Another technique that has been employed is the use of light scattering either in the form of dynamic light scattering (Jiang *et al*. 2005; Schuermann *et al*. 2008) or perpendicular light scattering. Use of light scattering allows monitoring of the activity of auxilin, Hsc70 and clathrin in real time without the need for fluorescent labelling. Perpendicular light scattering has been employed by the Smith group at Warwick has been to used to understand auxilin recycling (Rothine *et al*. 2011) and the role of clathrin light chains in disassembly (Young *et al*. 2013). More recently this technique was employed by Sousa *et al*. (2016) in their investigation into the mechanism of Hsc70 disassembly (Sousa *et al*. 2016). It is this method that was employed primarily in this chapter and subsequent chapters to answer a number of question on disassembly.

5.1.5 Clathrin-adaptor binding mutants in auxilin are implicated in disassembly

The role of auxilin as the recruiter and stimulator of Hsc70 binding to clathrin cage and subsequent disassembly has been well studied. Auxilin contains multiple binding motifs for both clathrin and adaptor proteins (Scheele *et al*. 2001). Scheele and colleagues investigated the role of a number of the DPF and DLL and motifs in auxilin binding and its effect on assembly and disassembly. In relation to disassembly they showed that mutating the ^{574}DPF motif to APA and both the ^{591}DLL and ^{781}DLL to ALA significantly reduced affinity to clathrin and the extent of disassembly of CCVs (Scheele *et al*. 2003). Extending the study initiated in this paper, we investigated the effect of these motifs on disassembly by creating the following mutants:

^{579} DPF->APA + ^{674} DPF->APA (hereafter referred to as auxilin APAx2)

^{781} DLL->ALA + ^{591} DLL->ALA (hereafter referred to as auxilin ALAx2)

^{781} DLL->DAA + ^{591} DLL->DAA (hereafter referred to as auxilin DAAx2)

A diagram of the location of the mutants in the auxilin construct is shown in Figure 5.01.

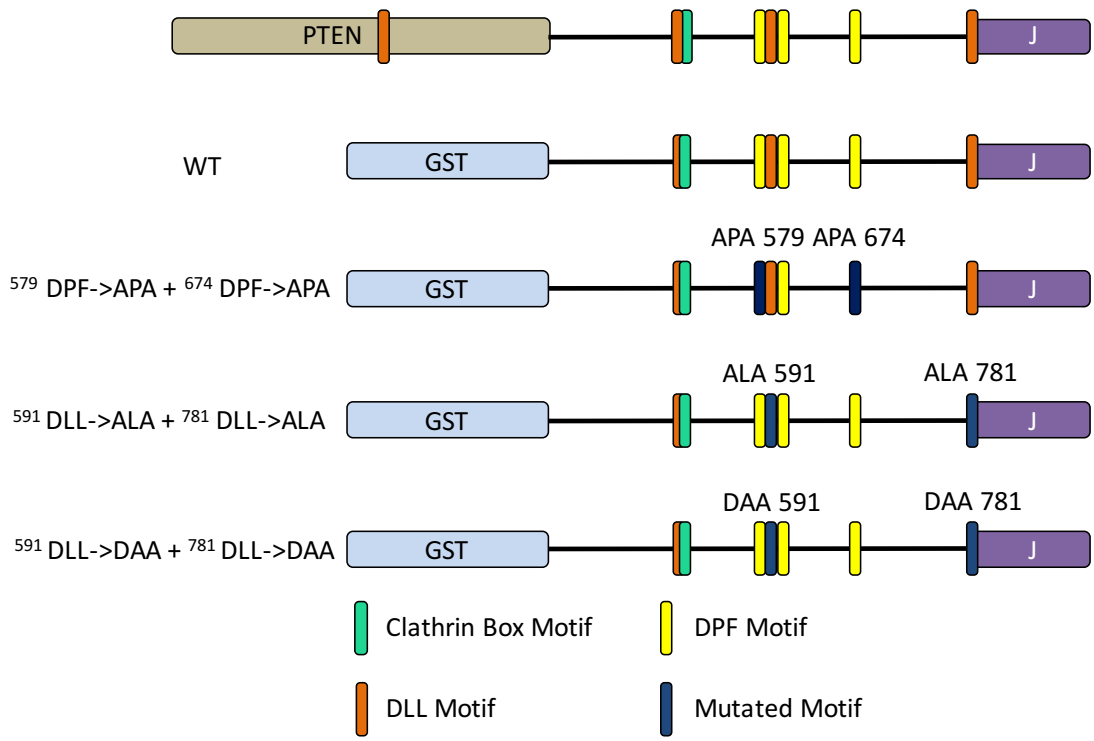

Figure 5.01 Diagrammatic representation of auxilin and the GST-auxilin$_{401-910}$ WT and clathrin/adaptor binding mutants generated for use in this investigation *with key binding motifs/mutations indicated. Auxilin contains a clathrin box motif at position 496 (cyan) along with the following DLL/DPF motifs (^{214}DLL, ^{495}DLL, ^{579}DPF, ^{591}DLL, ^{605}DPF, ^{674}DPF, ^{781}DLL). Mutations were made to GST-auxilin$_{401-910}$ to alter the DPF motifs at positions 579 and 674 to APA as per Scheele et al 2003 and is named as APAx2 (DPF 605 was not mutated as it was shown to have little effect on clathrin binding. Constructs were also designed with the DLL motifs at 591 and 781 mutated to ALA or DAA respectively. The DLL motif at 495 was excluded from the mutations as it is essentially a constituent of the clathrin box.*

These constructs take into account the subsequent discovery of a clathrin box motif (Smith *et al*. 2004) present at residue 495 that was not present in this previous study (residues 401-910 used here compared to 547-910 in Scheele *et al*. 2003) that binds the clathrin TD. These mutants retain this binding site whilst loosing the function of the other motifs and so will allow specific investigations of these motifs. In addition, the DPF and DLL motifs are addressed in isolation as opposed to in Scheele *et al*. 2003 where they are addressed together. Mutating these in isolation allowed interrogation of these motifs individually as they have been shown to have different functionality, with DLL being specific to clathrin binding where as DPF has dual specificity to clathrin and AP2 (Scheele *et al*. 2001). The final mutant (DAAx2) also addresses the functional importance of the aspartate and leucine residues in the motif and serves as a comparison to the ALAx2 mutant.

5.2.1 The effect of mutating auxilin-clathrin/adaptor binding motifs on clathrin cage disassembly

5.2.2 GST-auxilin mutants bind to clathrin cages

The ability of clathrin/adaptor binding auxilin mutants to bind cages was determined by incubation of GST-auxilin$_{401\text{-}910}$ WT, ALAx2, DAAx2 and APAx2 with clathrin cages. Pelleting of clathrin was then conducted using ultracentrifugation and samples from the supernatant and re-suspended pellet were analysed using SDS-PAGE. Data for WT, ALAx2, DAAx2 and APAx2GST-auxilin mutants is displayed in Figure 5.02. GST-auxilin in the absence of clathrin does not appear in the pellet due to its low mass (and therefore does not appear to be aggregating) but it does appear in the pellet in the presence of clathrin, suggesting that the protein is binding to clathrin as expected. Both ALAx2 and DAAx2 mutants also pellet with clathrin with no obvious loss of binding compared to WT, although this was only conducted at a single concentration so concentration dependent effects may occur. Binding of these constructs is expected as, unlike in Scheele *et al*., all contain the clathrin box motif at residue 496 as well as the other set of motifs (DPF in ALAx2 and DAAx2 mutants and DLL in the APAx2 mutant). This allows specific analysis of these motifs on cage disassembly without completely removing binding of the proteins to clathrin.

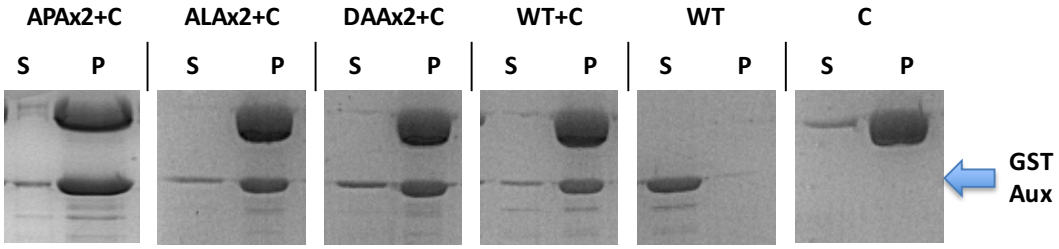

Figure 5.02: Binding of wild type (WT) and mutant GST-auxilin$_{401\text{-}910}$ to clathrin cages under ultracentrifugation as determined by SDS-PAGE*. WT, APAx2, ALAx2 and DAAx2 mutant GST-auxilin proteins incubated with clathrin at 1:1 molar ratio (3 μM) were pelleted at 140,000 x g and samples of the supernatant (S) and pellet (P) were analysed by SDS-PAGE. Clathrin (C) pellets as expected where as the GST-auxilin control (WT) is present in the supernatant. Mutants APAx2, ALAx2 and DAAx2 both bind to clathrin equally as well as WT auxilin with the majority of protein present with clathrin in the pellet.*

5.2.3 Disassembly of clathrin cages with auxilin mutants reveals differences in disassembly kinetics

The effect of auxilin clathrin binding mutants on disassembly was monitored using the perpendicular light scattering method that is described in section 2.4.6 and the data from these experiments is summarised in Figure 5.03. Disassembly was conducted using fixed clathrin and Hsc70 concentrations with concentrations of GST-auxilin varied with 100 nM, 50 nM, 25 nM and 10 nM being used. With WT-GST auxilin reducing the concentration reduces the rate of disassembly as determined by the t ½ (Figure 5.03 F) but does not significantly alter the final light scattering intensity which can be interpreted as the extent of disassembly. These results broadly correspond to the results obtained previously by Rothnie *et al*. (2011) where t ½ drops in a linear fashion below 50 nM GST-auxilin. However, there is a slight difference in the trend in end-scatter intensity where the general trend is a reduction in scattering intensity as the concentration of GST-auxilin used in the disassembly assay is reduced (with the exception of the ALAx2 mutant). The reason for this is not known but both observations are still in keeping with the hypothesis that auxilin is able to dissociate from triskelia and re-bind to clathrin cages to stimulate further disassembly.

In the case of the mutants there appears to be little difference in half life or final scatter at 100 nM but as the concentration is reduced the differences become more pronounced. At 10 nM the differences are particularly pronounced where both WT and the DAAx2 mutants have very similar t ½ (65.5±4.76 s and 75.25±4.75 s) and and final light scattering intensity (0.24±0.03 s and 0.18 ±0.03 s). However, with t ½ in the APAx2 and ALAx2 we can see that these values are significantly higher than compared to the WT (99.25±4.99 s and 126.25±4.6 s respectively). This suggests that at limiting concentrations of auxilin mutating these residues has an effect on the ability of auxilin to recycle back to clathrin to initiate further rounds of disassembly. The difference between the DAAx2 and ALAx2 mutants in this regard is also interesting as mutating out the aspartate residue of the DLL motif seems to have a greater effect on the rate of disassembly compared to mutating the two leucine residues. In addition, the greater reduction in t ½ of the ALAx2 mutant compared to the APAx2 mutant is also interesting. Scheele *et al*. and others have shown that the DPF motif has been shown to have dual binding affinity to clathrin and the α ear domain of the AP2

complex where as the DLL motif is specific to clathrin (Scheele *et al*. 2001). This therefore suggests that the clathrin binding specific motifs may be more important for auxilin disassembly than the dual function binding motifs. This may be due to a decrease in affinity of auxilin for clathrin, limiting the effectiveness of auxilin in disassembly.

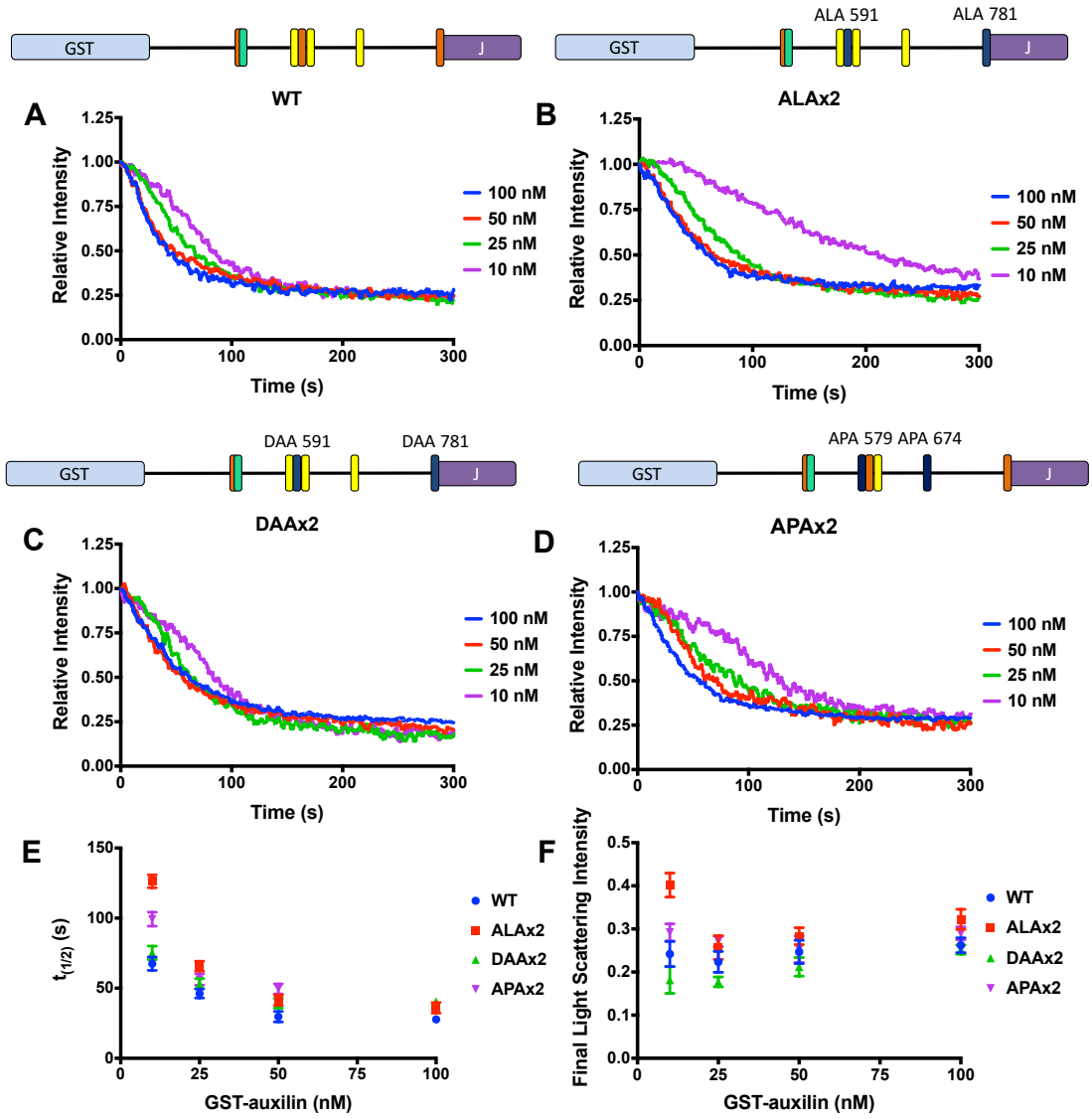

Figure 5.03 Disassembly of clathrin cages using varying concentrations of WT and clathrin/adaptor binding mutants of GST-auxilin$_{401-910}$ and Hsc70 as monitored by light scattering. *GST-auxilin was mixed with 250 nM of clathrin cages and an excess of ATP (500 μM). Disassembly was initiated through the addition of 1 μM Hsc70. Traces A-D show disassembly with WT (A), ALAx2 (B), DAAx2 (C), APAx2 (D) GST-auxilin at 100 nM, 50 nM, 25 nM and 10 nM respectively with a diagrammatic representation of the location of the mutants shown above. The half life (t ½) and final light scattering intensity as calculated for each auxilin variant are shown in E and F respectively. E) With t ½ the trend is for a longer half life with lower concentration of GST-auxilin as expected with difference becoming more apparent at lower concentrations of GST-auxilin. At low concentrations (10 nM) a major difference can be seen between the WT and the APAx2 and ALAx2 mutants which have a longer half life than the WT. This difference is not seen with the DAAx2 mutant. F) In terms of light scattering the end intensity is similar across all variants with the exception of the ALAx2 mutant at 10 nM which is significantly higher than the other mutants and WT.*

When looking at the final light scattering intensity as a measure of the extent of disassembly the general trend is for a drop in end intensity (an increase in the apparent extent of disassembly) with concentration of GST-auxilin. This observation is slightly at odds with results obtained previously where low concentrations of GST-auxilin (<25 nM) resulted in a slight reduction in the extent of disassembly at similar concentrations (Rothnie *et al*. 2011). This effect may be due to the apparent reduction in starting scattering intensity with lower concentration of auxilin (which is normalised in the analysis). Whilst this trend is not seen with any of the variants in this experiment the extent of disassembly is reduced again with all variants at 10 nM (with the exception of the DAAx2 mutant). The ALAx2 and APAx2 mutants show higher final light scattering values compared to the WT at 10 nM (0.402±0.028:ALAx2 and 0.292±0.020:APAx2 compared to 0.242±0.029:WT). This may indicate that these proteins are less able to disassemble clathrin due to reduced binding affinity at low concentrations.

5.5.1 Discussion

In this chapter I have described the use of the perpendicular light scattering assay developed by Alice Rothnie to differentiate the effects of mutant variants of auxilin during disassembly. The implications and conclusions from these observations are discussed here.

5.5.2 Implications for auxilin clathrin/adaptor binding motifs in cage disassembly

The role of auxilin in facilitating the disassembly of clathrin cages is linked to its ability to interact with Hsc70, clathrin and adaptor proteins through various binding motifs. By building on the work of Scheele *et al*. (2003) I have shown how mutating some of these motifs alters the ability of auxilin to disassemble clathrin and has implications for the function of auxilin.

5.5.3 Comparisons between DPF and DLL mutants

The results obtained here suggest a number of possibilities in relation to the DLL, DPF and clathrin box motifs. All mutations to the DPF and DLL motifs that were investigated here showed no obvious loss in binding as determined by pull downs using clathrin cages although it would be desirable in future work to determine binding

affinities using techniques such as ITC or SPR. Regardless, the fact that these proteins can bind through the retention of other binding motifs and the remaining clathrin box is notable.

The fact that the mutants do not seem to have a major effect on the extent of disassembly as determined by the final light scattering intensity, but do slow the rate of disassembly at low concentrations (particularly the ALAx2 and APAx2 mutants), seems to suggest that these motifs are important for the rate of auxilin recycling. Auxilin is believed to be able to dissociate from triskelia and re-bind to clathrin cages to facilitate further rounds of disassembly (Barouch *et al*. 1997; Ma *et al*. 2002; Rothnie *et al*. 2011). At high concentrations (100 nM) auxilin saturates clathrin and the rate is dependent on ATP-hydrolysis by Hsc70 and the subsequent dissociation of triskelia. At low concentrations (10 nM) the concentration of auxilin becomes a limiting factor and hence once dissociation of a triskelia has been initiated auxilin must be recycled to initiate another round of recruitment of Hsc70 to still intact clathrin cages. It would therefore suggest that mutations to the DPF and DLL motifs alter the ability of auxilin to either dissociate from free triskelia or to re-bind to cage structures and hence the rate of disassembly is reduced. One study that lends support to this is the work of Ma *et al.* (2002) who showed that an AP180 clathrin binding domain-auxilin J-domain chimera could disassemble clathrin (Ma *et al*. 2002). However, the construct had to be added in a 1:1 molar ratio to CHC, i.e. it did not act catalytically, suggesting that the clathrin binding domain of auxilin is not only important for the binding of auxilin but also for its ability to dissociate and act as a catalyst for disassembly.

When we look at the differences between the effects of the mutants the first observation is the difference in effect between the two sets of DLL mutants; that where DLL is mutated to ALA and those where DLL is mutated to DAA. The DAAx2 mutant showed a similar trend in t ½ relative to the WT where as the ALAx2 mutant was much slower at low concentrations of auxilin. This seems to suggest that the aspartate residue here is key to the interaction of of this motif with clathrin. There is an explanation in the context of the aspartate negative charge which seems to be implicated in clathrin and adaptor binding motifs. The mutation of leucine to alanine may be less significant due to the substitution of one hydrophobic amino acid for another, albeit with a shorter side chain.

The second observation is the difference between the DPF motifs and the DLL motifs. Again at low concentrations of auxilin the DPF->APAx2 mutant shows a reduction in t ½ relative to WT auxilin although not as much as the DLL->ALAx2 mutant (WT:65.5±4.76 s, APAx2: 99.25±4.99 s and ALAx2: 126.25±4.60 s respectively). An explanation for these observations may be attributable to the fact that the DPF motif is implicated in interactions with AP2 as well as (to a lesser extent) with clathrin where as the DLL motifs seems to be exclusively related to interactions with clathrin (Scheele *et al*. 2001). Scheele *et al*. (2003) also demonstrated that mutating different DPF motifs alters the binding to the α-adaptin ear domain more significantly than binding to the terminal domain (Scheele *et al*. 2003). This would suggest therefore that the exclusive binding of the DLL motif with clathrin is more important in relation to disassembly.

5.5.4 Explaining observed differences between this study and Scheele *et al.* (2003)

By further comparisons to the data obtained in this project with the work of Scheele and colleagues, from which this project was based, there are differences to explore. To determine the effect of the motif mutants on disassembly Scheele et al. (2003) used changes in clathrin heavy chain gel band intensity after incubating CCVs in the presence of Hsc70 and the varying auxilin constructs. Although direct comparisons are difficult (as the authors did not look specifically at the DPF and DLL mutants in isolation but rather in combination) their results show that the more mutations that were made to these motifs, the less clathrin was released from the CCVs. This is at odds with the observations made here where the rate of disassembly is significantly altered with these mutants rather than the extent. The differences in assay design as well as the constructs used can however provide explanations for these differences.

First the use in this study of a longer construct containing the additional clathrin box motif that was not present in the shorter constructs used in the Scheele study. Although the contribution of this motif's affinity to clathrin has not been specifically investigated it may be inferred that this motif is of greater importance for clathrin binding and facilitating Hsc70 recruitment during disassembly. Although it should be noted that auxilin can function in clathrin assembly without this motif as seen in Scheele *et al*. and many others (Ungewickell *et al*. 1995; Barouch *et al*. 1997; Ma *et al*. 2002; Bocking *et al.* 2011; Dannhauser and Ungewickell 2012; Sousa *et al*. 2016).

In addition, clathrin cages lacking the clathrin terminal domain (to which the clathrin box in auxilin binds (Smith *et al*. 2004)) can be disassembled and therefore, whilst this motif can interact with clathrin it is not crucial for disassembly (Ungewickell *et al*. 1995). However, the decay to a baseline intensity that is observed in the light scattering assay is consistent with the 'completion' of disassembly of clathrin cages as determined by EM (Rothnie *et al*. 2011) and hence completion may be more accurately confirmed through this assay than gel densitometry.

The second point is the use of lipid free clathrin cages in contrast to the CCVs used in the earlier, published, study. The light scattering assay is not compatible with lipids due to their high scattering intensity and hence a direct comparison is not possible using this technique. In addition, the CCVs themselves will contain adaptors that may alter that ability of auxilin to be recruited to clathrin, particularly where mutations to the DPF motifs would reduce any interactions with AP2 that might be required to destabilise the complex.

Finally, the difference in concentration of the components used should be taken into account. In effect the lowest ratio of clathrin to auxilin concentrations that was investigated in this study was 0.04 (10 nM GST-aux$_{401-910}$ to 250 nM clathrin) where as in Scheele the greatest effect were observed at a ratio of 0.0167 (15 nM aux$_{547-910}$ to 900 nM clathrin) and so reducing concentrations of our auxilin constructs to a lower concentration may reveal a more comparable effect, although issues concerning the accuracy of the concentration may become more prevalent.

5.5.5 Future work

To take this project further it would be interesting to look at these mutants in the context of a stopped-flow set-up. Stopped-flow has been used previously to study cage disassembly using light scattering and has significant advantages over the fluorimeter based assay. Using this form of set-up allows for a much more consistent mixing than is possible with manual pipetting (mixing is controlled by hydraulic syringes) and a greater amount of control over the mixing of components (up to 4 syringes can be mixed in varying ratios). As has been shown in Rothnie *et al*. (2011) and Sousa *et al*. (2016) the technique allows for the interrogation of the initial stages of recruitment of auxilin/Hsc70 to clathrin cages and the accurate fitting and modelling of the process. However, it does require large amounts of material in relation to volume which is of

particular relevance with Hsc70, which is difficult to express in large concentrations in insect cells. Further investigations using this technique would provide complementary information to the studies conducted and may reveal subtle differences between mutants that are not observable in the standard fluorimeter based assay (such as the differences between the ALAx2 and DAAx2 mutants).

Another major avenue of exploration would be to investigate the effect of mutating the clathrin box motif in auxilin with or without the associated DPF and DLL motif mutations. This would allow the interrogation of the contribution of this motif to the results observed here and shed light on the importance of this motif in relation to clathrin cage disassembly. As the DPF motifs are implicated with interacting with adaptors such as AP2 the effect and implications of adaptor proteins on how these mutations effect the disassembly of adaptor-cage complexes is explored in Chapter 7.

5.5.6 Conclusion

In conclusion in this chapter the effects of mutations on the disassembly protein auxilin and its ability to disassemble clathrin cages. Mutating auxilin clathrin/adaptor binding motifs result in a reduction in the rate of disassembly at low concentrations, suggesting that the motifs are important for the recycling of auxilin back to cages after the dissociation from triskelia. Further investigations into the role of these motifs in interactions of adaptors along with the use of stopped-flow will reveal the role of these motifs in greater detail.

Chapter 6: Disassembly of clathrin cages: investigating the effect of adaptor proteins

'The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" but "That's funny…"' Isaac Asimov

6.1.1 Introduction

6.1.2 Overview

In the previous chapter I investigated the role of auxilin binding motifs in clathrin cage disassembly. In this chapter I have extended the investigation of cage disassembly to the question of how the multiple adaptor proteins, which are involved in CME, might influence the clathrin disassembly process. By incubating adaptor proteins with clathrin cages I hoped to gain further insights into the role of auxilin in disassembly and its interaction with adaptors and determine if the promotion/stabilisation of cage complexes by adaptors affects the disassembly of clathrin cages by Hsc70 and auxilin.

6.1.3 Adaptors and clathrin cage disassembly

The role of adaptor proteins in facilitating interactions between clathrin, the plasma membrane, cargo, the cell cytoskeleton and other adaptors means they play an integral role in CME. As discussed in Chapter 4 many adaptor proteins have been shown to promote the formation of clathrin cages but the mechanisms by which they do this are not fully understood. One common proposed mechanism of assembly could be that adaptor proteins work mechanically, by linking multiple triskelia together and thereby stabilising the clathrin cage structure (Greene *et al*. 2000; Morgan *et al*. 2000; Drake *et al*. 2000; Drake and Traub 2001; Kalthoff *et al*. 2002). If this is the case, then it is possible that the binding of adaptor proteins would make it more difficult for auxilin and Hsc70 to disassemble the complex as a result of making it more stable.

The other question concerning adaptors is how the interactions between clathrin and adaptors behave during disassembly i.e. do the interactions spontaneously break as triskelia are removed from the complex or are they actively disrupted? One possibility is that auxilin and Hsc70 may interact or compete with adaptors to break these interactions. Adaptor proteins such as AP2 and epsin bind via clathrin box motifs (Owen *et al*. 2000; Drake *et al*. 2000; Drake and Traub 2001) which auxilin also shares (Smith *et al*. 2004). It has been shown that peptides containing the β2 HA clathrin box ot the auxilin clathrin box compete for binding to the clathrin terminal domain (Smith *et al*. 2004). In addition, other binding motifs such as the DLL and DPF/W motifs are shared between auxilin and adaptors such as epsin (Drake *et al*. 2000; Scheele *et al*. 2001; Kalthoff *et al*. 2002). These data and observations have lead to the hypothesis that auxilin may actively compete with adaptor binding to clathrin to displace the adaptors from binding to clathrin (Scheele *et al*. 2001; Smith *et al*. 2004). However, adaptors such as the Hip1 and Hip1R proteins interact directly with the CLC and so the mechanism by which these interactions might be disrupted (if this is indeed required) is not known.

In this chapter I show that the Hip1/1R CC domains and β2 HA adaptors have little effect on the ability of auxilin and Hsc70 to disassemble clathrin cages. However, with the adaptor protein epsin a strong inhibitory effect is observed that is dependent on the concentration of both epsin and auxilin. This effect is then investigated further in Chapters 7 and 8.

6.2.1 Disassembly with β2-adaptin-cages

6.2.2 β2-adaptin binds to clathrin cages in the presence or absence of GST-auxilin

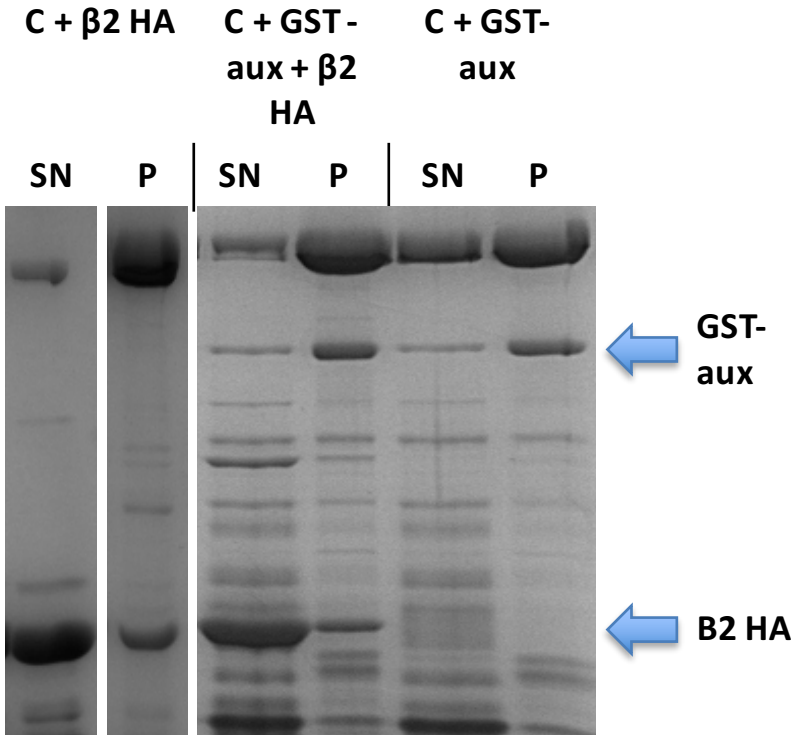

Figure 6.01. SDS-PAGE analysis of β2 HA and GST-auxilin binding to clathrin cages as determined by ultracentrifugation *indicates both proteins can bind to clathrin in concert. Incubation of 3 μM of cages with 15 uM of β2 HA (C+β2 HA) shows that β2 HA is present with clathrin in the pellet (P) suggesting binding as expected. Incubation of clathrin cages (3 μM) with 1:1 molar ratio of β2 HA and/or GST-aux. Both GST-aux and β2 HA can both bind to clathrin in concert (C+GST-aux+β2 HA) as both bands can be seen in the pellet with clathrin. This gel is representative of multiple experiments.*

Purified β2 HA was incubated with clathrin cages in the presence or absence of GST-auxilin and then pelleted to determine if both proteins are able to interact with clathrin at the same time (see Figure 6.01). β2 HA has previously been shown to bind in concert with auxilin (Scheele *et al.* 2003; Boecking *et al*. 2011) but other evidence using peptides of both clathrin boxes form auxilin and β2 HA show a level of competition for binding to the clathrin terminal domain (Smith *et al*. 2004). The results here confirm that both proteins interact with clathrin and are able to interact with clathrin at the same time. Again concentration titration of both proteins was not conducted and so it is possible that at certain concentrations there may be an element of competition for binding to clathrin.

6.2.3 β2 HA has no significant effect on cage disassembly

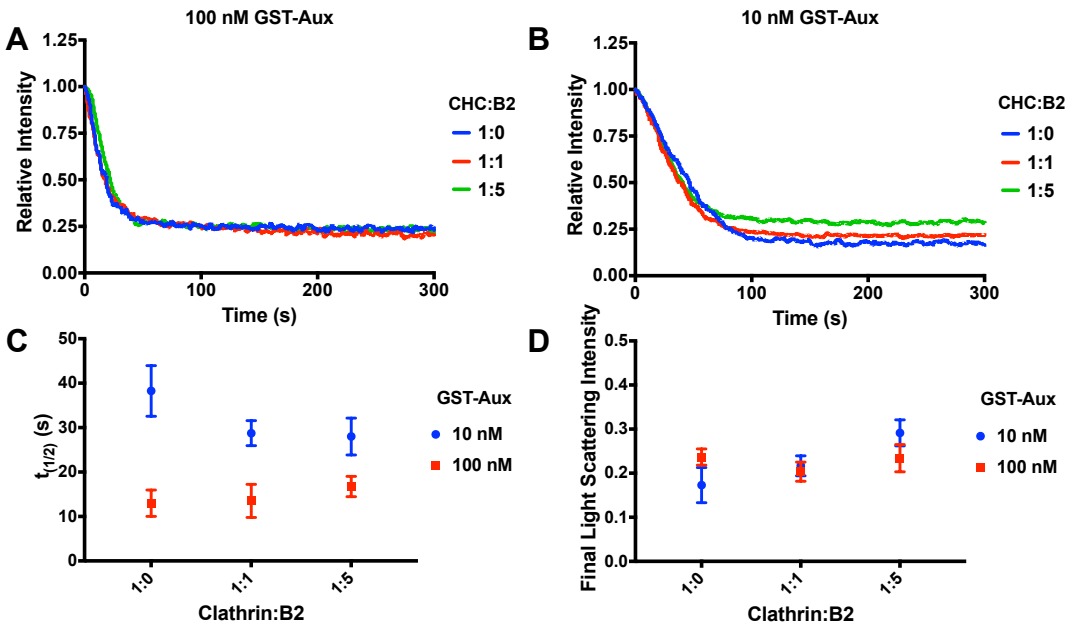

Figure 6.02. Disassembly of clathrin cages incubated with increasing concentrations of β2 HA. *Clathrin cages (3 μM) were incubated with molar ratios of 1:0, 1:1 and 1:5 excess of β2 HA and disassembled in the presence of 100 nM (A) or 10 nM (B) GST-auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Analysis of the t ½ indicates that β2 HA has no significant effect on the rate of cage disassembly at high concentrations of auxilin (100 nM) although at 10 nM auxilin there may be a slight increase in the rate of disassembly. D) There appears to be little change in final light scattering intensity in the presence of β2 HA at either 10 nM GST-auxilin or 100 nM GST-auxilin although with 10 nM GST-auxilin there is a trend to a higher light scattering intensity with increased concentration of β2 HA although this does not lie outside the error of the values obtained for 100 nM GST-auxilin.*

Disassembly of β2 HA-clathrin cage complexes was conducted after incubating clathrin cages at 3 μM with 1:1 and 1:5 molar ratios of clathrin to β2 HA. The adaptor-cage sample was diluted to 250 nM and disassembled with GST-auxilin at 100 nM and 10 nM in the presence of 1 μM Hsc70 and 500 μM ATP as described previously, the data for which is shown in Figure 6.02. At 100 nM GST-auxilin both final scattering intensity and t ½ do not change with the change in concentration of β2 HA. At 10 nM GST-auxilin a possible effect on the reduction in t ½ is observed (Figure 6.02 C). However this effect may be an artefact of the apparent reduction in final scattering intensity that is seen with increased concentrations of β2 HA (Figure 6.02 D). In conclusion it appears that β2 HA has no significant effect on clathrin cage disassembly.

6.3.1 Disassembly with Hip1CC and Hip1R CC cages

6.3.2 Hip1 CC and Hip1R CC have negligible effects on disassembly of clathrin cages

Disassembly of cages incubated with Hip1 CC and Hip1R CC at ratios of 1:1 and 1:5 molar excess was conducted as described previously and the data is displayed in Figure 6.03 and Figure 6.04 for Hip1 CC and Hip1R CC respectively. In the case of Hip1 CC there is little change in either t ½ or final scatter intensity with 100 nM GST-auxilin. With 10 nM auxilin there may be a slight increase in t ½ at a 1:5 excess of Hip1 CC and final scattering intensity increases slightly in the presence of Hip1 CC although in both cases the errors in these values indicate that there is little difference between these values and the control. The pattern for Hip1R CC is similar to Hip1 CC with possible slight increases in final scatter intensity at low auxilin concentrations but any difference is negligible.

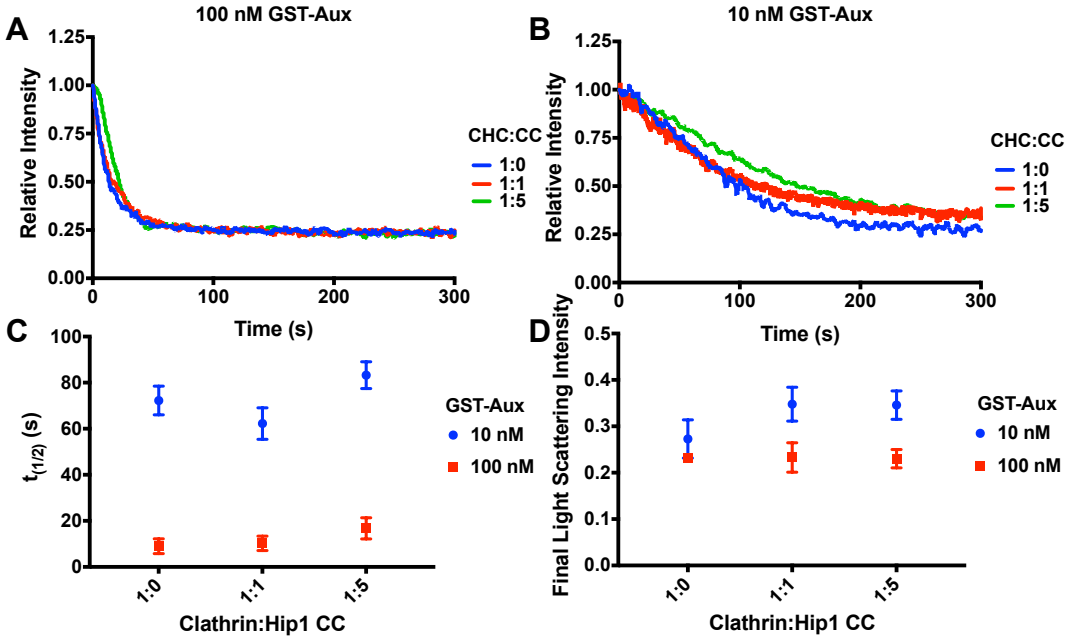

Figure 6.03 Disassembly of clathrin cages incubated with increasing concentrations of Hip1 CC. *Clathrin cages incubated at molar ratios of 1:0, 1:1 and 1:5 excess of Hip1 CC were disassembled in the presence of 100 nM (A) or 10 nM (B) GST-auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Analysis of the t ½ indicates that Hip1 CC has no significant effect on the rate of cage disassembly. D) However there appears to be an increase in final light scattering intensity in the presence of Hip1 CC at either 10 nM GST-auxilin relative to 100 nM GST-auxilin.*

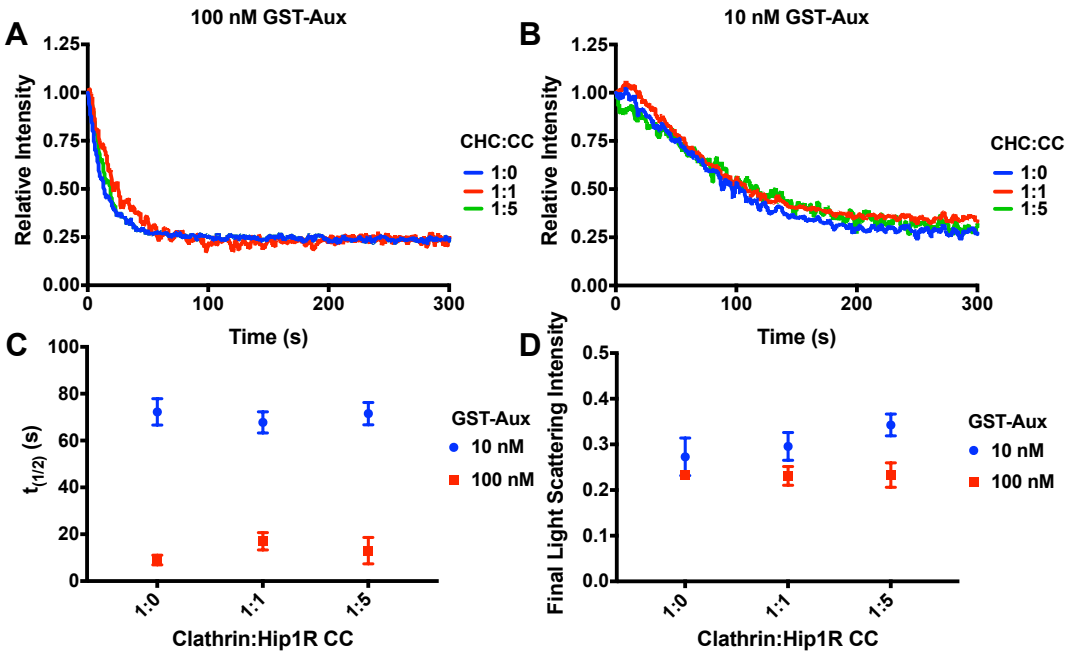

Figure 6.04 Disassembly of clathrin cages incubated with increasing concentrations of Hip1R CC. *Clathrin cages incubated at molar ratios of 1:0, 1:1 and 1:5 excess of Hip1R CC were disassembled in the presence of 100 nM (A) or 10 nM (B) GST-auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Analysis of the t ½ indicates that Hip1R CC has no significant effect on the rate of cage disassembly. D) There does seem to be a slight increase in final scattering intensity as the concentration of Hip1R CC is increased which is observed at 10 nM GST-auxilin but not 100 nM.*

6.4.1 Disassembly with epsin-clathrin cages

6.4.2 Epsin has a concentration dependent inhbitory effect on clathrin cage disassembly

Epsin-clathrin cage complexes were disassembled using the experimental procedures established in the previous sections (see Figure 6.05). The presence of epsin either at a 1:1 molar ratio or at a 1:5 excess shows a significantly different disassembly profile at either 100 nM GST-auxilin (A) or 10 nM GST-auxilin (B) with the curves reaching a plateau at a far higher intensity compared to the WT. When analysing the t ½ when epsin is present at 1:1 molar ratio both the rates for 100 nM GST-auxilin (71.5±4.75 s) and 10 nM (66.5±5.75 s) are comparable to the rate for the 10 nM control in the absence of epsin (72.25±4.0 s). At 1:5 there is a divergence from this pattern with the 100 nM value remaining high but the 10 nM apparently becoming much faster (61.25±3.75 s at 100 nM and 23.0±3.75 s). The reason for this seems to be the apparent difference in the curve shape of the 1:5 epsin sample incubated with 10 nM GST-auxilin. Alternatively, this could be indicative of epsin delaying the recruitment of Hsc70 by auxilin.

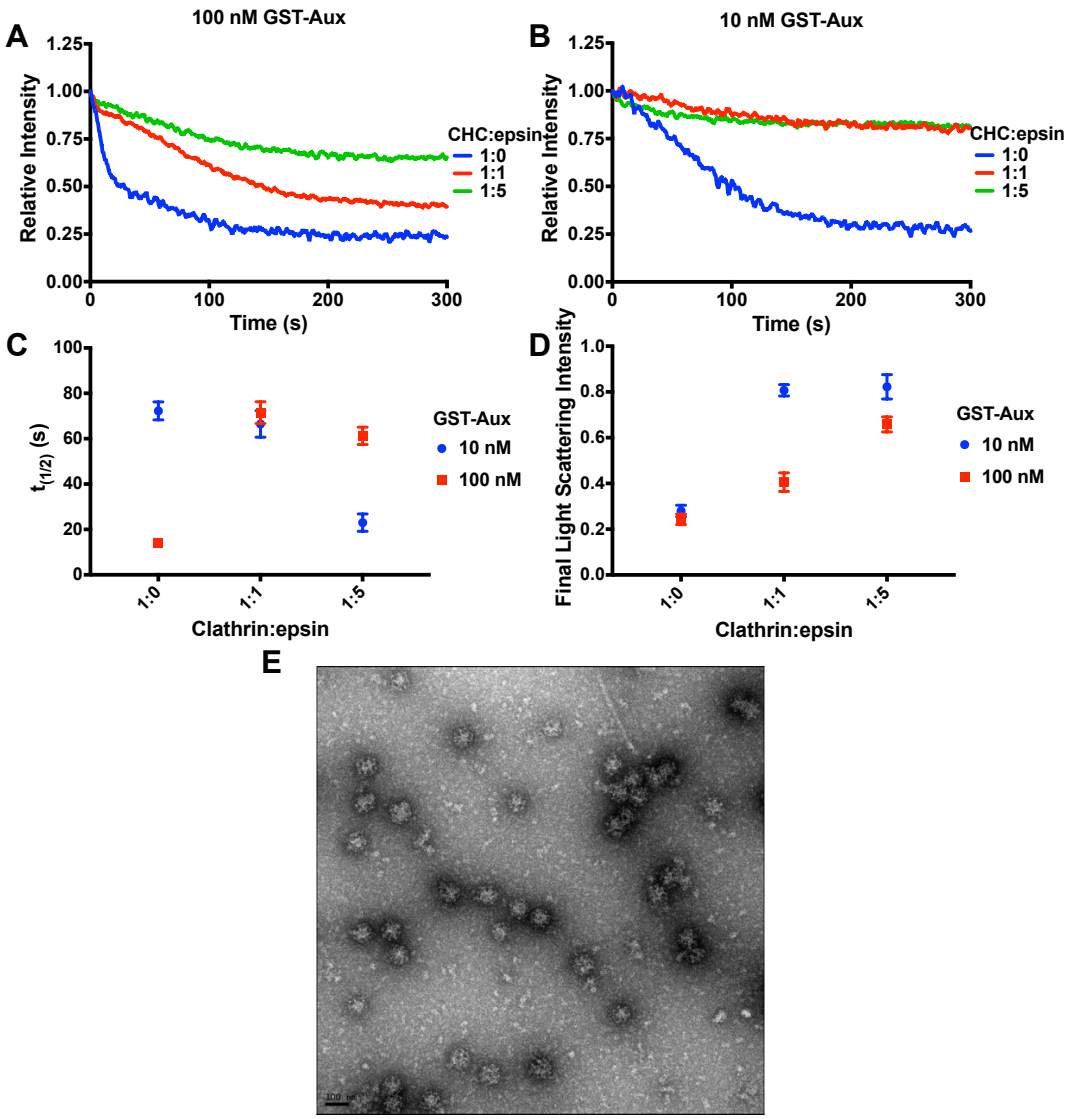

Figure 6.05 Disassembly of clathrin cages incubated with increasing concentrations of epsin. *Clathrin cages incubated at molar ratios of 1:0, 1:1 and 1:5 excess of epsin were disassembled in the presence of 100 nM (A) or 10 nM (B) GST-auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. With both concentrations of GST-auxilin an apparent increase in final light scattering intensity is observed. C) By analysing the t ½ of these traces the rate of disassembly is reduced in the presence of epsin with 100 nM GST-auxilin at both 1:1 and 1:5 ratios of clathrin:epsin. At 10 nM the trend seems to be the opposite although this is likely due to the minimal change in light scattering rather than due to an obvious change in rate. D) Final light scattering intensity increases with epsin concentration with both 10 nM and 100 nM GST-auxilin indicating that disassembly may have been inhibited. E) A sample from the 1:5 ratio 'disassembled' with 10 nM GST-auxilin was imaged by negative stain EM and shows the presence of clathrin cages that would not be expected after the addition of GST-auxilin, Hsc70 and ATP.*

Calculation of the final scattering intensity confirms that increasing epsin concentration results in an increase in final scatter intensity with 100 nM GST-auxilin (D). At 10 nM GST-auxilin a final scattering intensity of ~0.8 is seen at both concentrations of epsin. The large difference in final scatter intensity suggests that disassembly is being inhibited. This hypothesis was confirmed by taking a sample from cages incubated with 1:5 excess of epsin and disassembled with 10 nM GST-auxilin and imaging it using negative stain electron microscopy, a representative

image shown in (E). Imaging confirmed the presence of numerous clathrin cages with little obvious disassembly in the form of free triskelia. Taken together these data suggest that epsin inhibits clathrin cage disassembly in a manner dependent on auxilin concentration and epsin concentration. This effect and potential hypotheses are discussed and investigated in the following section (6.5.1) and Chapters 7 and 8.

6.5.1 Discussion

The effect of adaptor proteins on disassembly investigated in this chapter has yielded surprising differences between the effects of the adaptors tested here. In this section I discuss the implications of the results in the context of the proposed binding and assembly mechanisms of these proteins and in the context of the disassembly of the clathrin coat in the cell.

6.5.2 β2 HA effect on disassembly and interactions with auxilin

One outstanding question in relation to the disassembly of the clathrin cage is how adaptors are removed from the structure with auxilin competition for adaptor binding being one proposed mechanism. β2 HA was shown able to interact with clathrin cages at the same time as auxilin although previous results and hypotheses had suggested that both proteins may compete for clathrin binding and thereby provide a mechanism for the removal of adaptors from the CCV (Smith *et al*. 2004). The work by Smith *et al*. (2004) used peptides of both auxilin and β2 HA clathrin boxes to show that both peptides competed for binding to the terminal domain. However, it is likely that with protein constructs rather than peptides and with full length clathrin as opposed to the terminal domain only, binding of both proteins may not be significantly affected. First, auxilin contains DLL and DPF motifs in addition to its clathrin box, which provide affinity both to the terminal domain and the distal domain/ankle domain of clathrin (where as the clathrin box seems to be terminal domain specific) (Scheele *et al*. 2001). In addition β2 HA has been shown to interact with the ankle domain in addition to the hinge clathrin box binding to the clathrin terminal domain (Edeling *et al*. 2006a; Knuel *et al*. 2006). It is also possible that, given that 4 different binding sites on the terminal domain exist (Lemmon and Traub 2012) that at these concentrations these proteins bind to specific TD sites. The β2 HA clathrin box binding to the terminal domain has

been defined (ter Haar *et al*. 1998) but the auxilin clathrin box site has not. At high concentrations peptides have been shown to exhibit promiscuity across the multiple sites (Zhou *et al*. 2015) and hence it is possible that competition for binding as seen in Smith *et al*. 2004 might be explained by this phenomenon. At the concentrations of auxilin and β2 HA used here it is possible that both proteins preferentially occupy different spaces on the terminal domain and bind to additional regions on the CHC. Therefore it seems that, in the case of β2 HA, that auxilin does not directly displace the protein from clathrin.

As has been shown previously and as was demonstrated in section 4.4.3, β2 HA is able to promote clathrin cage formation although the change in size distribution previously noted was not reproduced (Greene *et al*. 2000). The proposed mechanism suggests that β2 HA is able to interact with the clathrin terminal domain through the clathrin box in the hinge and the neighbouring ankle region through the appendage domain and thereby promote interactions between triskelia (Greene *et al*. 2000, Edeling *et al*. 2006a; Kneul *et al*. 2006). Disassembly experiments with the AP2 complex have not noted a difference in the extent or apparent rate of disassembly using gel based techniques, single molecule imaging and EM (Rapoport *et al*. 2008; Boecking *et al*. 2011; Dannhauser and Ungewickell 2012) which fits largely with the data seen here (although there is a possibility that β2 HA marginally increases the rate). This would suggest that the interaction of β2 HA to promote cage formation does not significantly affect the rate or extent of disassembly. The first explanation for the lack of effect on disassembly is that the stabilising mechanism of β2 HA is too subtle for any effect on disassembly to be observed. A second possibility is that the binding of auxilin to clathrin does disrupt this proposed interaction of β2 HA without significantly effecting binding (at the concentrations used). Auxilin could potentially interact with a similar region on the ankle of the CHC to that occupied by β2 HA (Scheele *et al*. 2001). Given that auxilin in this assay is added approximately 1 minute before the initiation of disassembly through the addition of Hsc70 it is possible that binding of auxilin during this time is able to disrupt this interaction. Suggestions of further work to address these points are discussed in section 6.5.6.

6.5.3 Hip1 and Hip1R CC do not affect disassembly

As described previously the CC domains of Hip1 and Hip1R interact with the CLC of clathrin in contrast to the other adaptor proteins studied here which interact with the CHC. It has been proposed that CLC binding by Hip1/1R CC may alter the CLC interaction with the CHC and thereby promote assembly (Engqvist-Goldstein *et al*. 2001; Legendre-Guillemin *et al*. 2002; Legendre-Guillemin *et al*. 2005; Chen *et al*. 2005). It is therefore possible that binding of the CC domains of these proteins might stabilise cages and make them less susceptible to disassembly. The CLC is also likely to play a role in disassembly: DeLuca-Flaherty *et al*. showed that CLCa peptides/CLCa can stimulate ATP hydrolysis of Hsc70 (in the absence of auxilin) and CLCa can inhibit un-coating of CCVs (DeLuca-Flaherty *et al*. 1990). In addition, Young *et al.* (2013) showed that Hsc70 binding to clathrin induces conformational changes in the CLC interactions with the CHC (Young *et al*. 2013). It was also observed that the removal of CLCs required an increased concentration of auxilin to stimulate disassembly effectively. Given the evidence of the CLC's role it was postulated that interaction of the CC domains of Hip1 and Hip1R with the CLC, particularly if they are promoting assembly, might have a negative effect on the rate/extent of cage disassembly. In light of these results it seems that this is not the case and that whatever effect Hip1 CC and Hip1R CC have in relation to promoting cage formation this interaction does not have an effect on disassembly under these conditions.

6.5.4 The inhibitory effect of epsin

Epsin promotion of clathrin assembly has been shown, both in this thesis and in previous publications, to be similar to other adaptors with the formation of small cage particles (Kalthoff *et al*. 2002). This mechanism is proposed to occur through multiple interactions with neighbouring triskelia through the numerous clathrin/adaptor binding motifs of epsin (Drake *et al*. 2000; Drake and Traub 2001; Kalthoff *et al*. 2002). As with β2 HA it was postulated that this interaction could potentially alter the disassembly of clathrin cages through the physical linkage. The results here indicate that a strong inhibitory effect is observed on clathrin cage disassembly that is dependent both on the concentration of auxilin and the concentration of epsin. The mechanism by which this inhibition occurs is addressed in Chapters 7 and 8.

This inhibitory effect has not been detailed explicitly although previous work has likely observed this effect. Dannhauser and Ungewickell (2012) used AP180 and epsin clathrin/adaptor binding domains to assemble clathrin onto artificial liposomes using the poly histidine tags on the constructs through binding to Ni^{2+}on the Ni^{2+}-NTA-DOGS lipid (Dannhauser and Ungewickell 2012). Although Dannhauser and Ungewickell were able to disassemble clathrin from these coated vesicles disassembly was incomplete with only 30-40% of clathrin released from these structures in the presence of auxilin and Hsc70. Although direct comparison of components cannot be made due to the use of coated liposomes in contrast to clathrin cages, their experiment used similar concentrations of auxilin and Hsc70 as used in this study (1 uM Hsc70 and 147 uM auxilin) although disassembly was conducted at a higher temperature (37°C compared to 21°C) and measured through a pelleting assay (Dannhauser and Ungewickell 2012). Whilst disassembly is not complete the authors did observe that the addition of auxilin and Hsc70 did disrupt the formation of clathrin coated buds that form on the liposomes, suggesting that, whilst auxilin and Hsc70 do not completely remove clathrin from the complex they do partially disrupt the interaction that is occurring. This seems at odds with the apparent retention of cage structures seen in EM micrographs with low auxilin concentrations. Therefore, whilst there are similarities between the results obtained in this study and those obtained by Dannhauser and Ungewickell there are some discrepancies. One possibility is that the presence of lipid causes differences in the binding or recruitment of epsin/clathrin or auxilin/Hsc70. It should also be noted that the auxilin construct used is a shorter variant than the one used in this study and lacks the additional clathrin box motif at 495 ($aux_{401-910}$ compared to $aux_{547-910}$) which could contribute to the differences seen. However, it does appear that the effect of epsin as observed here may be a phenomenon of the protein itself rather than an artefact of the assay conditions. Further investigations into the inhibitory effect of epsin are explored in Chapters 7 and 8.

6.5.5 *In vivo* relevance: are adaptors present in the CCV?

Although many adaptors promote the assembly of the clathrin coat and are recruited during the maturation of the CCP the question as to the fate of adaptors during or after scission is not clear with contradictory evidence for many of the adaptors investigated here. This question is important as whether an adaptor is present in the CCV will determine whether it is likely to interact with auxilin/GAK or Hsc70 and therefore the relevance of these experiments (although adaptors may interact with the disassembly machinery during membrane remodelling (Jiang *et al*. 2000; Yim *et al*. 2005)).

With AP2 the presence of this protein (and therefore β2 HA) in CCVs is pretty clear cut. AP2 is found in purified CCVs (Blondeau *et al*. 2004) and *in vivo* tracking experiments suggest that AP2 is present primarily at the apex of the CCP as the pit moves into the cell and undergoes scission (Saffarian *et al*. 2009; Taylor *et al*. 2011). The interaction of the β2 HA with clathrin is therefore very likely to occur at the point of disassembly and hence the experiments conducted here are relevant to the situation *in vivo*. It is therefore interesting to note that the β2 HA interaction with the clathrin coat does not have a major effect on disassembly which may be important for uncoating *in vivo*.

The picture with Hip1/1R is less clear. Hip1R has been reported in CCVs (Engqvist-Goldstein *et al*. 1999; Engqvist-Goldstein *et al*. 2001; Blondeau *et al*. 2004) where as Hip1 has been reported to be absent from these structures (Gottfried *et al*. 2009) which could be indicative of possible differing functions of these proteins. In addition, the interaction of clathrin and Hip1/1R is proposed to occur to recruit these proteins to the site of endocytosis. Here the proteins are proposed to dissociate from the CLC to interact with the plasma membrane and cargo/actin at the neck of the CCP, although these proteins could remain bound to the clathrin coat away from the closing neck (Wilbur *et al*. 2008). It is therefore possible that these proteins could remain interacting with parts of the CCV after scission. As with β2 HA it is interesting that both Hip1/1R CC binding and promotion of assembly through CLC interactions does not significantly alter disassembly.

The picture with epsin is even less clear. The protein has been reported to be both present (Blondeau *et al*. 2004; Edeling *et al*. 2006a; Hawryluk *et al*. 2006; Rappoport *et al*. 2006) and absent in CCVs (Chen *et al*. 1998; Wang *et al*. 2006) with its location

during the formation of the CCP equally as unclear. Given its function with recruiting Hip1R (Brady *et al*. 2010), interaction with actin (Messa *et al*. 2014) and its role in membrane curvature and budding (Brooks *et al*. 2015) epsin has been proposed to locate primarily at the neck of the CCP and therefore away from the forming CCV (Praefke *et al*. 2004; Saffarian *et al*. 2009). It has also been suggested that epsin localises preferentially with its binding partner eps15 that is pushed to the edge of growing pits (Chen *et al*. 1998; Wang *et al*. 2006), also suggesting that epsin would be absent from mature CCVs. However, as mentioned epsin is detectable in CCVs and is present in CCPs throughout the structure (in EM images) and so is unlikely to be totally absent at the point of uncoating (Blondeau *et al*. 2004; Edeling *et al*. 2006a; Hawryluk *et al*. 2006; Rappoport *et al*. 2006). Therefore, it is possible that the effect seen here is related to CME *in vivo*. Further work and discussions in Chapters 7 and 8 addresses this topic again in the context of subsequent results.

6.5.6 Future work

The results obtained in this chapter open up a number of avenues of enquiry, with many of those relating to the inhibitory effect of epsin addressed in Chapters 7 and 8.

It would seem that for Hip1/1R CC and β2 HA that the question of how or whether auxilin/Hsc70 is needed to displace these adaptors has not been conclusively answered. Whilst their assembly promotion effects seem not to alter disassembly within the sensitivity of the assay used the other question as to whether auxilin actively competes for binding with adaptors was not fully addressed. The use of the auxilin clathrin binding mutants introduced in Chapter 5 would provide an ideal tool for investigating whether auxilin requires these motifs to compete with adaptors for binding. Although these mutants were used to interrogate the inhibitory effect of epsin (see Chapter 7) they were not applied to β2 HA or Hip1/1R CC and so extending the use of these mutants in the context of these proteins in the future may help to answer this remaining question.

An aspect that was not considered in the experiments conducted with these adaptors was the effect of Hsc70 concentration. Given that Hsc70 may interact with the CLC during disassembly it is possible that an effect of Hip1/1R CC binding may be observed at limiting concentrations of Hsc70 (DeLuca-Flaherty et. al. 1990). In light of this, disassembly with varied Hsc70 concentrations may reveal more about Hip1

CC and Hip1R CC roles during disassembly and would be an avenue for future investigation. On a related topic the CLC show differential effects on disassembly with an inhibition of disassembly with excess CLCa inhibiting disassembly to a greater extent the CLCb (Liu *et al*. 1995), (Ybe *et al*. 1998). It would therefore be interesting to conduct these disassembly experiments using CHC:CLCa and CHC:CLCb specific cages as opposed to cages with mixed CLC isoforms in the presence or absence of Hip1/1R CC to determine any effect, if any, on disassembly.

As discussed in Chapter 5 (see section 5.5.5) the use of stopped-flow apparatus when monitoring disassembly would allow for greater reproducibility and a much greater time resolution than is possible with the fluorimeter based assay. Subtle differences that adaptors may make during disassembly, particularly during the initial recruitment phase of auxilin and Hsc70, are lost due to low time resolution and reproducibility issues due to manual mixing. Stopped-flow would also allow for differential mixing of proteins so that, for example, the effect of pre-mixing or not pre-mixing auxilin and clathrin adaptor cages could be investigated. This would reveal if auxilin recruitment to cages is altered in the presence of adaptors which is not observable using the fluorimeter based system (Rothnie *et al*. 2011).

As discussed in Chapter 4 the adaptor protein AP180 has a similar structure to epsin and shows a similar, if not more potent, ability to promote cage assembly (see section 4.6.6 and 4.6.7) (Kalthoff *et al*. 2002). In addition, a similar effect on the inhibition of disassembly may have been noted previously (Jiang *et al*. 2000). If the ability of epsin to inhibit disassembly is related to its structure and clathrin binding (as suggested in Chapter 8) then it is likely that AP180 may show a similar effect. Expression and purification of AP180 was not pursued significantly during this project due to poor initial yields. However subsequent improvements in yield lead by various undergraduate and postgraduate students have allowed initial experiments on the effect of AP180 on disassembly to be conducted. These preliminary experiments subsequent to the completion of lab work for this thesis showed that AP180 may have a similar effect to epsin as an inhibitor of disassembly (data not shown). Conformation of this result and further investigation in light of the observations in Chapters 7 and 8 are therefore a priority for future work on this topic.

6.5.7 Conclusion

In conclusion in this chapter I have shown that disassembly of clathrin cages in the presence of β2 HA, Hip1R CC or Hip1 CC proteins does not significantly alter the rate or extent of disassembly. In contrast, epsin has an inhibitory effect that is increased at higher concentrations of epsin and with lower concentrations of GST-auxilin. The inhibition of epsin is investigated in further detail in Chapters 7 and 8.

Chapter 7: Further investigations into the effect of epsin on disassembly

''Tis strange - but true; for truth is always strange; Stranger than fiction'' Lord Byron, Don Juan

7.1.1 Introduction

7.1.2 Overview

In the previous chapter epsin was shown to have an inhibitory effect on clathrin cage disassembly that is dependent on both epsin and auxilin concentration. In this chapter I have investigated this effect further using the auxilin mutant constructs introduced in Chapter 5 , by varying Hsc70 concentrations and by investigating the effects of variations in protein batch and the presence or absence of affinity tags.

7.1.3 Probing epsin inhibitory effect of epsin

The inhibitory effect of epsin on disassembly has not been previously noted in the literature (Dannhauser and Ungewickell 2012; Holakr *et al.* 2015). Although, as described in section 6.5.4, Dannhauser and Ungewickell are likely to have noted a similar effect when disassembling clathrin from clathrin-epsin liposome structures although the authors did not pursue this observation further (Dannhauser and Ungewickell 2012).

A number of questions arose from the initial observations that epsin inhibited clathrin disassembly. The primary question was how does epsin cause this effect? Given that the concentration of auxilin was shown to affect the extent of disassembly in the presence of epsin, this indicated that some form of interaction between epsin, clathrin and auxilin may be occurring. Given that epsin binds to clathrin via clathrin box motifs and DPW motifs which are shared by auxilin this raised the possibility that these proteins may be interacting with clathrin in a competitive manner and this avenue of

enquiry was explored through the use of clathrin pull downs and the set of auxilin mutants analysed in Chapter 5.

The other variable that had not been addressed previously was the effect of Hsc70 concentration on the epsin effect. By varying Hsc70 concentrations during disassembly it may be possible to confirm whether the effect of epsin on disassembly was exclusively related to auxilin concentrations or whether a different mechanism was at work.

The final variable that is explored in this chapter is the effect of affinity tags of auxilin and epsin on disassembly and the issues related to variations in protein activity between purification batches. By addressing these issues, it would be possible to confirm that the epsin inhibitory effect was due to natural properties of the proteins used rather than an artefact of affinity tags.

In this chapter I demonstrate that epsin and auxilin compete for binding to clathrin and that auxilin clathrin/adaptor binding mutants are less able to disassemble clathrin-epsin cage complexes than WT auxilin. I demonstrate that the epsin inhibitory effect is also exacerbated by low concentrations of Hsc70, suggesting that epsin's role affects both Hsc70 and auxilin in their ability to initiate clathrin cage disassembly. Finally, I show that the inhibitory effect of epsin is due to its native properties rather than to its affinity tag but that variations in epsin inhibitory efficacy occur with differing batches of epsin and with different preparations of auxilin.

7.2.1 Auxilin and epsin compete for clathrin binding

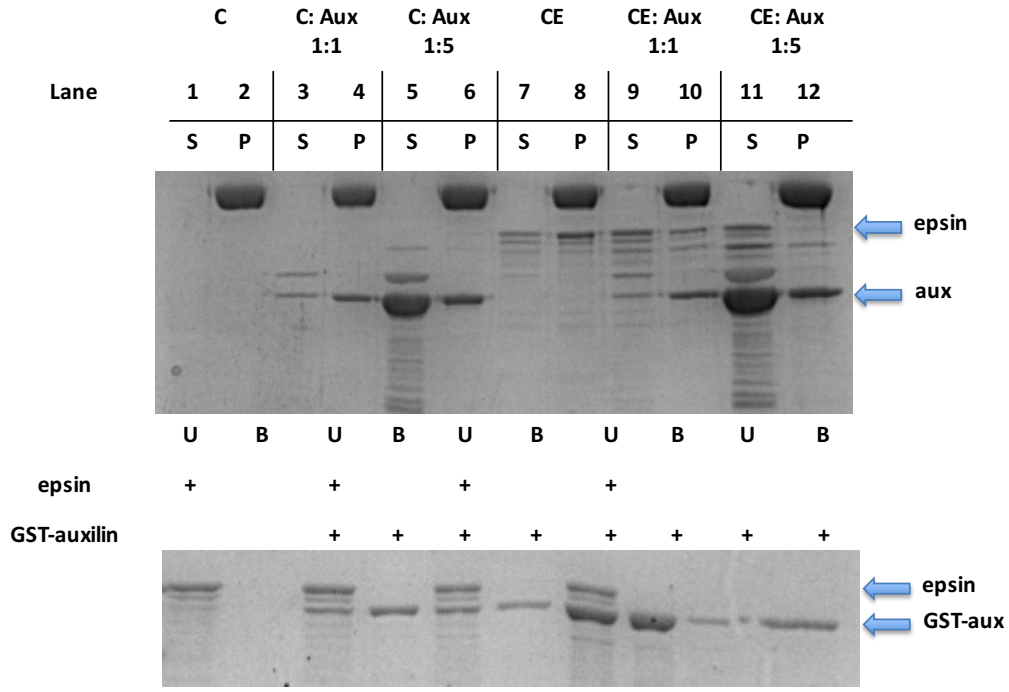

Figure 7.01 Analysis of epsin-clathrin-auxilin interactions by ultracentrifugation and GST-pull downs and SDS-PAGE. *A) 3 μM Clathrin cages incubated with increasing molar ratios of auxilin (aux) with or without the presence of epsin (3 μM) were pelleted by ultracentrifugation and pellet (P) and supernatant (S) fractions analysed by SDS-PAGE. Both auxilin (C:Aux 1:1 and 1:5) and epsin (CE) bind to clathrin as expected. By increasing the concentration of auxilin in the presence of epsin we can see a decrease in the epsin band present in the pellet fraction in the 1:1 ratio and the complete shift of epsin to the supernatant at the high concentration of auxilin (CE:Aux 1:5). B) Affinity pull down of GST-auxilin by GSH-beads was conducted in the presence or absence of epsin. Samples of the material associated both bound (B) and unbound (U) to the GSH-beads were analysed by SDS-PAGE. GST-auxilin is pulled down in the presence of beads where as epsin is not. When epsin and auxilin are mixed epsin remains in the unbound fraction and does not appear in the bound fraction with GST-auxilin.*

As shown in section 6.4.2 the epsin inhibitory effect was shown to be dependent on auxilin concentration and so further investigation of possible interactions between auxilin, epsin and clathrin were conducted. Binding assays were conducted with clathrin cages in the presence of auxilin$_{401\text{-}910}$ (cleaved) and epsin (see Figure 7.01 A). Cages incubated with a 1:1 molar ratio and 1:5 excess of auxilin show auxilin present with clathrin in the pellet fraction and excess protein in the supernatant. Incubation of cages with epsin at a 1:1 molar ratio show binding as expected (Lane 8). However, when both epsin and auxilin are incubated with cages together the intensity of the epsin band in the pellet fraction with clathrin diminishes and is almost removed at the highest concentration (Lane 12). This data suggests (albeit qualitative in nature) that auxilin is preventing epsin from binding to clathrin at high concentrations of auxilin. These results are consistent with the disassembly data and indicate that epsin and

auxilin are competing for binding to clathrin and that this a likely factor in epsin dependent inhibition of cage disassembly.

To investigate the possibility of any direct interactions between epsin and auxilin, GST-auxilin was incubated with GSH-beads at increasing concentrations with epsin and the beads spun out of solution at low g. SDS-PAGE confirmed epsin remains in the supernatant fraction where as GST-auxilin binds to the beads and pellets (see Figure 5.12 B). When both proteins are mixed no epsin is seen in the pellet fraction. This suggests that there is no strong interaction between these proteins, although it does not rule out the possibility of transient interactions or interactions that may only occur in the presence of clathrin. This observation would also suggest that the inhibition effect seen is not due to the presence of unbound epsin that is not interacting with clathrin and potentially sequestering auxilin in solution and preventing it from binding to clathrin.

7.2.2 Disassembly by clathrin binding mutants of auxilin are inhibited to a greater extent in the presence of epsin

To further investigate the interplay between epsin and auxilin that might occur in the disassembly reaction, disassembly experiments were conducted using the set of auxilin mutants with disrupted clathrin and adaptor binding domains that were investigated in Chapter 5: ALAx2 and APAx2. The DAAx2 mutant was not used as it had no significant effect on disassembly (see section 5.2.3). Data obtained so far from disassembly assays and pull downs suggest that auxilin and epsin compete for binding to clathrin. Epsin is known to bind to clathrin via an unstructured region containing two clathrin box motifs as well as a region of DPW motifs (Drake *et al*. 2000; Drake and Traub 2001). Auxilin binds to clathrin via a clathrin box and multiple DPF and DLL motifs (Scheele *et al*. 2001; Scheele *et al*. 2003; Smith *et al*. 2004) (see section 5.1.5). Using variants of auxilin with disrupted DPF and DLL motifs to disassemble epsin-clathrin cages would therefore provide an insight into which of these motifs is important for epsin inhibition.

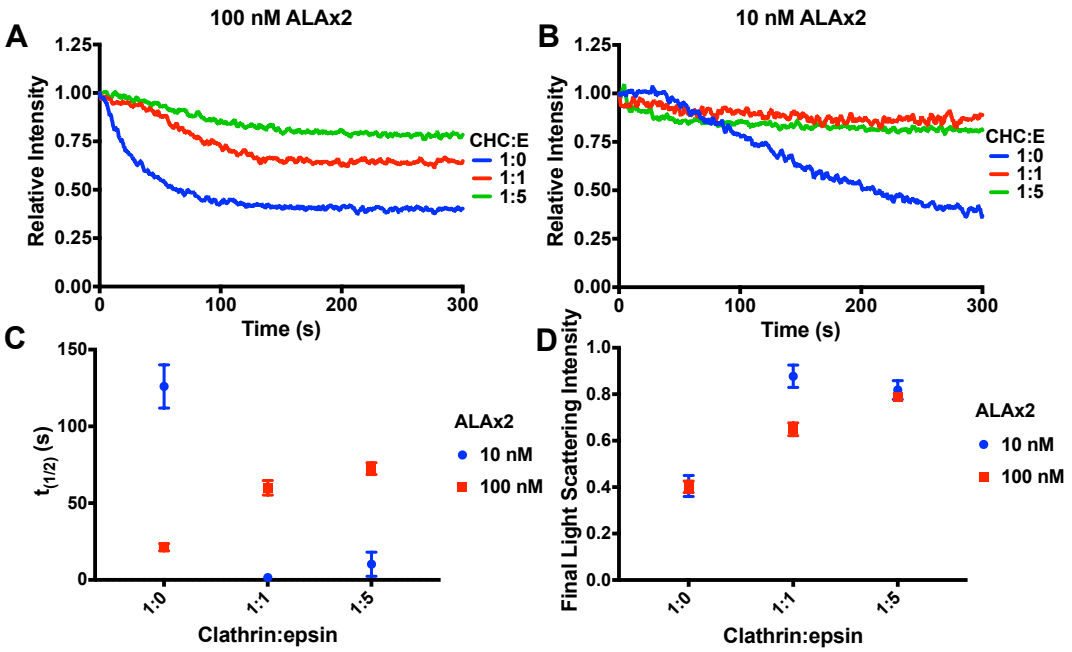

Figure 7.02 Disassembly of clathrin cages incubated with increasing concentrations of epsin using ALAx2 mutant GST-auxilin. *Clathrin cages incubated at molar ratios of 1:0, 1:1 and 1:5 excess of epsin (CHC:E) were disassembled in the presence of 100 nM (A) or 10 nM (B) ALAx2 GST-auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. With both concentrations of GST-auxilin an apparent increase in end-scatter intensity is observed. C) By analysing the t ½ of these traces the rate of disassembly is reduced in the presence of epsin when 100 nM GST-auxilin at both 1:1 and 1:5 ratios of clathrin:epsin. D) Final light scattering intensity increases with epsin concentration with both 10 nM and 100 nM GST-auxilin indicating that disassembly has been inhibited. The results from both t ½ and end scatter indicate that the mutations to auxilin exacerbate the inhibition of disassembly compared to WT GST-auxilin (see Figure 7.04 for comparison to WT).*

Clathrin cages incubated with epsin as previously described were disassembled in the presence of the GST-auxilin ALAx2 mutant at 100 nM and 10 nM (see Figure 7.02) with the same experiment conducted for the APAx2 mutant (see Figure 7.03). A composite of the t ½ and final light scattering intensity data for WT, ALAx2 and APAx2 is shown in Figure 7.04. As observed with WT GST-auxilin, epsin has an inhibitory effect that is increased at low concentrations of GST-auxilin (10 nM). The inhibitory effect appears to be more pronounced in the mutant variants of auxilin compared to the WT GST-auxilin but at high concentrations (100 nM) they are still able to disassemble clathrin-epsin cage complexes. This would suggest that these motifs are not integral to the ability of auxilin to counteract the inhibitory effect of epsin but the absence of the motifs simply make it more difficult for auxilin to counteract the effect, possibly through reduced affinity for clathrin (although this was not observed in pull down experiments in section 5.2.1). This might suggest that, given auxilin and epsin appear to compete for binding as seen in Figure 7.01, that these motifs are not directly required for this competition to occur. One binding motif remaining within the auxilin mutants is the clathrin box motif. These studies therefore

raise the question of whether the competition of epsin and auxilin for clathrin occurs via interaction with the terminal domain via these motifs.

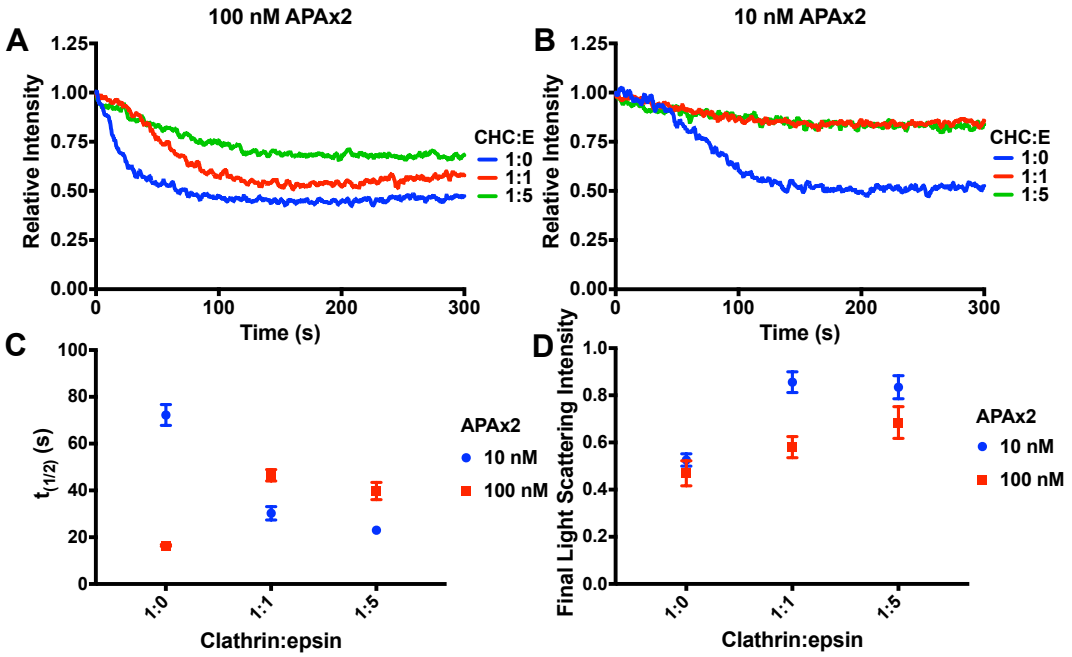

Figure 7.03 Disassembly of clathrin cages incubated with increasing concentrations of epsin using APAx2 mutant GST-auxilin. *Clathrin cages incubated at molar ratios of 1:0, 1:1 and 1:5 excess of epsin (CHC:E) were disassembled in the presence of 100 nM (A) or 10 nM (B) APAx2 GST-auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. With both concentrations of GST-auxilin an apparent increase in end-scatter intensity is observed. C) By analysing the t ½ of these traces the rate of disassembly is reduced in the presence of epsin when 100 nM GST-auxilin at both 1:1 and 1:5 ratios of clathrin:epsin. D) Final light scattering intensity increases with epsin concentration with both 10 nM and 100 nM GST-auxilin indicating that disassembly has been inhibited. The results from both t ½ and end scatter indicate that the mutations to auxilin exacerbate the inhibition of disassembly compared to WT GST-auxilin.*

There is an important issue to note however concerning the difference in disassembly activity (particularly in relation to the extent of disassembly) in the absence of epsin, with mutant variants of auxilin and the values observed here compared to the initial observations made in section 5.2.3. In particular, the final light scattering intensity is seen to increase in the controls relative to the WT control. However, it should be noted that the end scatter intensity for both mutants was effectively unchanged with concentration (ALAx2 100 nM = 0.401±0.025, 10 nM = 0.405±0.045, APAx2 100 nM = 0.469±0.053, 10 nM = 0.526±0.026) suggesting that this may not be an effect of auxilin itself as one might expect the end scatter intensity to vary with concentration. The other component that may influence the effects that we have seen is the clathrin itself. Previous work by the Smith group has shown that the removal of the CLC from clathrin cages makes them more resistant to disassembly (Young *et al.* 2013). Given that the data for WT GST-auxilin was collected using a different sample

of clathrin it is possible therefore that, although this was not noted at the time, that loss of CLC may have contributed to this effect.

Given these observations it may be questionable therefore to make specific comparisons regarding the activity of the WT and mutant forms of auxilin in the presence of epsin. Despite this it can be concluded that the mutant forms of auxilin are still able to disassemble clathrin cages and hence the DPF and DLL motifs are unlikely to play a significant role in the interplay between auxilin and epsin during disassembly.

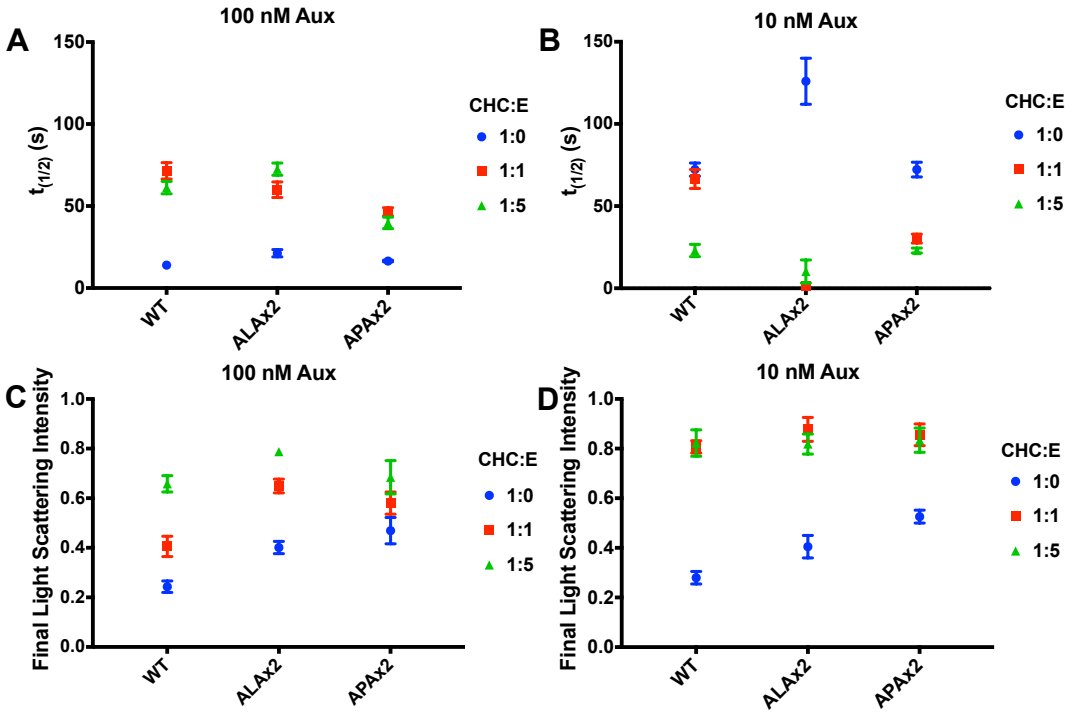

Figure 7.04 Composite figure of t ½ and final light scattering intensity values for the disassembly of epsin-clathrin cage complexes in the presence of WT, ALAx2 or APAx2 GST-auxilin *(see Figures 6.05, 7.02 and 7.03). Graph titles indicate the concentration of the auxilin construct used. A) t ½ with 100 nM GST-auxilin shows how the presence of epsin slows the rate of disassembly across all variants although the APAx2 mutant appears to be less effected than both the WT and ALAx2 mutants. B) At 10 nM both mutants exhibit a faster t ½ in the presence of epsin compared to the WT although this might be attributable to the slightly higher end scatter intensity observed (see D). With final light scattering intensity both mutants show a shift to higher values compared to the WT at both 100 nM (C) and 10 nM (D) in either the presence or absence of epsin. The reason for this is not known but it may be due to differences in the clathrin used in the mutant disassembly experiments compared to the WT experiments.*

7.2.3 Hsc70 concentration also affects the extent of clathrin cage disassembly in the presence of epsin

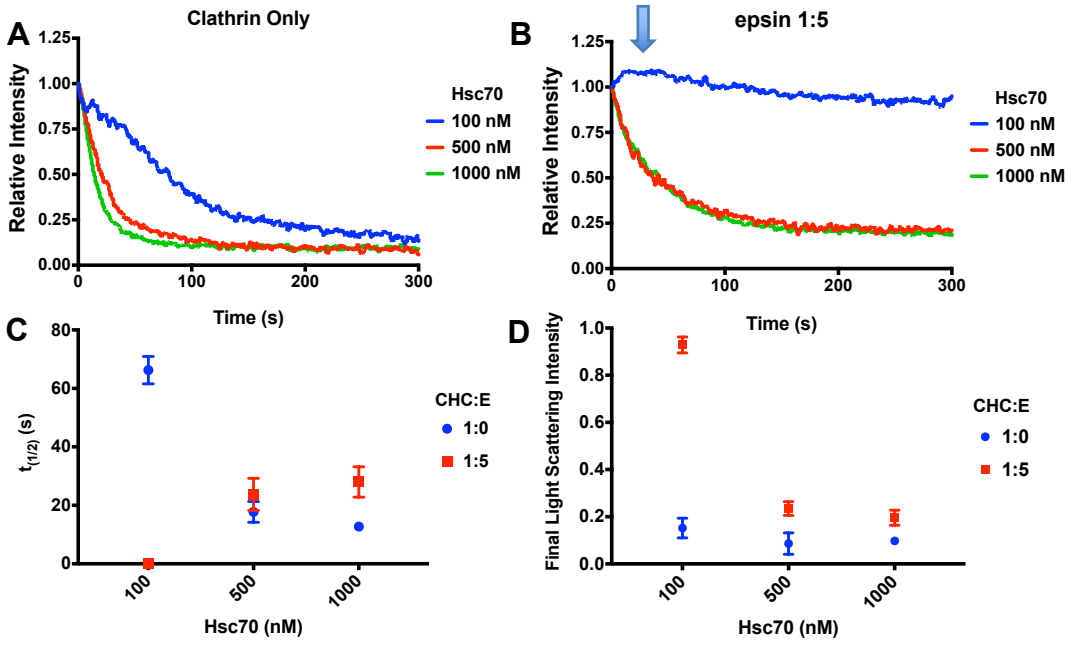

Figure 7.05 Disassembly of clathrin cages incubated with increasing concentrations of epsin using varying concentrations of Hsc70. *Clathrin cages incubated in the absence (A) or presence (B) of a 1:5 molar excess of epsin (CHC:E) were disassembled with 100 nM auxilin, 500 μM ATP and initiated with 1000, 500 or 100 nM Hsc70. Reducing concentrations of Hsc70 results in a slower rate of disassembly which is exacerbated in the presence of epsin where at 100 nM Hsc70 disassembly is inhibited. C) t ½ in the presence of epsin is greater compared to its absence with the exception of at 100 nM Hsc70 with the epsin-clathrin complex where an apparent increase in scattering intensity above the starting intensity was noted and hence t ½ is not an applicable measure in this instance (recorded a 0). D) Final light scattering increases with low concentrations of Hsc70 and increases dramatically in the presence of epsin, suggesting inhibition of disassembly in the presence of epsin.*

The final variable in relation to epsin inhibition and disassembly that had not been investigated at this point was the effect of Hsc70 concentration. Cages in the presence or absence of epsin (1:5 molar excess clathrin:epsin) were disassembled with varying concentrations of Hsc70 (1000 nM, 500 nM and 100 nM) (see Figure 7.05). At both 1000 nM and 500 nM there is little difference in t ½ both in the presence or absence of epsin but at 100 nM a dramatic difference is noted. In the absence of epsin disassembly occurs with 100 nM of Hsc70 but, as is demonstrated by the end scatter intensity, disassembly is severely inhibited with epsin at 100 nM Hsc70. Interestingly at 100 nM Hsc70 an initial increase in light scattering above the original intensity can be seen. This increase in intensity has been studied in a stopped-flow set up and corresponds to the initial recruitment of auxilin and Hsc70 to clathrin cages that increases the relative size/density of the cage particles and hence increases the scattering signal (Rothnie *et al.* 2011; Sousa *et al.* 2016). At these concentrations of

epsin, Hsc70 and GST-auxilin it seems that the length of the 'recruitment phase' is dramatically increased suggesting that the initial recruitment of Hsc70 via auxilin has been affected (indicated by arrow (Figure 7.05)). As a result, Hsc70 may be unable to bind effectively and so dissociates causing the subsequent decay in signal back down to a base line.

Taken together with the auxilin results this suggests that high concentrations of both auxilin and Hsc70 can overcome the inhibitory effect of epsin.

7.2.3 Protein batch variation in auxilin and epsin alters disassembly inhibition and kinetics

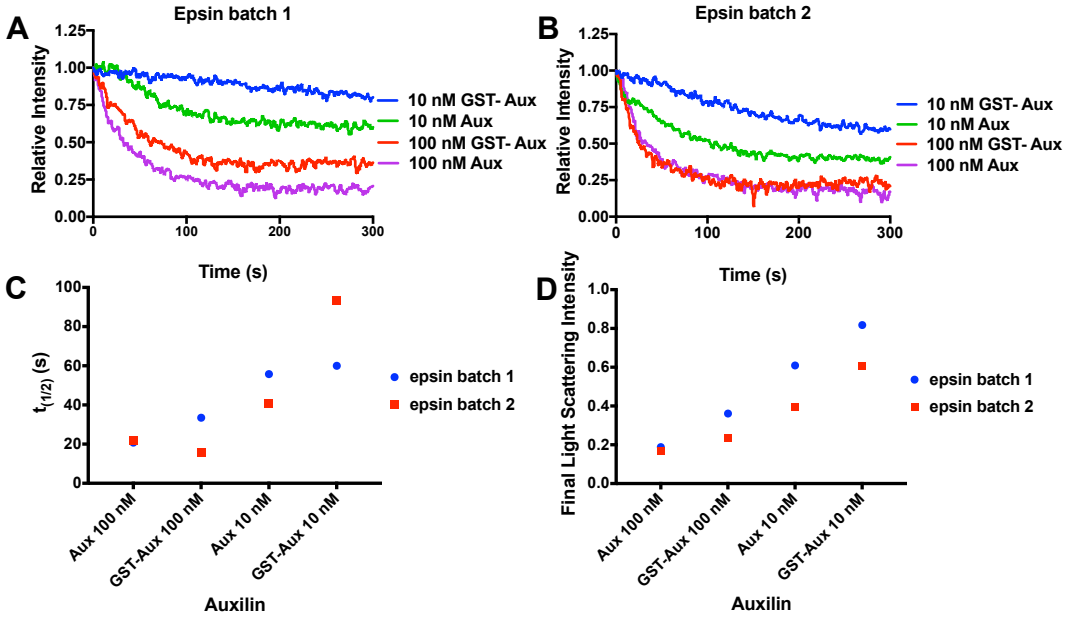

Figure 7.06 Disassembly of clathrin cages incubated with different batches of epsin and using GST-auxilin and cleaved auxilin. *3 μM clathrin cages incubated with batch 2 epsin (A) or batch 1 epsin (B) at a 1:5 fold excess of epsin were disassembled in the presence of 100 nM or 10 nM GST-auxilin or auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. The data indicate that the epsin batch shown in (B) shows a greater level of inhibition compared to that shown in (A). In addition, cleavage of the GST-tag seems to reduce the level of disassembly/increase the activity of auxilin. C) By analysing the t ½ of these traces we can see that values differ between epsin batches and between the activity of GST-auxilin and cleaved auxilin. D) Final light scattering intensity increases with epsin concentration with both batches of epsin but is higher at the same concentration of (GST)-auxilin with batch 1 compared to batch 2 indicating. The results from both t ½ and end scatter indicate variations between protein prep. Note that each condition was only tested once due to a lack of material and hence error bars are omitted.*

Different protein preparations of both auxilin and epsin were noted to alter disassembly profiles although with the same overall trend. This observation was noted with a new epsin prep (epsin batch 2) which showed a lower level of inhibition of disassembly compared to the first preparation (epsin batch 1). Direct comparisons were made between these two protein preps and between two batches of auxilin, one

retaining the GST tag and the other with the tag cleaved (see Figure 7.06). Due to limiting amounts of batch 1 epsin, each condition was only conducted once and hence no error bars are present for t ½ and end scatter calculations. However, the observations from these experiments are that the batch 1 epsin shows a greater level of inhibition than batch 2 as determined by final light scattering intensity and causes a slower rate of disassembly (with the exception of disassembly with 10 nM GST-auxilin).

Cleavage of the GST tag from auxilin also results in a greater extent and rate of disassembly. The reasons for this could be due to an increase in the relative amount of 'active' protein in the cleaved auxilin preparation. Although the presence of the GST-tag was taken into account for the calculation of protein concentration in the un-cleaved construct, it was observed that cleavage of the GST tag and re-purification of the protein on a GST-affinity column results in a reduction in the amount of contaminating protein bands (see section 3.4.2), increasing the proportion of active auxilin in the assays. Another possibility is that, although the presence of the GST-tag has been shown previously to make little difference to the rate of disassembly (Rothnie *et al*. 2011) (and that free GST does not inhibit disassembly (see Appendix A.2)), it is possible that the tag provides some form of steric hindrance in the presence of epsin. This is particularly relevant as the GST tag on the C-terminus of the auxilin construct and is relatively close to the clathrin box of auxilin (the GST tag is attached at auxilin residue 401 with the clathrin box starting at residue 495). Given that the data suggest that epsin competes for clathrin binding, it is possible that the proximity of the tag to this binding motif hinders the ability of auxilin to interact with clathrin in the presence of epsin. GST is known to dimerise and although dissociation constants for dimerization seem to vary (between μM and <1nM) (Fabrini *et al*. 2009) it seems likely that at the concentrations used in this study that a proportion of the GST tags may form dimers (and hence GST-auxilin dimers). This in turn could exacerbate any possible steric hindrance affect that epsin has on auxilin.

7.2.4 Cleavage of epsin affinity tag has little effect on inhibition of disassembly

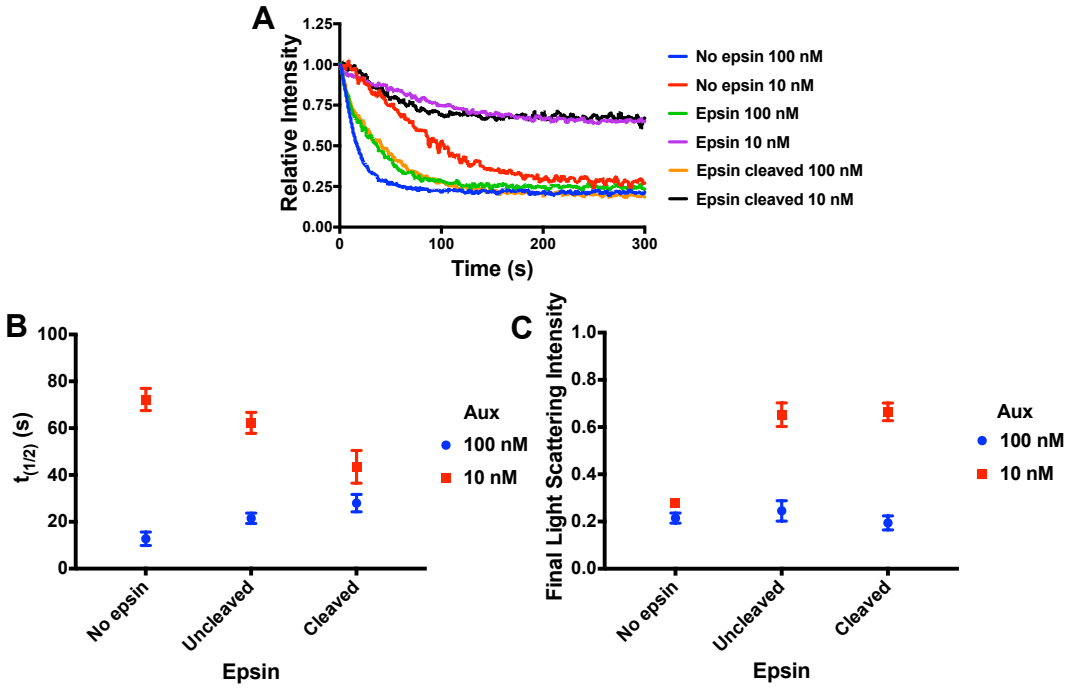

Figure 7.07 Disassembly of clathrin cages incubated in the presence or absence of epsin with its His_6thioredoxin tag cleaved or retained. *A) Clathrin cages incubated in the presence or absence of a 1:5 molar excess of cleaved or un-cleaved epsin were disassembled with 100 nM or 10 nM GST-auxilin, 500 μM ATP and initiated with 1μM Hsc70. Data indicates there is little difference between cleaved or un-cleaved epsin. B) t ½ analysis shows some difference between cleaved and un-cleaved epsin which is likely due to slight differences in the disassembly curves. C) End scatter indicates there is no difference in the extent of disassembly in the presence of absence of the affinity tag.*

Epsin preparations were for the most part conducted without cleavage of the affinity tag. To confirm that the affinity tag was not causing the inhibitory effect on disassembly the tag was removed by digestion with the protease thrombin and purified away from remaining cleaved tag and un-cleaved protein as described in section 3.5.2. The cleaved epsin was then incubated with clathrin and disassembled as previously with the comparison to the WT shown in Figure 7.07. Cleaved epsin behaves the same as WT epsin in terms of end scatter intensity at equivalent concentrations of epsin and hence the tag does not seem to play a role in the extent of disassembly. However, there does appear to be a difference in terms of t ½ at 10 nM GST-auxilin concentrations with a faster rate of disassembly compared to the un-cleaved variant. The difference for this is not known but could be down to subtle differences in concentrations. Another possibility is that the tag (His_6Thioredoxin) is sizeable at 14 kDa and hence may partially hinder recruitment of auxilin or Hsc70 to their binding sites although the

effect here seems to be minimal. In conclusion it seems that inhibition of disassembly by epsin is attributable to the protein itself rather than the affinity tag.

7.3.1 Discussion

In this chapter I have expanded upon the initial observations in relation to the epsin inhibition of clathrin cage disassembly that sheds light on what the precise mechanism behind this effect may be which is further explored in Chapter 8.

7.3.2 The epsin inhibitory effect is dependent on a number of factors

Gel pull-down experiments clearly show that auxilin is capable of displacing epsin from clathrin cages at high concentrations. This displacement of epsin from clathrin by auxilin is consistent with the concentration dependent effects of epsin and auxilin on the extent of disassembly. These observations would suggest that the inhibitory effect of epsin is due to direct competition for binding to clathrin. As both proteins share similar binding motifs and are likely to interact primarily with the clathrin terminal domain then this would appear to be a strong possibility. The fact that auxilin DLL and DPF mutants (ALAx2 and APAx2) are more greatly affected by the presence of epsin than WT auxilin also suggests that competition may be a component in the inhibition of disassembly. However, these mutants are still able to cause disassembly suggesting that neither the DLL or the DPF motifs are crucial for any competition. Given that these mutants retain the other binding motif in addition to the clathrin box means that, if competition for epsin binding is key, that each of these motifs may contribute in part to competition with epsin. This is in part supported by Dannhauser and Ungewickell's study, where incomplete disassembly of epsin-clathrin structures was observed using a shorter version of auxilin lacking the clathrin box motif present in the construct used here (Dannhauser and Ungewickell 2012). Therefore, a construct containing the DLL and DPF motifs is able to, at least in part, disassemble epsin-clathrin structures without the clathrin box motif.

An interesting counter observation to the auxilin competition hypothesis, is that by varying Hsc70 concentrations in the disassembly of clathrin-epsin cages the inhibitory effect of epsin is increased at low concentrations of Hsc70. Therefore, the inhibitory effect can be said to be dependent on both the concentration of auxilin and Hsc70.

Auxilin and epsin compete for binding to clathrin but the fact that increasing Hsc70 reduces the effect of epsin suggests that the mechanism of inhibition is not soley dependent on competition for protein binding. This is because Hsc70 interacts with clathrin via an exclusive site that it is not known to bind any other adaptor proteins (Rapoport *et al*. 2008). It is therefore likely another mechanism is at play. This is further supported by other observations of auxilin binding. Although the DPF and DLL motifs appear to be able to bind to multiple sites on the CHC the region of auxilin located nearer to C-terminal of auxilin and the J-domain appears to preferentially bind to the distal domain of clathrin (Scheele *et al*. 2001). This is consistent with the role of this region of the protein for recruitment of Hsc70 to the hub of clathrin and therefore preferential binding to this region would facilitate recruitment of Hsc70 to its binding site. Given that epsin seems to interact exclusively with the clathrin terminal domain (Drake *et al*. 2000), it is therefore likely that auxilin and epsin do not compete for clathrin binding on all regions of the CHC. This again supports the idea that epsin disassembly inhibition is not entirely dependent on auxilin competition with epsin for clathrin binding.

In Chapter 8 I discuss in detail possible epsin-clathrin interaction hypotheses and how these relate to both the assembly and disassembly effects of epsin interaction with clathrin cages. I explain the experiments used to investigate these hypotheses by attempting to disrupt these interactions through the use of mutations to epsin and through peptide/adaptor competition.

7.3.3 Variability in protein does not alter trends in the data.

The data in this chapter has highlighted issues in sample variation and protein composition can alter the kinetics of clathrin cage disassembly. However, this is limited to variation in absolute values for light scattering after disassembly and small variations in t ½. The trends observed remain consistent between batches. In relation to the cleavage of the auxilin GST tag the removal of this tag increases its apparent ability to counter the inhibitory effect of epsin. There are a number of explanations for this ranging from steric hindrance to increased activity (as discussed in 7.2.3). However, as stated, the overall trend of disassembly is retained and therefore epsin inhibition of disassembly is not caused primarily by the presence of the GST tag on

auxilin. In the case of epsin itself no obvious differences were noted in binding affinity with SDS-PAGE samples, although as can be seen in purification gels (see section 3.5.2) 'purified' epsin contains a number of degradation products that could vary between the different batches. In light of results described in Chapter 8 it appears that epsin inhibition is attributable to the full length protein rather than any contaminants or degradation products.

7.3.4 Conclusion

In this chapter I have investigated in greater detail the epsin inhibitory effect on clathrin cage disassembly. I have shown that epsin and auxilin compete for binding to clathrin and are unlikely to interact directly with each other in the absence of clathrin. Auxilin DPF and DLL motif mutants are also inhibited by epsin but are still able to disassemble clathrin-epsin complexes and so are not crucial for auxilin competition with epsin. Hsc70 also shows a concentration dependent inhibition in the presence of epsin, suggesting that the mechanism of inhibition is linked to the activity of both auxilin and Hsc70. Finally, I have demonstrated that cleavage of the His_6thioredoxin affinity tag from epsin does not significantly alter the inhibitory effect of epsin.

Chapter 8: Disrupting the epsin effect on assembly and disassembly through mutations and binding competition

'As our circle of knowledge expands, so does the circumference of darkness surrounding it' Albert Einstein

8.1.1 Introduction

8.1.2 Overview

The adaptor protein epsin has been shown during this project to have dual effects on promoting the formation of smaller clathrin cages and inhibiting the disassembly of clathrin cages by auxilin and Hsc70. In this chapter I propose hypotheses for how epsin interacts with clathrin and how these interactions give rise to the observed effects. These hypotheses are tested through the disruption of clathrin binding through mutants in epsin and through the use of competition for clathrin binding with known clathrin box peptides and adaptor proteins.

8.1.3 Proposing hypotheses for epsin-clathrin interactions and their effect on clathrin cage assembly and disassembly

Of the adaptors investigated in this project epsin has had the most marked effect on both clathrin cage assembly and disassembly. In Chapter 4 epsin was shown to both reduce clathrin cage size distribution and shift the size distribution to a smaller particle range, in a manner similar to that previously observed for other adaptors. Later, in Chapters 6 and 7, epsin was shown to inhibit clathrin cage disassembly in a manner that is dependent on the concentration of epsin itself and the disassembly proteins

auxilin and Hsc70. The question that arises from these observations is, what are the properties of epsin that drive these phenomena? I propose that both of these effects are due to binding of the clathrin box motifs of epsin to neighbouring triskelia, thus bringing together their terminal domains (TD). This 'linking' hypothesis is based both on the experimental observations made during this investigation and on previously published work and hypotheses.

Epsin interacts with clathrin cages through a large unstructured region containing two clathrin box motifs and 8 DPW motifs that also contribute to its binding affinity for clathrin (Drake *et al*. 2000; Drake and Traub 2001). The two clathrin box motifs of epsin are separated by a distance of 233 amino acids and given that this region has been shown to be essentially unstructured (Kalthoff *et al*. 2002; Dafforn and Smith 2004). It is estimated that this domain could stretch 150 to 180 Å into the cytoplasm (Kalthoff *et al*. 2002; Dafforn and Smith 2004) and therefore facilitate multiple interactions with clathrin and adaptors.

A crystal structure of the clathrin TD was published by Miele *et al*. (2004) in complex with a binding motif (W-box) from the adaptor protein amphiphysin that interacts with a different binding pocket to the standard clathrin box (Miele *et al*. 2004). Amphiphysin also contains a canonical clathrin box motif and given the short distance between the two motifs the authors proposed that the two motifs are able to interact with the same TD (see Figure 8.01 B). The clathrin box motif in epsin at residue 257 (LMDLA) has been proposed to be similar to the W-box motif of amphiphysin (WLDWP) and hence may interact with the TD at the same site (Drake and Traub 2001; Miele *et al*. 2004).

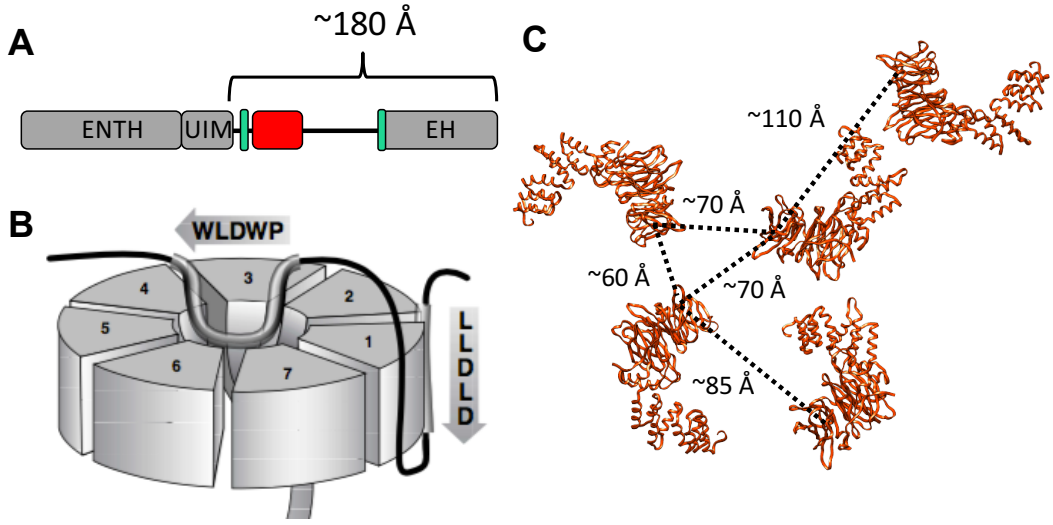

Figure 8.01 Representations of possible epsin-clathrin interactions*. A) A diagrammatic representation of the structure of epsin with the globular membrane binding ENTH domain the ubiquitin interacting motif region (UIM), the epsin hand (EH) domains indicated. The clathrin binding region is bounded by two clathrin box motifs (shown in cyan) with the region containing 8 DPW motifs indicated in red. The unstructured region of epsin has been proposed to stretch up to 180 Å in solution (Kalthoff et al. 2002), (Dafforn and Smith 2004). B) Model of amphiphysin to the clathrin TD as reproduced from (Miele et al. 2004). The second clathrin box or W-box (WLDWP) of amphiphysin is proposed to be similar to the LMDLA clathrin box motif in epsin (Drake and Traub 2001) and therefore epsin could potentially interact with the TD in a similar fashion. C) Distances between clathrin TD (Ile 80) in a clathrin cage as measured from structures reproduced from 3IVY and 1XI4 using UCSF chimera. Many of the distances between the TD are compatible with multiple interactions with a single epsin molecule.*

With these two pieces of evidence, and the fact that the TD contains a potential 4 binding sites for adaptor proteins (Lemmon and Traub 2012), I propose two options for epsin-clathrin interactions (summarised in Figure 8.01). The first is that a single epsin molecule interacts with a single clathrin TD in a manner similar to amphiphysin, with both clathrin boxes contacting the same TD and the intervening DPW region either contacting the same TD through the other regions on the TD or left free in solution to interact with AP2. This proposed 'occupation' hypothesis would explain the epsin inhibitory effect on disassembly though competition with auxilin. In this situation epsin effectively blocks the preferred binding sites of auxilin at low concentrations. At high concentrations auxilin is either able to interact with other sites on the TD or the CHC (see section 7.3.2) or outcompete epsin for these binding sites and therefore induce disassembly. This hypothesis explains the observed auxilin-epsin competition for clathrin binding (see section 7.2.1) but does not explain the inhibitory effect of reduced Hsc70 concentrations (see section 7.2.3). Nor does it provide a

satisfactory explanation for epsin induced assembly as seen in Chapter 4 (see section 4.5.3).

An alternative hypothesis (termed here as the 'linking' hypothesis) is that epsin is able to contact multiple clathrin box motifs. This has in the past been suggested for epsin and other adaptors as a mechanism for assembling clathrin-adaptor complexes (Morgan *et al*. 2000; Greene *et al*. 2000; Drake *et al*. 2000; Drake and Traub 2001; Kalthoff *et al*. 2002; Dafforn and Smith 2004). In this hypothesis the unstructured clathrin-adaptor binding domain is able to contact neighbouring clathrin TDs through its two clathrin box motifs/DPW motifs. This binding would promote interactions between triskelia, thereby bringing them together to help form clathrin cages as seen in Chapter 4. Both clathrin boxes have been shown to work co-operatively to assemble clathrin (Drake and Traub 2001) and this hypothesis is also supported by recent evidence that demonstrated that both clathrin boxes in epsin are required to induce clathrin assembly on liposomes (Holkar *et al*. 2015). Secondly this interaction would potentially stabilise clathrin cages through the extra interactions between triskelia. This would therefore require a greater input of energy to disrupt the cage structure in the form of increased recruitment of auxilin and Hsc70 as seen in Chapters 6 and 7.

8.1.4 Probing clathrin-epsin interactions through epsin clathrin box motif mutants, adaptors and clathrin box peptides

To investigate these hypotheses mutant constructs of epsin were designed in line with Drake *et al*. (2000) and Holkar *et al*. (2015) where the residues of the clathrin boxes were mutated to alanine in each clathrin box individually and together in one construct (constructs are shown in diagrammatic form in Figure 8.02):

^{257} LMDLA -> AAAAA (hereafter referred to epsin Δ257)

^{480} LVDLD -> AAAAA (hereafter referred to as epsin Δ480)

^{257} LMDLA -> AAAAA and ^{480} LVDLD -> AAAAA (hereafter referred to epsin Double Knock Out or DKO)

By generating these 3 combinations of mutants I was able to probe the effect of each motif in turn. If the hypothesis that epsin links triskelia together through its clathrin

box motifs then mutating either of these motifs individually should negate both the assembly and disassembly effects.

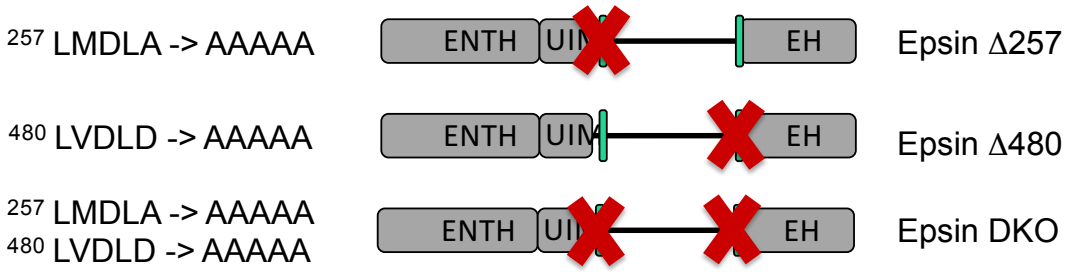

Figure 8.02 Diagram showing the 3 mutated constructs of epsin generated to study the role of the epsin clathrin box motifs on clathrin assembly and disassembly*. Mutations made to epsin clathrin box motifs (cyan) and the locations of the motifs mutated are indicated by a cross. The epsin Δ257 mutant is mutated at the 257 clathrin box but is unchanged at the 480 clathrin box and visa versa for the epsin Δ480 mutant. The double knock out mutant (DKO) has both mutations combined simultaneously in the same construct.*

The other avenue of approach to disrupt epsin-clathrin interactions was to use competition from other adaptor proteins that may interact with the same motifs as epsin or interact with epsin in such a way as to disrupt binding. Both the α HA and β2 HA can interact with epsin through the DPW region (Owen *et al*. 1999; Owen *et al*. 2000; Traub *et al*. 2000; Drake *et al*. 2000; Praefcke *et al*. 2004; Edeling *et al*. 2006a; Schmidt *et al*. 2006) and could potentially inhibit or enhance the inhibitory effect of epsin on disassembly (or the effect on assembly). Therefore clathrin cages were incubated with epsin and β2 HA or α HA to determine if the presence of either of these proteins affects disassembly. Incubation of clathrin with epsin and GST-TD was also conducted in order to see if the addition of free TD would disrupt epsin interactions with clathrin cages by providing alternative binding sites for epsin or auxilin and therefore potentially disrupting inhibition.

The final approach used to probe epsin inhibition of disassembly was through the use of clathrin box peptides. The following 3 peptides containing each of the epsin clathrin box peptides along with a control peptide containing the β2 clathrin box were designed and synthesised (Alta Bioscience, UK) with the clathrin box residues highlighted in red:

Epsin 257 Cbox: ESSLMDLADVF

Epsin 480 Cbox: NAALVDLDSLV

β2 Cbox: LGDLLNLDLGP

By adding excess peptide, it was postulated that epsin-clathrin interactions could be perturbed. Given that β2 HA was shown not to compete for binding to clathrin with epsin (see 8.5.2) the β2 Cbox peptide was included as a control for any effects that the epsin clathrin box peptides may have. Both the epsin 257 Cbox and the β2 Cbox have been used previously to determine interaction affinities with the clathrin TD using fluorescence anisotropy and have been shown to bind to the clathrin TD with an affinity of ~ 100 μM (Sarah Baston, University of Warwick, unpublished data). Binding of the epsin 480 Cbox peptide has not been previously studied and so no value for binding affinity (if any) has been determined.

In this chapter I show that mutating the clathrin box motifs in epsin reduces binding to clathrin cages but does not eliminate binding. I show how mutating clathrin box motifs alters both the assembly and disassembly effects of epsin in a manner that is consistent with the linking hypothesis. I show that attempts to disrupt the epsin inhibitory effect on disassembly through interactions with the α and β2 HA produces results consistent with previously published data. Finally, I show that attempted disruption through the use of GST-TD and peptides leads to unexpected results that require further investigation to confirm the observations made. These results are then put into context with the proposed hypothesis for epsin-clathrin interaction and related to possible implications for the role of epsin *in vivo*.

8.2.1 Epsin clathrin box mutant effect on clathrin cage assembly

8.2.2 Epsin clathrin box mutants are still able to bind to clathrin

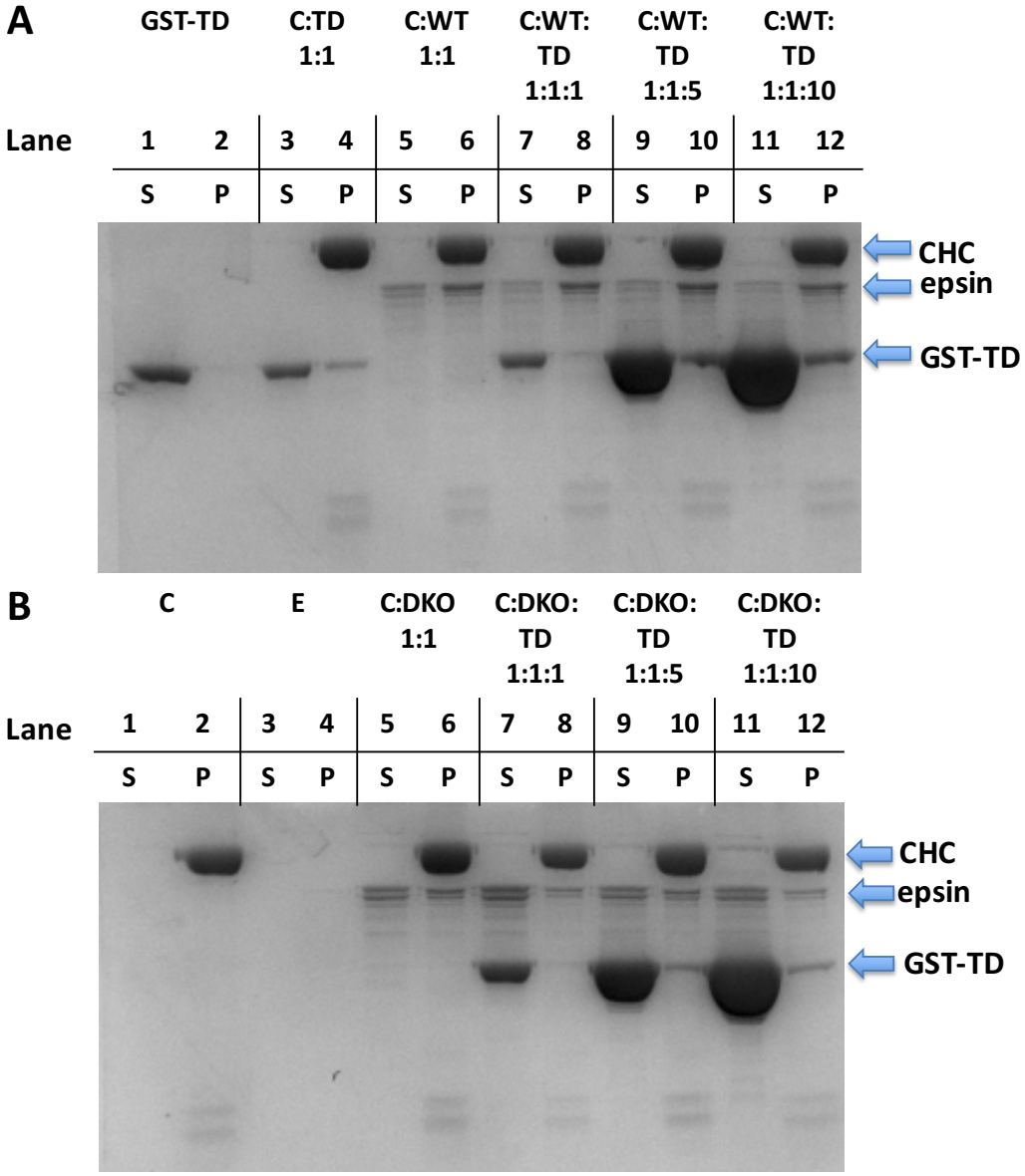

Figure 8.03 Binding of WT and clathrin box mutant epsin to clathrin cages as determined by ultracentrifugation and SDS-PAGE. *Epsin constructs were incubated in the presence or absence of 3 μM clathrin at 1:1 and 1:5 molar ratio of clathrin:epsin for 1 hour before centrifugation with supernatant (S) and re-suspended pelleted (P) material analysed by SDS-PAGE. A) WT epsin does not pellet in the absence of clathrin but does with clathrin as expected. The DKO mutant also seems to pellet but with a notable loss of intensity in a 5-fold excess (Lanes 8 and 12). B) Again the Δ257 and Δ480 mutants both seem to bind to clathrin with a possible reduction in binding compared to WT.*

In order to determine the effect of clathrin box mutations on the ability of epsin to bind to clathrin pull downs of WT, Δ257, Δ480 and DKO epsin were conducted in the presence of clathrin cages and the supernatant and pellet fractions analysed by SDS-PAGE (see Figure 8.03). The major observation made from these pull downs is that all of the mutant variants of epsin show some form of binding to clathrin, indicating that binding has not been completely eliminated. However, whilst all of the mutants bind to clathrin there does appear to be a reduction in binding that is most pronounced between WT and DKO epsin. This is most apparent when comparing the 5 fold excess conditions (See Figure 8.03 A Lanes 8 and 14). Here we notice an apparent qualitative drop in the amount of epsin present in the pellet relative to the WT which could be suggestive of a reduction in binding of the DKO mutant. When comparing the single clathrin box mutants Δ257 and Δ480 there may also be a change in binding intensity (particularly with Δ480 see Figure 8.03 B Lanes 6 and 12). These observations are of course qualitative (although observed in multiple gels experiments) and a more quantitative assessment of the affinity of the clathrin mutants through a technique such as ITC would provide a quantitative measure of the changes in binding affinity. None the less the observations that all of the mutants interact with clathrin to a greater or lesser degree is key to later observations.

To confirm that loss of binding affinity was due to loss of epsin binding to the TD both WT and DKO epsin were pelleted with GST-TD (see Figure 8.04). Pull downs with GST-TD confirm that WT epsin interacts with the TD and that the loss of the clathrin boxes in the DKO mutant significantly affects its ability to bind to the TD.

The fact that all of the mutants are still able to interact with clathrin is not unexpected. Drake *et al*. (2000) demonstrated that epsin contains two clathrin box motifs and that the region between the clathrin box motifs containing the DPF motifs provides affinity to clathrin. Progressively shortening a construct containing the 257 clathrin box (but not the motif at 480) resulted in a reduction in binding of clathrin (Drake *et al*. 2000). The retention of binding seen here fits with these previous results.

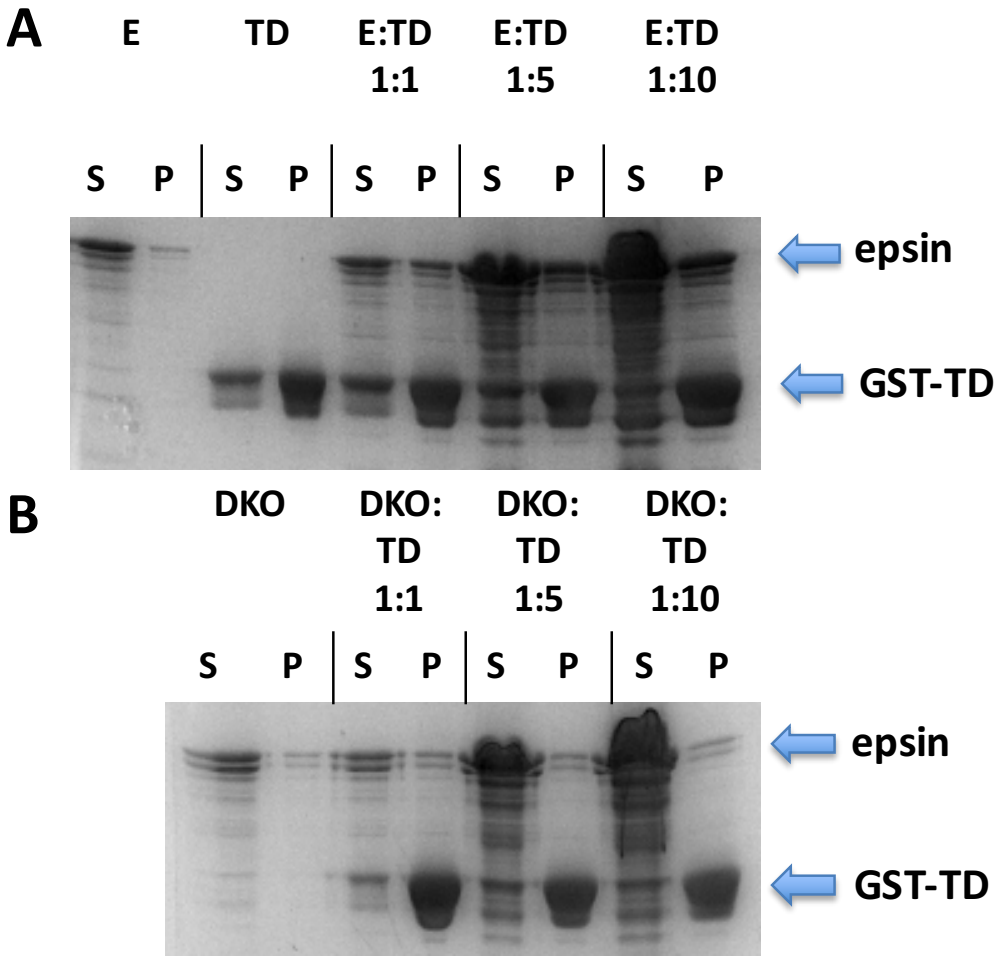

Figure 8.04 Binding of WT and DKO clathrin box mutant epsin to clathrin GST-TD as determined by GSH affinity pull downs and SDS-PAGE. *Epsin constructs were incubated in the presence or absence of 3 μM GST-TD at 1:1 and 1:5 and 1:10 molar ratio of GST-TD:epsin with GSH-sepharose beads for 1 hour before centrifugation with supernatant (S) and bound (P) material analysed by SDS-PAGE. A) WT epsin shows increased binding to the GST-TD with higher concentrations of epsin but the DKO mutant (B) shows almost no binding above the background binding in the control in the absence of GST-TD.*

8.2.3 Epsin clathrin box mutants show differential size distributions as determined by EM and DLS

Polymerisation of clathrin in the presence of epsin showed a dramatic shift to smaller cages (see section 4.5.3). To determine the role of the epsin clathrin box in this effect clathrin was polymerised in the presence of Δ257, Δ480, DKO clathrin box mutant epsin and WT epsin as described previously and size distribution determined through use of DLS and EM as described in Chapter 4. Note that, unlike in Chapter 4 where EM data and DLS are compared directly the EM data and DLS data here come from different samples. This might explain some of the differences in the observed distributions between samples and hence any conclusions drawn from these data must be approached with this in mind.

Using the size distribution from the EM data the evidence suggests that clathrin box mutant epsin is still able to change the distribution of clathrin cages in a manner similar to WT epsin although with some subtle differences. Figure 8.05 shows the distributions of cage radii as measured with cages polymerised with a 1:1 or 1:5 molar excess of epsin as conducted previously. Compared to the clathrin control WT epsin again shifts the distribution seen to smaller cages with a peak distribution of around 35 nm when incubated at a 1:1 molar ratio. A similar distribution is seen with all of the clathrin box mutants with the exception of the DKO mutant where a shoulder consisting of larger particles up to a radius of 50 nm is seen. At the 1:5 ratio the single clathrin box mutants both retain a similar distribution with a peak frequency at around 35-40 nm where as the distribution for WT epsin continues to shift to smaller cages with an increase in cages with a radius of 30 nm and an almost complete loss of cages at 40 nm. Interestingly the DKO mutant shares an almost identical distribution to that shown by the WT. Taken together these observations seem to suggest that mutating epsin clathrin box motifs has little effect on the cage size.

These results differ slightly compared to the distribution seen in the initial epsin experiments where the distribution in the Δ257 and Δ480 mutants more closely matches the WT distribution in those experiments. Both the DKO and WT epsin in this set of experiments are shifted to a smaller size distribution than expected. As will be discussed with the DLS results in this section, clathrin concentration is an important factor in the shift in size distribution (clathrin concentration is itself a factor in size distribution) and hence changes in concentration may contribute to the variation in distribution seen here. And as highlighted in Chapter 4, the EM analysis only measures ‘cage like’ particles and hence aggregates and small particles are ignored. Although not quantified, it was noted that grids containing cages polymerised with the DKO mutant showed fewer cages in EM micrographs compared to the other epsin variants.

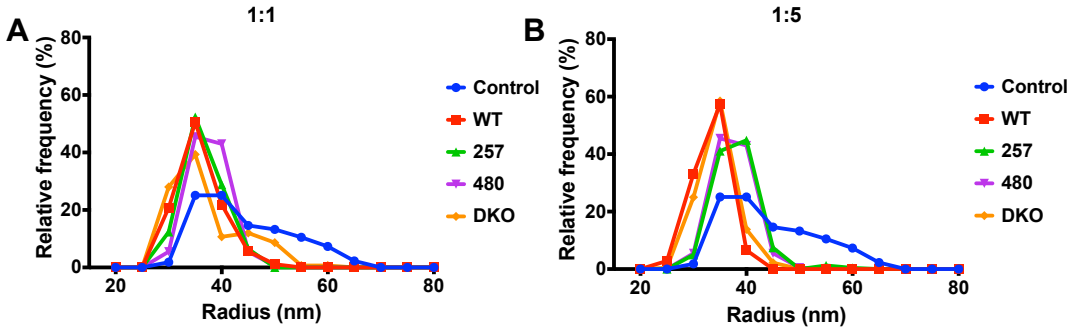

Figure 8.05 Histogram of the radius of clathrin cages polymerised in the presence of WT and clathrin box mutant epsin as imaged by negative stain EM and measured in ImageJ*. The number of particles measured for each distribution was 170<n<210. A) Shows the distribution for epsin polymerised with clathrin at a 1:1 molar ratio. All cages polymerised in the presence of epsin, whether mutant or WT show a shift in distribution to a smaller cage radius around 35 nm although the DKO and 480 mutants show a slightly wider distribution. B) At the 1:5 ratio both theΔ 257 and Δ480 mutants show a similar distribution to each other with the DKO mutant and WT epsin continuing to show a similar distribution to at the 1:1 ratio.*

When the same experiment was conducted by DLS a difference in apparent effect was noted. This effect is likely exacerbated by the apparent loss of clathrin during the experiment with an apparently lower concentration of clathrin present in all samples compared to the expected amount (see Table 8.01). Although the clathrin sample after depolymerisation was re-quantified and changes in volume after dialysis taken into account the apparent concentration of clathrin calculated during the analysis appears to be significantly lower than expected and vary substantially between samples.

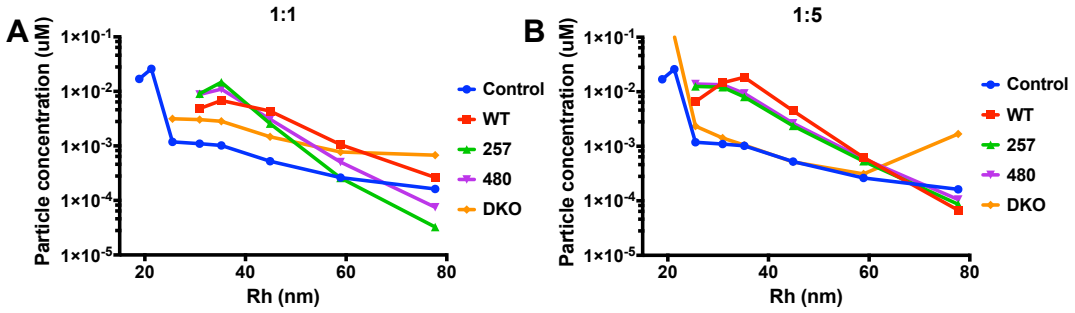

Figure 8.06 Hydrodynamic radius (Rh nm) of clathrin particles polymerised in the presence of WT and clathrin box mutant epsin and their concentrations (μM) as determined by DLS*. A) At a 1:1 ratio WT epsin and the single clathrin box mutants 257 and 480 show distributions indicating polymerisation with a preponderance of smaller particles as seen previously with epsin. With the control however we see little evidence of polymerisation with peaks present in the 21.3 nm indicative of small oligomers. A similar distribution is noted with the DKO mutant. B) At the 1:5 ratio the effects seen with all variants of epsin are repeated and exacerbated with the WT and single clathrin box mutants all continuing to shift towards smaller cages. With the DKO mutant we also see an increase in particle concentration at the extremes of the distribution, suggesting the formation of small particles and aggregates.*

In Figure 8.06 we see the data collected on the clathrin cages polymerised in the presence of mutant and WT epsin at a 1:1 (A) and 1:5 (B) ratio of clathrin: epsin as used previously. Both WT epsin and the single clathrin box mutants Δ257 and Δ480 all exhibit polymerisation of clathrin cages with an apparent shift in Rh towards cages

of smaller radii which is largely consistent with the EM data. However, the clathrin only control does not show any obvious polymerisation which is also exhibited in the DKO polymerisation sample. Taken together with the calculated clathrin concentrations given in Table 8.01 we can make some interesting observations about these apparent effects.

The apparent clathrin concentration calculated from the DLS data is derived from the theoretical scattering intensity of the polymerised clathrin and intensity observed. Whilst these values are an estimate based on a theoretical scattering intensity the values seen for this experiment tend to show much lower values than expected, which ties in with the observed distributions in the DLS. At an apparent concentration of 0.3 μM as opposed to 3 μM it is likely that clathrin failed to polymerise (clathrin concentrations <0.5 μM are unlikely to polymerise). The reason for the loss of clathrin is unknown but it is likely due to loss of clathrin stability, such as through the loss of the CLC. With WT epsin and the Δ257 and Δ480 clathrin box mutants at a 1:1 ratio the calculated concentration is higher at between 0.8-1.0 μM with the DKO mutant showing a substantially lower concentration at 0.5 μM. Whether this is because of random changes in concentration it is not known but it is tempting to explain the variation in concentration and apparent polymerisation in terms of the ability of each of the epsin variants to bind. It is possible that as the DKO mutant shows a noticeable reduction in binding (see Figure 8.03) it is unable to efficiently polymerise cages and stabilise clathrin complexes at this concentration whereas the single mutants and WT epsin are. Although at the 1:5 ratio it appears that the apparent clathrin concentration is higher in the DKO sample than with the WT and other mutants, it is still of note that little assembly is observed in the DLS (Figure 8.05 B). It is possible that, at the concentration used, the clathrin is not able to assemble efficiently alone but in the presence of epsin (with the exception of the DKO mutant) assembly is promoted. Repeating this experiment to specifically interrogate the effect of clathrin concentration would provide evidence for or against this hypothesis.

Sample & Ratio (C:E)	Nominal [C] μM	Calculated [C] μM
Control	3.3	0.3
WT 1:1	3.0	0.8
Δ257 1:1	3.0	1
Δ480 1:1	3.0	0.9
DKO 1:1	3.0	0.5
WT 1:5	3.0	1.6
Δ257 1:5	3.0	1.1
Δ480 1:5	3.0	1.2
DKO 1:5	3.3	2.1

Table 8.01 Table of clathrin-epsin polymerised samples as analysed by DLS for which the concentration curves are displayed in Figure 8.05*. The nominal concentration is given for all samples with the correction for volume changes after dialysis (all samples were initially at 3 μM). Apparent concentrations calculated during the analysis of the DLS data indicate that the apparent (Calculated) concentrations used during polymerisation vary significantly between samples and hence may explain the observations made during the DLS experiments.*

In conclusion the preliminary assembly experiments using the mutant clathrin box variants of epsin show some interesting results. It is tempting to conclude that the assembly effect of epsin is dependant on the extent of epsin binding, i.e. the affinity of epsin for clathrin cages, as opposed to the mechanism of binding i.e. the requirement of both clathrin boxes to link cages. This would count against the linking hypothesis for epsin binding (at least in relation to clathrin box interactions) although a far greater data set should be generated before specific conclusions can be made.

8.3.1 Epsin inhibition of disassembly: investigating the effect of clathrin box mutants and protein/peptide competition

8.3.2 Epsin clathrin box mutants do not inhibit disassembly

To determine the theory that the epsin clathrin boxes play a key role in the inhibitory effect in relation to disassembly the clathrin box mutants were incubated with cages as previously described (using a 1:5 molar ratio) and disassembled as per previous experiments (see Figure 8.07). As described in section 7.2.3 epsin inhibition of cage disassembly is in part dependent on the protein batch and the activity of auxilin and in this experiment WT epsin seems to inhibit to a lesser extent than has been seen previously. However, the key observation is that all of the clathrin box mutant variants of epsin show almost no significant increase in final light scattering intensity, suggesting little to no inhibition of disassembly is occurring. This is consistent with the hypothesis that the clathrin boxes are required for the inhibition of disassembly and that both boxes are required simultaneously for the effect to occur.

Whilst there is a clear difference between all of the mutants in terms of final light scattering intensity compared to WT epsin it is possible that the Δ480 mutant may be exhibiting a slight amount of inhibition. This mutant retains the clathrin box at Δ257 and hence may indicate that this motif may play a slightly more important role in the inhibition of disassembly although a greater amount of data would need to be collected to confirm or deny this observation.

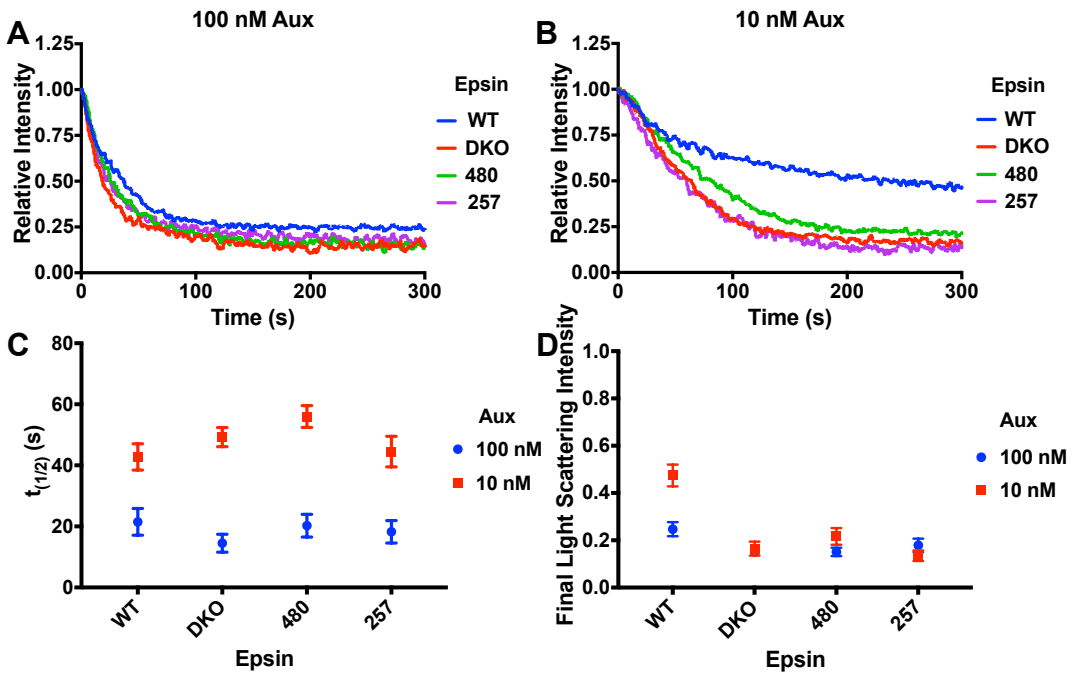

Figure 8.07 Disassembly of clathrin cages incubated with a 1:5 Molar excess of WT epsin or the clathrin box mutant epsin constructs DKO, Δ480 or Δ257. *Clathrin cages disassembled in the presence of 100 nM (A) or 10 nM (B) auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Rates of disassembly for all epsin constructs are consistent at 100 nM although at 10 nM there appears to be an increase in t ½ relative to WT and the other two mutants. D) As shown previously WT epsin results in a higher final light scattering intensity at low concentrations of auxilin (10 nM) but this effect is not observed with any of the mutants with the exception of epsin Δ480 which exhibits a slightly higher end scatter intensity. This indicates that mutating any of the clathrin boxes seems to remove the inhibitory effect of epsin.*

8.4.1 The effect of α HA on epsin-clathrin disassembly

8.4.2 GST-α HA binds to clathrin more efficiently in the presence of epsin

Having pursued the use of mutants to disrupt the epsin inhibition of disassembly an alternative approach was used: that of disruption of binding through competition. Although evidence from the clathrin box motif mutants confirmed that the clathrin box motif is key to the inhibition effect by epsin the protein also interacts with clathrin via the DPW region between the two clathrin boxes (Drake *et al*. 2000). DPW motifs, including that of epsin, have been shown to interact with the the α HA domain from the AP2 complex (Brett *et al*. 2002). Incubating α HA with epsin-clathrin complexes during disassembly would indicate whether these motifs are important for the inhibition effect as potential interactions with α HA might disrupt this interaction with clathrin. In addition the α HA can interact with auxilin via its DPW domains (Scheele *et al*. 2003). The α appendage contains two sites for interacting with DPF/DPW motifs although the side site shows preferential binding for DPW where as the platform site

provides a more general adaptor binding site (Brett *et al*. 2002; Praefcke *et al*. 2004; Schmidt *et al*. 2006) (see section 1.6.4). It is therefore possible that both auxilin and epsin could interact with α-HA at the same time, with epsin preferentially interacting with the side site and auxilin interacting with the platform site.

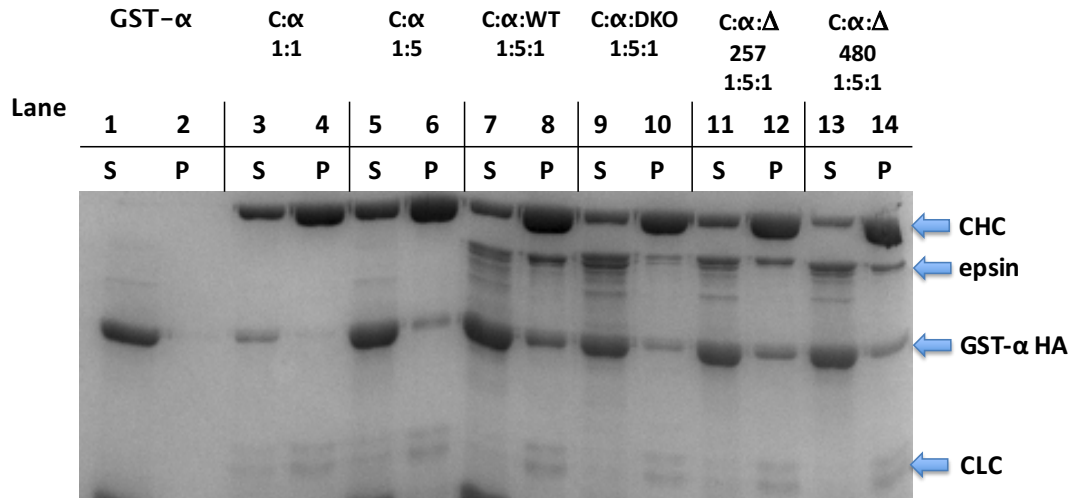

Figure 8.08 Binding of GST-α HA to clathrin cages (3 μM) in the presence or absence of WT and clathrin box mutant epsin as determined by ultracentrifugation and SDS-PAGE. *GST-α HA does not pellet on its own (GST-α) and binds to clathrin poorly to clathrin at both 1:1 and 1:5 molar excess of GST-α HA as has been previously shown. In the presence of WT epsin a greater amount of GST-α HA can be seen in the pellet (Lane 8). In the presence of the DKO mutant, which binds to clathrin less efficiently than the WT, an increase in GST-α HA in the pellet is not observed (Lane 14) but is observed to an extent in the Δ257 and Δ480 mutants that both bind to clathrin, if not as effectively. This suggests that GST-α HA is being pulled down by epsin.*

To investigate potential interactions between epsin and α-HA, GST-α HA was incubated with clathrin in the presence or absence of epsin before pelleting and analysis of the pellet and supernatant fractions by SDS-PAGE (See Figure 8.08). α-HA is not known to contain any specific clathrin binding motifs but may interact with the ankle domain in a similar way to the β2 HA (Knuehl *et al*. 2006). As such it does bind to clathrin as is shown here although with apparent low affinity. In the presence of WT epsin however the intensity of the GST-α HA band in the pellet increases (C:α:WT 1:5:1). This effect is concordant with the amount of epsin bound to clathrin: when epsin is instead incubated with the DKO mutant construct (which binds less efficiently than the WT) the intensity of the GST-α HA band is equivalent to the pellet in the absence of epsin (C:α:DKO 1:5:1). With the single clathrin box mutants the effect is intermediate. This suggests that GST-α HA is interacting with epsin. It also suggests that the binding of GST-α HA does not significantly effect the ability of epsin to interact with clathrin. GST itself does not appear to interact with clathrin and so it is likely that this effect is due to α HA interactions with epsin (see Appendix).

One possible explanation would be that interactions with the DPW motifs of epsin and α HA are able to occur in tandem to clathrin binding. When Drake *et. al.* shortened the clathrin binding region of epsin they showed that truncating from the C-terminal end (the regions furthest from the DPW motifs and the 257 clathrin box) reduced affinity to clathrin (Drake *et al*. 2000). This would suggest that this region provides clathrin binding affinity despite not containing any recognised motifs important for clathrin binding. Therefore if α HA is interacting with the DPW motif(s) of epsin this may not significantly effect clathrin binding.

8.4.3 GST-α HA does not change epsin inhibition of disassembly

Investigating the effect of the α-HA interaction on epsin dependent inhibition of clathrin cage disassembly was conducted by incubating clathrin cages with varying GST α HA concentrations in the presence or absence of epsin (see Figure 8.09). The results indicate that GST α HA has no effect on disassembly in isolation and neither does it have an effect on the inhibitory effect of epsin. Interestingly the rate of disassembly in the presence of both epsin and GST α HA is decreased. The explanation for this in not clear, however, it is possible that the presence of GST α HA binding to epsin in some way contributes a form of steric hindrance to disassembly. Note that disassembly here was conducted with cleaved auxilin without the GST tag and hence any possible effects of dimerization between auxilin and GST-α HA were avoided. Disassembly of clathrin cages incubated in the presence of GST does not alter disassembly (see Appendix).

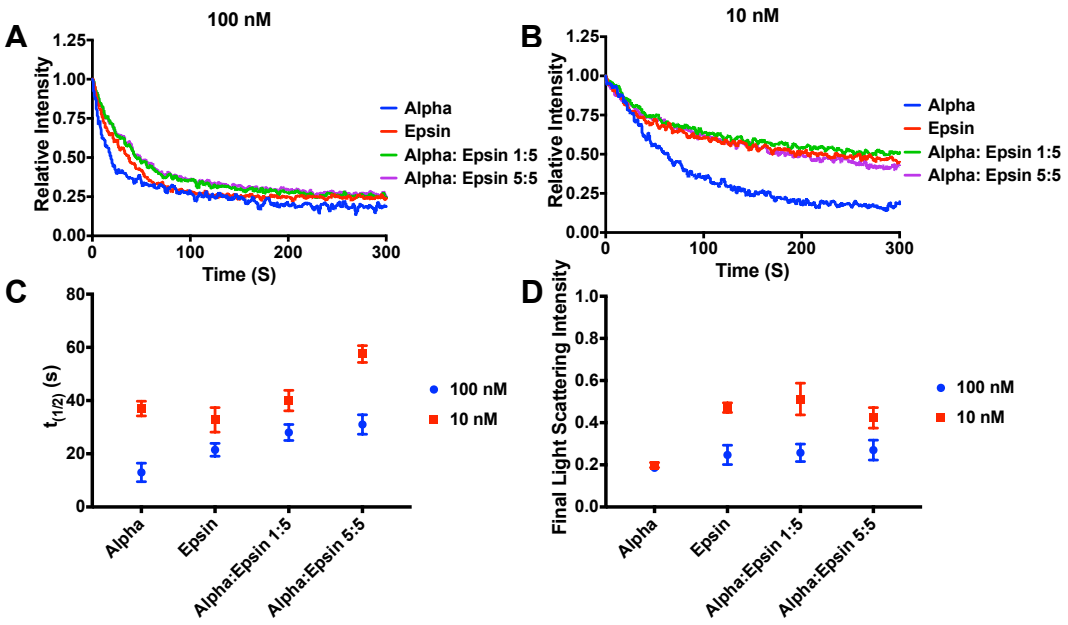

Figure 8.09. Disassembly of clathrin cages incubated with α-HA in the presence or absence of a 1:5 molar excess of WT epsin. *Clathrin cages disassembled in the presence of 100 nM (A) or 10 nM (B) auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Rates of disassembly as determined by t ½ indicate that GST-α HA has no effect on the rate of disassembly. in the presence of both GST-α HA and epsin the t ½ increases suggesting having both proteins bound to clathrin may have a negative effect on disassembly. D) Final light scattering indicates that the extent of disassembly is not effected by the presence of GST-α HA and the inhibitory effect of epsin is retained in the presence of this protein.*

The fact that the presentation of a possible extra binding site for auxilin within the clathrin cage in the form of α HA does not alter the extent of disassembly is not without support. Dannhauser and Ungewickell explored the disassembly of CCVs formed using epsin in the presence or absence of the AP2 complex. As described, the authors noted that release of clathrin was never complete and that the presence of AP2 made no difference to this effect (Dannhauser and Ungewickell 2012). The presence of AP2 made no difference to this effect, which supports the observations made here. Therefore, if auxilin is indeed able to bind to α HA in this complex, this binding does not significantly effect the ability of auxilin to counter the inhibition of disassembly by epsin.

8.5.1 The effect of β2 HA on epsin-clathrin disassembly

8.5.2 Epsin and β2 HA are able to bind to clathrin in concert

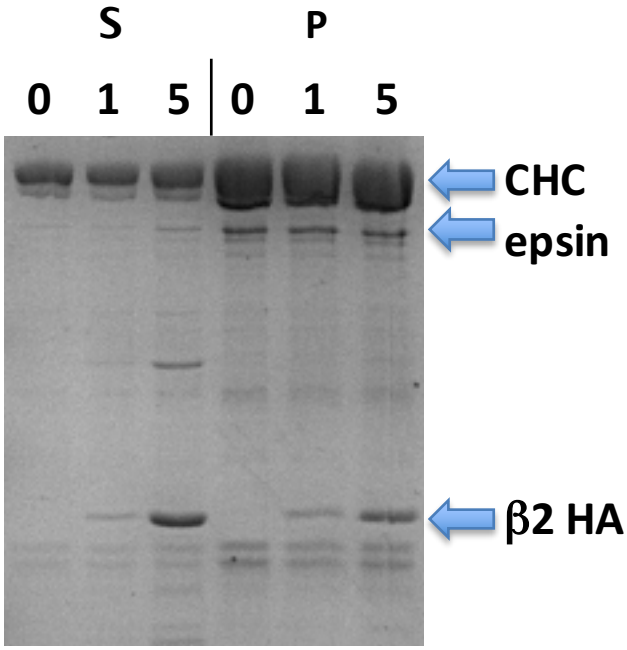

Figure 8.10 Binding of β2 HA (β2) to clathrin cages in the presence or absence of epsin as determined by ultracentrifugation and SDS-PAGE. *3 μM clathrin cages were incubated with 3 μM epsin and with 0, 1 and 5 molar excess of β2 HA and pelleted 140,000 x g and supernatant (S) and pellet (P) fractions analysed by SDS-PAGE. The results indicate that increasing concentrations of β2 HA does not reduce the amount of epsin present in the pellet fraction, suggesting that these proteins do not compete for binding.*

Another component of the AP2 complex was also investigated to determine if it could disrupt the epsin inhibitory effect: β2 HA. As this domain binds to clathrin primarily through a clathrin box motif it was hypothesised that this motif could compete with epsin for binding to clathrin in much the same way that auxilin does. To confirm whether these two proteins might compete for binding, clathrin was incubated with epsin in the presence or absence of β2 HA and clathrin cages pelleted by centrifugation (see Figure 8.10). SDS-PAGE analysis of the pellet and supernatant fractions indicated that both proteins are able to interact with clathrin without significantly loss of binding. This fits with observations made by Drake *et al*. where the epsin clathrin/adaptor binding domain was able to pull down both clathrin CHC and CLC as well as components of the AP2 complex (Drake *et al*. 2000). This would lend further support to the hypothesis that β2 HA and epsin are binding to discrete regions on clathrin. At the concentrations used the clathrin box of β2 HA could potentially be interacting with a different TD binding site to epsin. Alternatively the appendage

binding site on β2 HA, through interaction with the ankle region of the CHC, may be enough to retain binding in the presence of epsin.

8.5.3 β2 HA increases the rate of clathrin-epsin cage disassembly but not the extent of disassembly

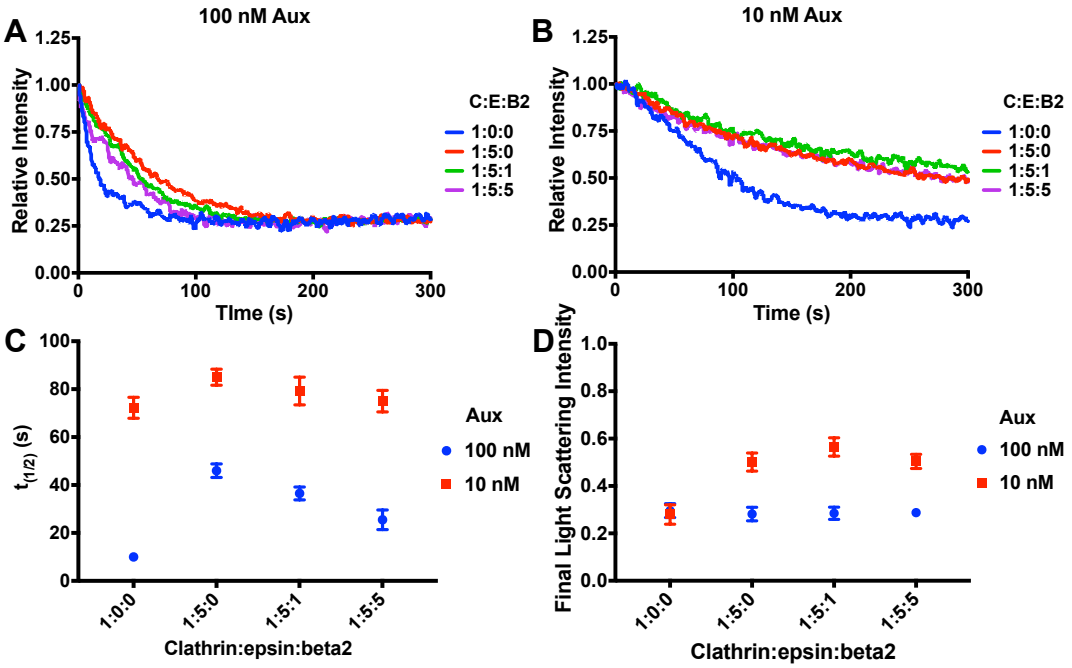

Figure 8.11 Disassembly of clathrin cages incubated with epsin in the presence of increasing concentrations of β2 HA. *3 μM of clathrin cages were incubated with a 5 fold molar excess of epsin (1:5:0) with either 3 μM of β2 HA (1:5:1) or 15 μM β2 HA (1:5:5) and disassembled in the presence of 100 nM (A) or 10 nM (B) auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Rates of disassembly as determined by t ½ indicate that increasing concentrations of β2 HA increases the rate of disassembly in the presence of epsin at 100 nM. D) β2 HA does not significantly alter the extent of disassembly in the presence of epsin, suggesting that it does not disrupt the inhibitory effect of epsin.*

In Chapter 6 β2 HA was shown to have little effect on disassembly although a possible increase in rate was observed in the presence of the protein. As demonstrated in the previous section, β2 HA does not compete directly with epsin for clathrin binding and so there is little to suggest that β2 HA would counter the epsin inhibitory effect. Disassembly of clathrin-epsin cages was conducted in the presence of increasing concentrations of β2 HA with the data indicating that β2 HA does not have a significant effect on the inhibition of disassembly as determined by final light scattering intensity (see Figure 8.11). Interestingly there does appear to be to be a trend that, in the presence of β2 HA, the rate of disassembly increases. This is most notable at 100 nM auxilin and could occur at 10 nM auxilin although the increase in rate at this concentration of auxilin is within the error for each of the data points. The

mechanism for this observed effect is not known but as β2 HA appeared to increase the rate of disassembly slightly in the absence of epsin (see section 6.2.3) it could be that this effect is as a consequence of β2 HA binding and is not countered by the presence of epsin. These observations again fit with the observations made by Dannhauser and Ungewickell (2012) that the extent of disassembly of epsin CCVs does not vary in the presence of AP2.

8.6.1 The effect of clathrin TD on epsin-clathrin disassembly

8.6.2 Epsin pulls down GST-TD when bound to clathrin

The clathrin TD is the primary binding site for adaptor proteins and in particular contains the binding sites for clathrin-box/W-box containing proteins (Lemmon and Traub 2012). Although the affinity of adaptor proteins for the TD is often considerably lower than that for clathrin cages adding an excess of TD may disrupt adaptor binding to clathrin by providing alternative binding sites. WT and DKO epsin were incubated in the presence or absence of GST-TD prior to incubation with clathrin cages and then pelleted by ultracentrifugation. SDS-PAGE analysis of the supernatant and pellet fractions indicates that GST-TD interacts with clathrin cages (see Figure 8.12). The reason for this is unknown but it could possibly be due to non-specific interactions as a consequence of the GST-tag. However, purified GST does not appear to interact with clathrin as determined via clathrin cage-GST pull downs (see Appendix). When WT epsin is also added, epsin continues to be present in the pellet fraction and therefore does not appear to be disrupted from clathrin binding by the large addition of GST-TD (C:WT:TD 1:1:5 and 1:1:10). Interestingly the amount of GST-TD in these two conditions does increase in the presence of epsin. This is contrasted in the equivalent experiment with DKO mutant epsin where the intensity of the GST-TD band in the pellet fraction does not increase (C:DKO:TD 1:1:5 and 1:1:10). Given that DKO epsin binds less efficiently to clathrin and combined with observations from GST-TD pull downs with these proteins (see Figure 8.04) this would tend to suggest that epsin is interacting with the TD. Given the large protein loading it could be argued that material could have spilled over from the neighbouring lane. This experiment was conducted twice and the same pattern observed and so it is reasonable to state that the results observed here are as a consequence of protein interactions. In addition, GST

incubation with clathrin suggested that GST itself does not interact with clathrin (see Appendix).

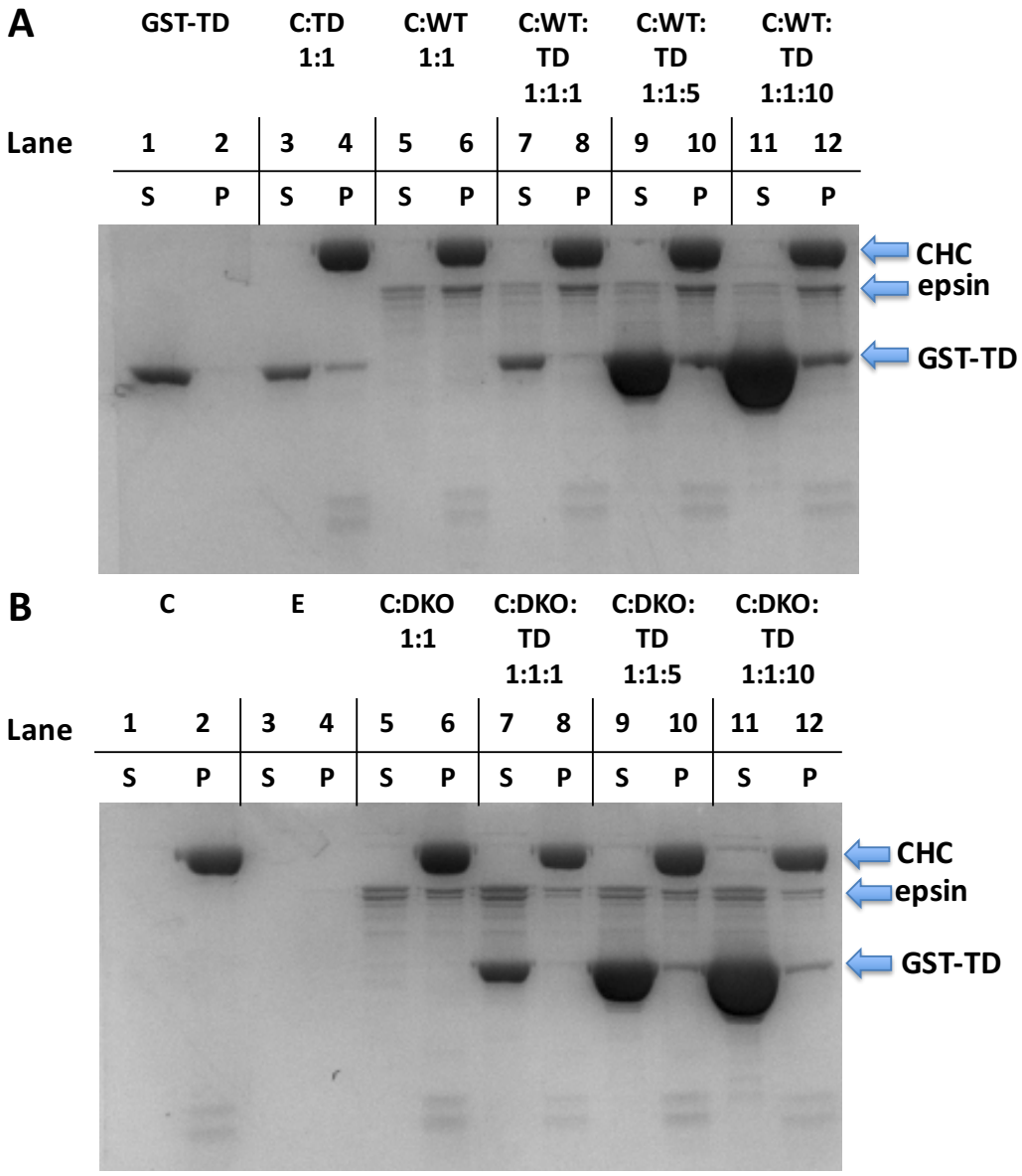

Figure 8.12 Interactions of GST-TD with clathrin cages in the presence or absence of WT epsin or clathrin box mutant epsin DKO as determined by ultracentrifugation and SDS-PAGE*. A) GST-TD does not pellet on its own (GST-TD Lane 2) but does appear to interact mildly with clathrin (C:TD 1:1 Lane 4). In the presence of WT epsin a greater amount of GST-TD can be seen in the pellet (C:WT:TD 1:1:5 and 1:1:10, Lane 10 and Lane 12). B) In the presence of the DKO mutant, which binds to clathrin less efficiently than the WT, the amount of GST-TD present in the pellet fractions of these samples does not significantly increase (Lanes 10 and 12). This suggests that GST-TD is being pulled down through interactions with epsin.*

Interestingly, this interaction does not appear to significantly alter the association of epsin for clathrin cages. One possibility is that GST-TD is interacting with free sites along the clathrin binding domain of epsin, possibly through the DPW motif region as hypothesised for α HA. However, as the clathrin box motifs are integral to the inhibitory effect of epsin, if the TD is interacting with an epsin clathrin box then one

would expect an interaction of epsin with GST-TD via this motif to prevent epsin inhibition of disassembly. As this is not observed either GST-TD does not interact via an epsin clathrin box motif or the clathrin box motifs of epsin are not crucial to inhibition (which is contrary to the observed effect of clathrin box mutants in epsin).

8.6.3 GST-TD reduces disassembly in the presence of epsin

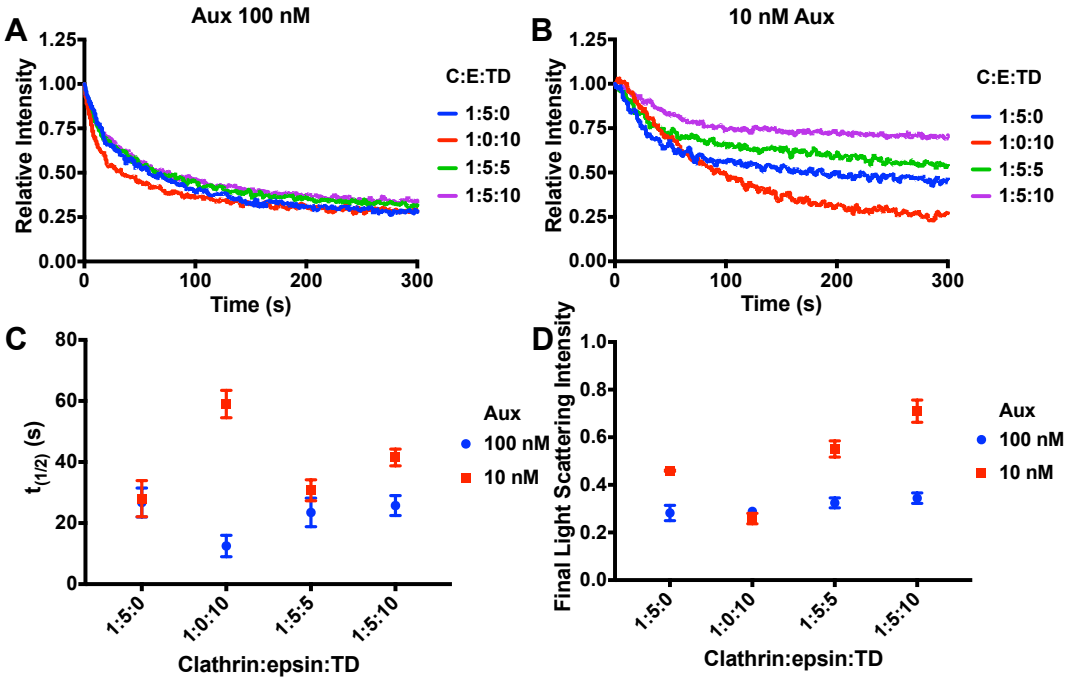

Figure 8.13 Disassembly of clathrin cages incubated with WT epsin and GST-TD in various combinations to determine their combinatorial effect on disassembly. *3 μM of clathrin cages were incubated with a 5 fold molar excess of WT epsin (1:5:0) or 10 fold GST-TD (1:0:10). WT epsin cages were also incubated with 5 fold or 10 fold excess of GST-TD to clathrin (1:5:5 and 1:5:10) and disassembled with either A) 100 nM auxilin or (B) 10 nM auxilin as initiated with addition of 500 μM ATP and 1 μM Hsc70. C) Rates of disassembly as determined by t ½ indicate that GST-TD has no effect on disassembly. When incubated with epsin an increase in the t ½ can be seen with the highest concentration of epsin at 10 nM auxilin (1:5:10). D) When looking at the final light scattering intensity we can see that at 10 nM auxilin the presence of GST-TD seems to increase the end scattering intensity which is concentration dependent. This indicates that the inhibitory effect of epsin is increased in the presence of GST-TD.*

To gain an insight into how a TD-epsin interaction might affect disassembly, clathrin cages incubated with epsin and/or TD were disassembled as previously described (see Figure 8.13). GST-TD does not appear to have an effect on disassembly on its own. Interestingly the presence of GST-TD with epsin seems to have a negative effect on the final light scattering intensity, increasing the final light scattering intensity at 10 nM auxilin relative to epsin cages without TD, indicating that the presence of the TD is having a negative effect on disassembly. This would suggest that the TD is not interacting with epsin or clathrin in a way that disrupts the epsin inhibitory effect i.e. the epsin clathrin boxes are still able to bind to clathrin and inhibit disassembly. It is

again worth noting that disassembly was conducted with cleaved auxilin lacking the GST tag and hence any possible effects from dimerization of auxilin with TD were avoided.

There are several possible explanations for the exacerbation of the inhibitory effect seen with the addition of GST-TD. The first is that the TD is creating some form of steric hindrance through its binding. Neither GST-TD nor GST itself interact significantly with clathrin or affect disassembly (see Appendix) and so it is possible that the recruitment of GST-TD by epsin interferes with auxilin binding. The second possibility is that auxilin is binding to GST-TD rather than to the CHC and hence is unable to recruit Hsc70 to the correct location in the clathrin cage. GST-TD does not significantly alter disassembly in the absence of epsin so it is unlikely that the effect is due to auxilin binding to GST-TD in solution and therefore sequestering the protein away from clathrin cages. Pull downs of these complexes involving clathrin, GST-TD, auxilin and epsin may help reveal the method of inhibition.

8.7.1 Disassembling clathrin-epsin cages in the presence of clathrin box peptides is inconclusive

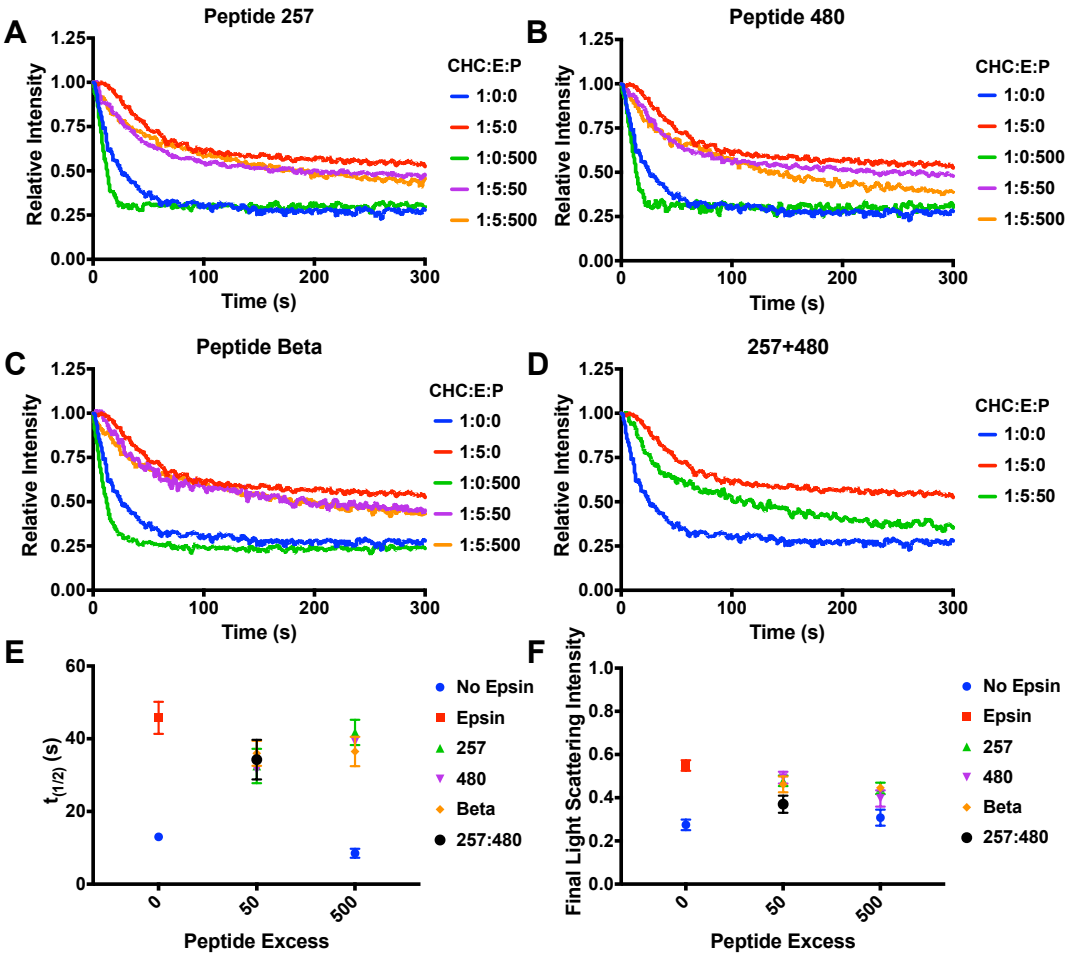

Figure 8.14 Disassembly of clathrin cages incubated with WT epsin and clathrin box peptides in various combinations to determine their combinatorial effect on disassembly. *3 μM of clathrin cages were incubated with a 5 fold molar excess of WT epsin (1:5:0) with a 50 (1:5:50) or 500 fold(1:5:500) excess of clathrin box peptide. (A-D) show disassembly in the presence of Cbox peptides 257, 480, Beta and a combination of 257 and 480 respectively. E) Epsin inhibits disassembly as shown previously but in the presence of peptide at all concentrations the t ½ is reduced slightly, suggesting a faster rate of disassembly. F) With all peptides a lower final light scattering intensity is observed that is dependent on increased concentrations of peptide and suggests that the presence of any of the peptides results in a reduction of epsin inhibition. It should be noted that all 3 peptides increased the apparent rate of disassembly in the absence of epsin.*

As shown in 8.3.2 both clathrin boxes in epsin are required for the inhibition of disassembly. Therefore, it should be possible to disrupt epsin inhibition through competition for binding with either of these motifs. Clathrin cages were therefore incubated in the presence or absence of epsin and/or Cbox peptides corresponding to the clathrin boxes from epsin (257 and 480) or β2 HA (Beta) (see section 8.1.4) and disassembled (see Figure 8.14). Previous work using the epsin 257 Cbox and β2 Cbox clathrin box peptides had shown that the affinities of the peptides from the TD was in

the region of 100 μM (Kd). In light of these values, incubation of clathrin box peptides was therefore conducted at 50 and 500 molar excess to clathrin (150 μM and 1500 μM) in order to ensure binding by these peptides.

Disassembly of epsin-clathrin cages in the presence of all 3 peptides shows a slight reduction in the inhibition of disassembly with a drop in final light scattering intensity. This effect is at least partially concentration dependent with increased concentrations of peptide (peptide 480 Cbox in particular) resulting in a greater extent of disassembly and the effect is greater when both epsin clathrin boxes are used in tandem (8.14 D). These observations would suggest that these peptides are able to disrupt epsin-clathrin interactions and therefore reduce the inhibitory effect of epsin.

However, there is an alternative explanation for these results that likely lies with an issue with the peptide buffer rather than as a consequence of peptide interactions. The peptides were reconstituted in 0.2 M ammonium sulphate and PBS at pH 7.9 (as recommended for optimal solubilisation). This buffer (peptide buffer) is significantly different to the polymerisation buffer in which the clathrin cages were incubated with a much higher pH and high salt content. Both the high salt and high pH are not conducive to clathrin polymerisation. With the 500 fold excess of peptide (1500 μM) a significant proportion of the total incubation volume containing clathrin, epsin and peptide consisted of this buffer and therefore conditions were not ideal for the retention of clathrin cages. Therefore, increased volumes of this buffer were likely leading to a reduction in stability of the clathrin cages in solution and hence the reduction in inhibition seen in the presence of peptides. Further evidence for this comes from disassembly with the combined 500-fold excess of 257 and 480 Cbox peptides and through the use of a peptide buffer control where the incubation volume contained 60% and 100% of the peptide buffer respectively (data not shown). When cages incubated under these conditions were placed into the fluorimeter the initial scattering signal was approximately 5-10 fold lower than would be expected of clathrin cages at 250 nM. This would suggest the loss of cages during the 1 hour incubation period. A similar slight drop was noticed for other cages incubated with peptide, although these differences were not initially noted as being significantly different due to inherent variability in scattering intensity within samples. This buffer contribution would also explain the observation that the presence of the clathrin box peptides increased the rate of disassembly compared to the clathrin only control (as

these cages are destabilised by the increase in pH and ionic strength of the buffer). In addition, given that β2 HA does not significantly compete with epsin for clathrin binding or reduce epsin inhibition (see section 8.5.3) one would not expect a peptide derived from this construct to have the same effect as the epsin clathrin box peptides (although peptides could compete at high concentrations (e.g. Smith *et al.* 2004). Note that all other adaptors were dialysed into polymerisation buffer prior to incubation with clathrin cages to avoid the buffer issues described here.

In conclusion, given that all 3 peptides show a similar trend in reducing epsin inhibition and due to the adverse effect on clathrin of high levels of the peptide buffer it is likely that the effects observed are as a consequence of the buffer (at high concentrations) rather than the peptides themselves, although the effects seen at low concentrations of peptide may be genuine. In future, variations of this experiment attempts should be made to reconstitute these peptides in polymerisation buffer to confirm whether these peptides are indeed able to counter epsin inhibition independent of the buffer conditions.

8.8.1 Discussion

In this chapter I have investigated the role of the clathrin boxes present in epsin on assembly and disassembly to test hypotheses about epsin interaction with clathrin. At the beginning of this chapter I proposed that the effects of epsin on assembly and disassembly may be linked to the same physical properties of epsin. If epsin molecules link together the TDs of triskelia and therefore mechanically induce smaller cages, this same mechanical effect would also make it more difficult for auxilin and Hsc70 to induce dissociation of these structures. The results obtained from this chapter confirm the importance of the clathrin box in the inhibitory activity of epsin in relation to disassembly. Preliminary results from the assembly assay indicate that the linking hypothesis may not be responsible for both assembly and disassembly effects of epsin. In this discussion I address the findings and propose alternative suggestions in light of the evidence obtained and in light of other work. Finally, I will discuss the implications for the role of epsin *in vivo* and the future work arising from the results obtained in this chapter.

8.8.2 Assembly with clathrin box mutant epsin suggests that binding efficiency of epsin is key to change in size distribution

The preliminary results from assembly assays using clathrin box mutant epsin raise some interesting points in relation to the epsin binding hypotheses introduced at the beginning of this chapter but also in relation to the sensitivity of clathrin assembly to changes in clathrin concentration.

Both measurements of cage sizes from DLS and EM suggest that mutating clathrin boxes in epsin alters the way that the protein promotes assembly with slight differences in the observations made from the different techniques. EM measurements indicate that all clathrin box mutants are able to assemble clathrin with a distribution similar to WT epsin. This would suggest that the linkage hypothesis for assembly is incorrect i.e. that epsin links together triskelia via the clathrin box motifs to promote assembly. However, as discussed in Chapter 4 (see section 4.6.2) measuring cage size by EM biases the measurements to 'cage like' particles due to it being unfeasible to measure the size of amorphous aggregates or cage intermediates. The data is therefore a reflection of the size distribution of the 'measurable' fraction of the sample and does not take into account the actual number of each particle size. Given the large size of an EM grid and random variations in concentration and protein staining/fixing across the grid it would not be feasible to get an accurate value for the true number of different cage/particle sizes. Despite this it was observed that (in the areas imaged) that the clathrin box epsin mutants produced fewer cages for the given concentration than compared to WT epsin. The Δ257 and Δ480 mutants were intermediate in cage number where as with the DKO mutant it was more difficult to find cages to measure. Despite the qualitative nature of these statements this observation ties in with the observed DLS results and allows us to draw some inferences as to epsin promotion of clathrin assembly.

With DLS the results suggest that neither the clathrin only control, nor the DKO epsin-clathrin sample, were able to fully polymerise into clathrin cages. In contrast both the single clathrin-box mutants and the WT were able to assemble with a distribution similar to that seen in Chapter 4 (see section 4.5.3) although both the single clathrin box mutants were not as effective as the WT. The results are likely to have been

affected by an apparently low concentration of clathrin (which reduces the conversion to cages and reduces cage size (Jones 2016)) either due to errors in calculation or poor sample stability. Despite this some interesting observations can be made. Although all of the mutants are still able to bind to clathrin the DKO mutant appears to show the least affinity for clathrin and concurrently had the weakest effect on assembly. The single clathrin box mutants appear to be intermediate in their binding and also intermediate in their effect on assembly compared to the WT and DKO mutants. It could be speculated therefore that the effect of epsin promotion on clathrin assembly is dependent on the amount of epsin bound to clathrin, rather than a physical interaction between triskelia. This would also tie in with the observations for the EM micrographs described in the previous paragraph that mutations to clathrin boxes results in fewer clathrin cages.

These results are at odds with previous hypotheses regarding the assembly mechanism of adaptors and a recent paper where mutations to epsin clathrin boxes motifs reduced the ability of epsin to recruit and assemble clathrin on lipids (Holkar *et al.* 2015). Holkar *et al*. (2015) demonstrated that mutations to either clathrin box motif in epsin (as used in this investigation) severely inhibits recruitment of clathrin and subsequent formation of a clathrin coat whereas the epsin Δ257 and Δ480 mutants used here are still able to assemble clathrin in a similar way to WT (although not as efficiently). The recruitment of clathrin to a membrane by epsin may be more severely affected by these mutations and may therefore explain the difference in severity of the effect seen. However, as highlighted both in Chapter 4 and in Jones (2016) thesis the assembly of clathrin is sensitive to a large number of factors. Therefore, a large number of replicates under various conditions such as varied clathrin and epsin concentration should be conducted to gain a complete picture of the interactions between these two proteins during assembly.

In conclusion the preliminary results described here suggest that epsin binding to clathrin is the primary driver of epsin-mediated clathrin assembly and subsequent smaller cage size distribution as opposed to the physical linkage of multiple domains via the clathrin box motifs. However, these results must be replicated before firm conclusions can be drawn.

8.8.3 Mutating clathrin box motifs in epsin indicates that they are key to the inhibitory effect of epsin

Clathrin box mutants of epsin show a clear effect on disassembly since the removal of either or both clathrin boxes prevents epsin inhibition of disassembly. Therefore, both clathrin boxes are required for the inhibitory effect. This observation does not discriminate clearly between either hypothesis; that epsin is either physically holding together cages or that epsin is occupying auxilin binding sites on clathrin and therefore inhibiting interactions with clathrin. In the linking hypothesis both clathrin boxes are required for interactions with neighbouring triskelia and in the occupying hypothesis the loss of a clathrin box interaction with the TD would leave auxilin free to bind to the TD. However, taking into account other factors of auxilin-clathrin and epsin-clathrin interactions a preferred model emerges.

The first point to make is that auxilin does not require interactions with the TD to disassemble clathrin as cages lacking the clathrin TD can be disassembled (Ungewickell *et al*. 1995) and auxilin residues 715-901, that primarily interact with the distal domain of the CHC rather than the TD, are sufficient to recruit Hsc70 and disassemble cages (Scheele *et al*. 2001). Epsin interacts primarily through the TD (Drake *et al*. 2000; Drake and Traub 2001) and so, although epsin and auxilin clearly compete for clathrin binding as shown (see section 7.2.1), it is unlikely that this competition is essential for the inhibition of disassembly. Finally, the fact that limiting Hsc70 concentrations also inhibits disassembly (see section 7.2.3) (and is known to bind to clathrin at a site far from any known epsin interaction) lends further support to the linking hypothesis. This is possibly at odds with the preliminary results from the assembly experiments as discussed in section 8.8.2. However, it is possible that further investigations into this aspect of epsin-clathrin interactions will lend support to the linking hypothesis.

8.8.4 Competition for epsin-clathrin binding

The fact that neither the α nor β2 HA domains significantly alter the disassembly of epsin-clathrin cages is not unexpected given the observations made previously by Dannhauser and Ungewickell (2012), where the addition of AP2 made no difference to the extent of disassembly of clathrin from coated vesicles (Dannhauser and Ungewickell 2012). In the case of the α HA interactions the absence of any effect is

significant for two reasons. The first is that the α HA has been suggested as an alternative recruitment site for auxilin to the clathrin coat (Dannhauser and Ungewickell 2012; Traub 2005) and given that the platform and side site might allow α HA to bind to both auxilin and epsin together (Brett *et al*. 2002; Praefcke *et al*. 2004; Schmidt *et al*. 2006) this would be a plausible suggestion. Secondly, interaction of α HA with the DPW region of epsin could potentially reduce the interaction of this region with clathrin. Given that neither of these situations appears to have arisen suggests that, if auxilin is able to bind to the α HA in this complex this recruitment does not result in any change in epsin inhibition of disassembly (see Figure 8.15 A). And with regards to the second point this observation is consistent with epsin inhibition of disassembly being dependent on the clathrin box motifs. If α HA interaction with DPW motifs breaks the interaction of this region with clathrin it does not significantly alter the disassembly of epsin-clathrin cages and neither does it seem to significantly alter binding. The increased interaction of GST α HA with clathrin in the presence of epsin does support the role of epsin in CME as an important recruiter of AP2 and clathrin during the maturation of the CCP. Again with the β2 HA the observation that β2 HA and epsin can bind to clathrin in concert and that the presence of β2 HA does not alter epsin inhibition of cage disassembly fits with previously published data. The binding region on β2 HA for epsin (the platform site) is also implicated in the interaction of this region with the ankle domain of clathrin and hence in the presence of clathrin it is possible that these two proteins do not interact (Owen *et al*. 2000; Edeling *et al*. 2006a). This observation would therefore fit with the suggestion that epsin interacts with both HA domains of AP2 in the absence of clathrin but is dissociated from the β2 HA in the presence of clathrin. This would act to facilitate early stronger AP2-epsin interactions that might facilitate recruitment to the plasma membrane (Saffarian *et al*. 2009).

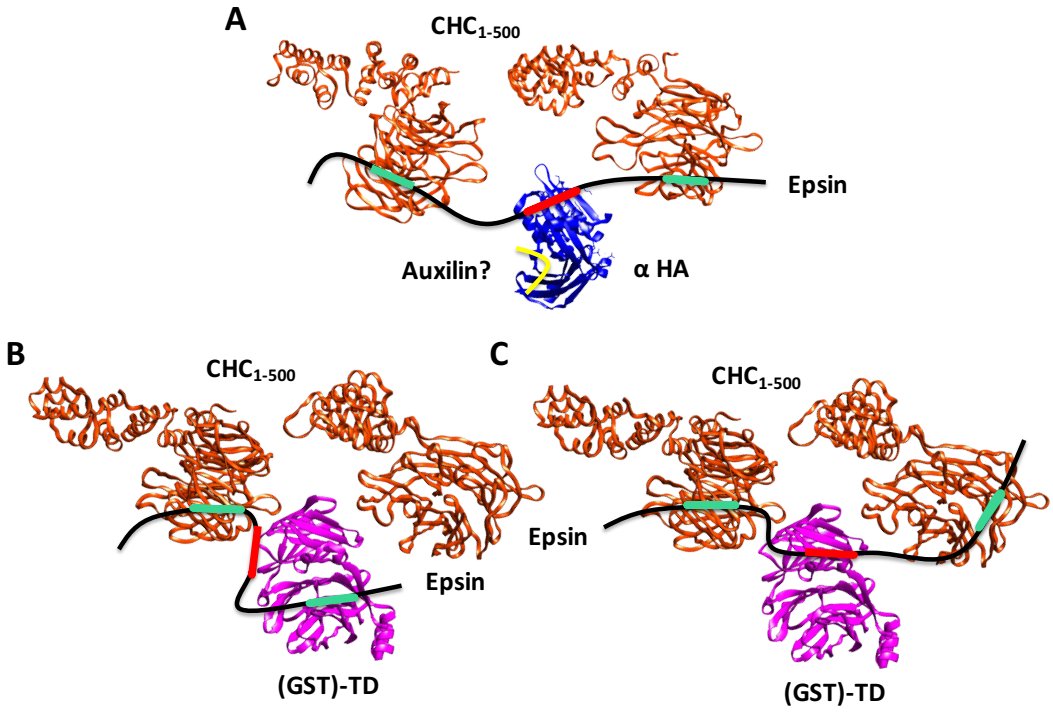

Figure 8.15 Proposed binding modes for (A) clathrin:epsin,GST α HA and (B and C) clathrin:epsin:GST-TD in a clathrin cage. *A) The α-HA (blue) appears to bind to epsin (black) when epsin is bound to clathrin (orange). Epsin interactions motifs are shown as cyan (clathrin box) and red (DPW) motifs. Given that this interaction does not alter the ability of epsin to interact with clathrin (see Figure 8.08) or the ability of epsin to inhibit disassembly (see Figure 8.09) this supports an interaction as shown. This could potentially retain interaction sites for auxilin but this does not appear to alter disassembly, at least in the presence of epsin (see Figure 8.09). GST-TD interaction with epsin appears to interact with epsin in complex with clathrin (see Figure 8.12) but appears to exacerbate the inhibition of disassembly by epsin (see Figure 8.13). This interaction does not seem to disrupt the epsin clathrin box interaction with clathrin (B) as this would break the inhibitory effect of epsin. Potentially an interaction is occurring between GST-TD and the epsin DPW region (C) although how this interaction exacerbates the inhibitory effect is unclear. Terminal domain structures highlighted from structure 3IVY and α HA from IKY6 and manipulated in UCSF Chimera.*

Both pull-downs and disassembly with GST-TD produced some interesting results. GST-TD, instead of disrupting epsin-clathrin binding as hypothesised, appears to interact with clathrin-epsin cages and subsequently enhance the inhibitory effect of epsin. The interaction appears to occur without disrupting epsin-clathrin binding and appears not to occur through interactions with the clathrin box motifs as this would potentially disrupt the epsin inhibitory effect on disassembly. It is possible that the interaction occurs with the DPW region of epsin although why this would occur in preference to interactions with the clathrin box motifs is not clear. As for the mechanism of inhibition this is again unclear, but could potentially be as a consequence of auxilin interacting with the GST-TD rather than the CHC. However,

the presence of free GST-TD in the absence of epsin has no negative effect on disassembly and so why auxilin would interact with GST-TD only when it is interacting with the epsin-clathrin complex is not clear (see Figure 8.15 B and C).

Finally, clathrin box peptides may be able to counter the inhibition of epsin but given the issues highlighted with the contribution of buffer components to the stability of clathrin cages this result requires additional work to be confirmed.

8.8.5 Biological relevance: possible roles of epsin in CME

Epsin has been shown to have an effect both on the size distribution of clathrin cages and also on the ability of Hsc70 and auxilin to disassemble clathrin cages. The question remains however, as to whether these phenomena are related to functions of epsin *in vivo* or whether these are simply artefacts of the *in vitro* systems used. The role of epsin in controlling the size of vesicles *in vitro* and *in vivo* has been well studied (Kalthoff *et al*. 2002; Jakobsson *et al*. 2008) but the inhibitory effect of epsin on cage disassembly has not been specifically noted with the possible exception of work by Dannhauser and Ungewickell (2012). The fact that a similar effect was noted by these authors using clathrin coat structures on lipids would suggest that the lack of lipids in our assay does not contribute to the inhibitory effect seen.

The role of epsin *in vivo,* in addition to interacting with ubiquitinated proteins, appears to be related both to the regulation of vesicle size and the regulation of actin polymerisation (Jakbsson *et al*. 2008; Messa *et al*. 2014) (see section 1.8.1). As part of that role it is likely present at the neck of the CCP to interact with the Hip1R and other components of the actin machinery (Saffarian *et al*. 2009). As discussed in Chapter 6 (see section 6.5.5) the question as to whether epsin locates entirely to the neck (and is therefore not present in the CCV) or is retained throughout the CCP is not clear. However, it seems that on balance that the evidence points towards at least some epsin being present in the CCV at the point of disassembly (Rappoport *et al*. 2006; Hawryluk *et al*. 2006; Edeling *et al*. 2006a). Epsin may therefore play a role in making cages more resistant to disassembly *in vivo*. This may be to stabilise vesicles containing certain cargo, such as ubiquitinated proteins that interact with epsin. Although epsin is present at around 90% of endocytic events in various cell lines (Taylor *et al*. 2011) it is possible that epsin is present in greater amounts within

specific endocytic sites containing these cargos and could therefore stabilise these structures. Alternatively, the amount of epsin present in the mature CCV may be too low to significantly effect disassembly in the manner seen in our assays and would therefore be an artefact of the high concentrations of epsin used in this assay.

The inhibitory properties of epsin in disassembly may also play a role in the proposed actions of auxilin and Hsc70 during the maturation of the CCP. Hsc70 and auxilin/GAK have been implicated in the remodelling of the CCP by facilitating the exchange of clathrin and adaptors as the pit matures (Jiang *et al*. 2000; Wu *et al*. 2001; Yim *et al*. 2005; Holakr 2015). It is possible that the interaction of epsin with clathrin may potentially stabilise the clathrin structure on the plasma membrane to reduce the rate of exchange of clathrin and therefore regulate the maturation of the structure.

In conclusion the *in vivo* significance of the inhibitory effect of epsin on clathrin cage disassembly remains to be established. Given the uncertainty about the fate of epsin during CCP maturation concrete assertions cannot yet be made. Despite this the effect gives us further information about how epsin interacts with clathrin cages and how auxilin and Hsc70 drive the disassembly of clathrin structures.

8.8.6 Future work

In this chapter a large number of questions in relation to epsin-clathrin interactions have been addressed but much of the work is preliminary and must be expanded upon in order to draw firm conclusions.

The use of DLS to study epsin clathrin box mutants during polymerisation has yielded interesting preliminary observations but requires a number of replications with varied conditions including; epsin concentration, clathrin concentration and buffer conditions. This work would be a priority to confirm or deny the epsin linkage hypothesis.

Although a number of interactions between clathrin, epsin and adaptors or peptides have been conducted a number of experiments should be completed to lend further support to these effects. Although the inhibition of disassembly by epsin was initially confirmed by negative stain EM (see section 6.4.2) this was not subsequently repeated for other assays. The use of EM would confirm inhibition in these experiments and could also provide conformation as to the stability of clathrin cages which was suspect in the presence of the peptide buffer. Equally the use of centrifugation-gel based assays

would also provide important information as to the extent of cage disassembly and to potential interactions between the proteins present in the disassembly assay. For example the use of pull down experiments with clathrin-epsin-GST α HA and auxilin could be used to confirm if α HA is interacting with epsin as increased concentrations of auxilin would prevent epsin binding (see section 7.2.1) and therefore GST α HA binding would also be reduced.

The change in binding interactions of the epsin clathrin box mutants with clathrin was only measured qualitatively using gel-based pelleting assays. It would therefore be of interest to see if specific binding constants could be attributed to each of the clathrin boxes in epsin and to the DKO mutant which is still able to interact with clathrin but with reduced affinity. The use of techniques such as ITC or SPR would help provide quantitative measures for the binding of each of these components of epsin. These techniques could be used in conjunction with mutations to the clathrin TD to determine if the epsin clathrin box motifs preferentially target one of the 4 binding sites on the TD or if they are able to bind multiple different sites.

The sourcing of clathrin from porcine brain, whilst providing large yields, does not allow for control over the precise nature of the protein material and of course does not allow for manipulation of the clathrin structure through mutation. Expressing recombinant CHC is possible in insect cells and has recently been successfully implemented in *E.coli* allowing for rapid and easy genetic manipulation (Sousa *et al*. 2016). Mutations in the CHC would provide complimentary work to the epsin clathrin box mutants with mutations to the TD (as described in the previous paragraph) providing important information on how epsin interacts with the clathrin cage structure.

In addition to mutations to clathrin a further set of mutants in epsin would also help to probe its interactions with clathrin during assembly and disassembly. If the distances between the clathrin boxes is indeed important for epsin to link multiple clathrin TDs then shortening of the region between the two clathrin boxes would result in abrogation of both assembly and disassembly effects. Additional support for the importance of the separation of the clathrin boxes comes from communications with Stephen Royle at the CMCB, University of Warwick. His group have used artificial constructs to initiate endocytosis in cells. The separation of clathrin boxes in these

constructs appears to be important as a construct containing 3 epsin clathrin boxes close together in sequence did not efficiently promote endocytosis (private communications). This lends further support to the hypothesis that the distance between these motifs is crucial and the use of shortened epsin constructs in both the assembly and disassembly assays would be crucial to confirming these observations.

As discussed in Chapters 6 and 7, AP180 has a natively unfolded clathrin binding region similar to epsin and has been proposed to interact with clathrin in a similar fashion. Preliminary work conducted during the writing of this thesis suggests that AP180 may have an inhibitory effect similar to that of epsin and AP180 has also been shown to be displaced from clathrin by auxilin (Scheele *et al*. 2001). It would be interesting to confirm these initial observations as this would suggest that the effects seen in epsin are a shared across other adaptors and may therefore point to a common *in vivo* function.

8.8.7 Conclusion

In conclusion the clathrin box motifs in epsin have been shown to be important in altering the effect of the protein on clathrin cage polymerisation and in clathrin cage disassembly. The data points towards epsin facilitating interactions between clathrin triskelia as hypothesised in previous publications, resulting in the inhibition of disassembly and in the possibly the promotion of small clathrin cages. Repeating assays and extending the range of experiments through an increased range of conditions and mutations would allow firm conclusions to be drawn as to the mechanism of epsin-clathrin binding and its relevance to the role of epsin in CME.

Chapter 9: Discussion

'Open your mind, we can understand it' Suzuka Nakamoto, The One, Babymetal

9.1.1 Overview

Since the discovery of clathrin and coated vesicles great strides have been made in understanding the role and mechanism of CME. From the initial description of clathrin by Barbra Pearse (Pearse 1975) to Browne and Goldstein that discovered the link between receptor internalisation and endocytosis (Goldstein *et al.* 1985). And from the understanding of the clathrin coat structure (Fotin *et al.* 2004 a and b) to the *in vivo* and *in vitro* assays that have revealed the temporal regulation and mechanisms behind this process (Taylor *et al.* 2011) we have gained great insights into this biological system. However, as our understanding has increased so too has the realisation of the incredible complexity of this system that is dependent on cargo, cell, tissue, organism, disease and developmental state. A great deal therefore remains to understand about this process and its role in cell biology.

In this thesis I have attempted to gain a greater understanding behind the mechanisms involved in the interactions of some of the many proteins involved in the assembly and disassembly of clathrin cages. In the introduction (see section 1.11.1) I set out my aims to understand how adaptor proteins interact with clathrin in assembly and how these proteins interact with the disassembly machinery. This work has expanded upon previous work and has yielded unexpected results that are none the less consistent with theories regarding the role of these proteins *in vivo*. There are however a large number of outstanding questions arising from this work. In this final chapter I summarise the work presented in this thesis, the implications of the results and the future avenues of enquiry that have arisen from this work.

9.2.1 Dynamic light scattering as a tool for assaying the assembly of clathrin-adaptor cage complexes

The first aim or question that was set out in the introduction to this thesis was whether an alternative or complimentary technique could be employed to provide a quantitative measure of clathrin cage sizes which could then be employed to answer how adaptors promote the formation of clathrin cages. Previous approaches using gel densitometry and EM have been used but have several drawbacks (see section 4.1.1). Dynamic Light Scattering (DLS) was employed in collaboration with Joseph Jones to provide preliminary data on the effect of adaptors on clathrin cage polymerisation as detailed in Chapter 4 and Chapter 8. This technique provided some interesting initial observations on the effect of adaptors to assemble clathrin cages and has proved the proof of concept for the use of this technique in analysing this system.

DLS determines the size of particles in solution through the motion of these particles and their subsequent interaction with light passed through the solution. It is therefore able to determine the size distribution of a significant proportion of the population in solution (as opposed to measuring from negative stain EM micrographs). A modified fitting algorithm developed by Joseph Jones was shown to more accurately represent the polymerised and depolymerised clathrin samples (Jones 2016). In the case of adaptor-clathrin polymerisation it was noted that samples measured in DLS largely matched the distributions imaged with EM, with the exception of samples where aggregation had occurred. In these cases, such as with Hip1 (see Figure 4.03), cage measurements were still possible using EM but the cages found represented a small proportion of the clathrin present i.e. a significant proportion was found in non 'cage-like' particles or aggregates which cannot be accurately measured in EM micrographs. This observation highlights both a strength and weakness of DLS: that it can determine that a sample has aggregated but that the technique is an average of the distribution of particles in the cuvette. The technique is biased towards large particles as these scatter a greater proportion of light than smaller particles, therefore in a sample with a few aggregates the presence of smaller cages may be dominated by the presence of larger particles. The effect of concentration is also important both for the assembly of clathrin cages, as clathrin concentration alters size distribution (Jones 2016). Therefore,

changes in the concentration of clathrin that is able to polymerise (which may be altered by aggregation or the presence of adaptors themselves) plays an important role in the final distribution seen. Taken together these observations suggest that DLS is an appropriate tool for studying clathrin assembly but highlight the importance of collecting a large amount of data from DLS polymerisation given the sensitivity and inherent variability of the sample used.

9.2.2 Adaptor-clathrin assembly

DLS was employed in this thesis to see if it was possible to gain a greater understanding of the mechanisms behind adaptor promotion of clathrin cage assembly. The adaptors and adaptor domains used in this investigation (β2 HA, Hip1 CC, Hip1R CC and epsin) had all previously been shown to promote cage assembly either through EM or gel based assays but this effect had not necessarily been measured quantitatively (Greene *et al.* 2000; Engqvist-Goldstein *et al.* 2001; Kalthoff *et al.* 2002; Legendre-Guillemin *et al.* 2002; Chen *et al.* 2005). Each of these adaptors has different structures and varied interaction sites on clathrin and subsequently different proposed mechanisms of the promotion of clathrin cages.

Although the results on this topic of this investigation are preliminary they do suggest that the different binding mechanisms of adaptors, and therefore their promotion of assembly, have differential effects on assembly. In the case of Hip1 CC and Hip1R CC the proposed mechanism of assembly is markedly different to other adaptors: that of binding to the CLC and neutralising a patch of acidic residues that negatively regulate assembly (Legendre-Guillemin *et al.* 2005; Ybe *et al.* 2007; Ybe *et al.* 2009). It is interesting to note that the general effect seen by polymerisation with Hip1/1R CC was the formation of a population of larger cages (although this appears to be in contrast to the small cages reported previously for Hip1R (Engqvist-Goldstein *et al.* 2001). Given that many other adaptors appear to promote small cages (such as shown with epsin) it is possible to speculate that the effect is due the unique assembly mechanism of these proteins. The interaction with the CLC is likely to cause the same change in CHC conformation that was noted by Wilbur *et al*. (2010). It is possible that this bent knee conformation (See section 1.4.4) is more conducive to the formation of larger cage structures, where as the interactions with the terminal domain as seen with other adaptors is more conducive to smaller cages. Crystal structures of the hub

domain as per Wilbur *et al.* (2010) with the Hip1/1R CC domains would provide conformation of this effect and may further explain the ability of these proteins to promote clathrin cage assembly.

β2 HA and epsin in contrast are both proposed to link triskelia through interactions with the terminal domain although through different mechanisms that potentially explain the differences in size distribution seen in these experiments. β2 HA, both in isolation and as part of the AP2 complex, has been implicated in assembling clathrin cages by forming interactions between the clathrin terminal domain and the ankle domain of a neighbouring triskelion (Greene *et al.* 2000; Knuehl *et al.* 2006). Epsin's interaction with clathrin is proposed to involve multiple contacts with neighbouring triskelia and has been previously shown to promote the formation of smaller clathrin cages as seen with other adaptors (Drake *et al.* 2000; Drake and Traub 2001; Kalthoff *et al.* 2002). In the case of epsin the preliminary use of the clathrin box motif mutants in assembly would suggest that the epsin effect on polymerisation is driven by the amount of epsin that is able to bind rather than as a consequence of multiple interactions. With β2 HA similar mutants to the platform site and clathrin box motifs that are proposed to interact with clathrin could be used to interrogate the interaction mechanism in assembly (Shih *et al.* 1995; Clairmont *et al.* 1997; Edeling *et al.* 2006a; Kneuhl *et al.* 2006).

The observation that adaptors bind to clathrin through different mechanisms and potentially promote similar or different cage structures is of note. It may be that the differences in the polymerisation seen here are due to these different modes of interaction and relate to their different functions *in vivo*. With Hip1/1R CC it could be that the preferential promotion of large cage structures is related to their role in initial recruitment with clathrin before dissociation from clathrin during interactions with the cytoskeleton (Wilbur *et al.* 2008). Where as the interactions of AP2 and epsin in the interior of the clathrin coat to promote smaller structures may be required to regulate the amount of cargo present in the new vesicle. The use of functional mutants as used here with epsin could be extended to other adaptors and applied to *in vivo* systems to gain further insights into the role of these interactions.

9.3.1 Adaptors, auxilin and Hsc70 in disassembly

The role of Hsc70 and auxilin in disassembly has been recognised for over a decade but the precise mechanism, particularly in relation to how Hsc70 drives disassembly, has not been understood until recently. And many questions still remain to be answered in relation to the role of Hsc70 and auxilin in the interaction with adaptors during disassembly. The second two aims of this thesis were; to gain a further understanding of how Hsc70 and auxilin function in clathrin cage disassembly using functional mutants of these proteins and to understand if and how adaptors interact with the disassembly machinery.

9.3.2 Auxilin mutants in disassembly

The use of auxilin clathrin-adaptor binding motifs mutants proved an interesting tool for understanding auxilin disassembly and for understanding the inhibitory effect of epsin on disassembly. Mutations to the DLL and DPF motifs were investigated by Scheele *et al.* (2003) in assembly and disassembly and similar constructs were again investigated using disassembly in real time with light scattering as opposed to centrifugation and gel densitometry as used in the previous study (Scheele *et al.* 2003). The results presented in Chapter 5 indicate that all of these mutants are able to interact with clathrin and do not show an obvious loss of binding (although binding was only determined qualitatively) but do result in slower rates of disassembly. This would indicate that these residues are key to the catalytic action of auxilin i.e. its ability to re-bind to cages after the dissociation of the triskelion to facilitate further release of triskelia from the cage structure. This is slightly at odds with the original results from Scheele *et al.* (2003) where the mutations reduced binding and disassembly at low concentrations. The likely reason for this difference is the presence of the additional clathrin binding motif used in this study that was not present in the Scheele study: the clathrin box. This allows the protein used in this study to retain interactions with clathrin despite the loss of the other motifs. These observations do however match well with those of Ma *et al.* (2002) that support the assertion that the clathrin binding motifs of auxilin near to the J-domain are key to recruitment of Hsc70 but motifs nearer the N-terminus are more essential for the catalytic action of auxilin (Ma *et al.* 2002). In this investigation mutations to the DPF and DLL motifs were grouped as per motif type as opposed to spatial grouping. It would therefore be interesting to create

auxilin mutants based on these groupings to investigate their roles in disassembly in real time.

9.3.4 Adaptor interactions and disassembly

Two questions still remain from these observations. Why does auxilin contain multiple motifs for clathrin and adaptor binding and how and why do the same motifs apparently target different regions of the CHC? The first of these questions was partially investigated through the use of adaptors in disassembly and also ties in with the discussion as to if and how auxilin is required to disrupt adaptor binding and potential stabilisation of the clathrin cage structure. It would appear that, in the case of Hip1/1R CC and β2 HA that the effect of these proteins on the promotion of clathrin cages does not extend to significantly altering disassembly kinetics, although more subtle effects might be observed through the use of a stopped-flow apparatus (see section 9.5.1). It is likely that this is desirable *in vivo* as the cell might not necessarily desire cages structures that are too stable and therefore difficult to disassemble. In the case of Hip1/1R CC given their unique binding interaction with the CLC it is unlikely that either protein interacts with auxilin although Hsc70 could still potentially interact with the CLC (DeLuca-Flaherty *et al.* 1990). And as discussed in detail in section 6.5.5 it is debatable as to whether these proteins are present in the CCV at the point of disassembly. In terms of the promotion effect of β2 HA it is possible that the effect is too weak to make a significant difference to disassembly observable in the fluorimeter based assay. Again the observation that this effect is likely weak is also significant *in vivo* as AP2 is very likely present in CCVs at the time of uncoating and so a negative effect on disassembly may not be desirable (Saffarian *et al.* 2009; Taylor *et al.* 2011).

9.4.1 Epsin-clathrin interactions and disassembly

The inhibitory effect of epsin on disassembly was unexpected but in general appears to fit with previous data and hypotheses of how epsin interacts with clathrin (Drake *et al.* 2000; Drake and Traub 2001; Kalthoff *et al.* 2002; Holkar *et al.* 2015) and previous data on disassembly (Dannhauser and Ungewickell 2012). The effect serves as an interesting discussion point as to how adaptor proteins interact with clathrin and how auxilin and Hsc70 disassemble the clathrin cage.

Epsin inhibition of disassembly was shown in Chapters 6 and 7 to be dependent on both auxilin and Hsc70 concentrations and that auxilin could compete for clathrin binding with epsin. It was noted that auxilin can compete for epsin binding for clathrin, an effect that has been seen with the adaptor AP180 (Scheele *et al.* 2001), which would potentially explain the need for higher concentrations of auxilin in disassembly. However, the fact that disassembly is dependent on the concentration of both auxilin and Hsc70 would suggest a mechanical explanation as Hsc70 is not known to interact with any known site on clathrin shared by epsin or other adaptors (Rapoport *et al.* 2008). Epsin has been suggested to form multiple interactions with clathrin to induce assembly (as seen in Chapter 4) (Drake *et al.* 2000; Drake and Traub 2001; Kalthoff *et al.* 2002) and so this hypothesis was used to explain the observed effects of epsin both in relation to assembly and disassembly. Using clathrin box motifs mutants supported this hypothesis in relation to disassembly; that removal of either or both clathrin boxes results in the loss of inhibition. However, as discussed in section 9.2.2 the preliminary results of the assembly experiments appear to suggest that the amount of epsin that is able to bind clathrin cages is what drives assembly of cages in the presence of epsin. This would tend to contradict the epsin linkage hypothesis as loss of a single clathrin box motif would result in epsin being unable to assemble cages through linking clathrin terminal domains. Despite the preliminary nature of the experiments the implications of the results should not be dismissed until further data is obtained.

It is of course possible that a large number of factors contribute to epsin's effect on clathrin assembly and disassembly. It is certainly possible that epsin is able to assemble clathrin cages through linking clathrin triskelia but it could also be the case that epsin's interaction with the terminal domain is sufficient to drive the assembly of cages through an alternative mechanism, such as by reducing the mobility of the terminal domain and subsequently causing the triskelion to adopt a conformation more conducive to assembly. Alternatively, the assembly effect could also be driven in part by the affinity of the DPW region between the clathrin boxes. Mutation of the clathrin boxes does not completely remove binding of epsin with clathrin (see section 8.2.2) and so this interaction could contribute to promotion of assembly by epsin (although the DKO clathrin box mutant of epsin showed almost no assembly in DLS (see section 8.2.3). The importance of the clathrin boxes in epsin in relation to assembly was also

by Holkar *et al.* (2015) whose work strongly suggests that both clathrin boxes in epsin are required to assemble clathrin on liposomes as mutation of either of these motifs (as used in this investigation) inhibits assembly (Holkar *et al.* 2015). In the case of disassembly, it could be that both competition of epsin and auxilin for clathrin binding and stabilisation of triskelia interactions through epsin binding could lead to the observed inhibition of disassembly. In both cases the use of an epsin truncation mutant, that contains a shortened DPW region between the clathrin box motifs, would prove conclusively whether epsin binding to multiple triskelia is the driving force behind both observed effects.

Epsin competition for clathrin binding with auxilin, the effects of adaptor competition and previous peptide competition experiments combined with *in vivo* experiments, all depend on the way in which adaptors interact with the terminal domain of clathrin. The observation that epsin and auxilin compete for clathrin binding but β2 HA does not compete with auxilin or epsin for clathrin binding (despite containing clathrin box motifs) suggests that these proteins bind to specific sites on the terminal domain. The fact that a single clathrin terminal domain binding site is sufficient for the normal functioning of endocytosis *in vivo* (Willox *et al.* 2012) would suggest that specific interactions at the terminal domain are not necessarily crucial. This is supported by observations that peptides containing clathrin interaction motifs are able to interact with multiple sites on the terminals domain. Whether competition or specific binding of adaptors is a part of CME will be further understood through further use of mutations and chemical inhibitors (PITSTOP) *in vivo* and *in vitro* (von Kleist *et al.* 2011).

The final point to raise in conclusion to this discussion relates to whether the inhibitory effect is relevant to the process *in vivo.* As discussed in section 6.5.5 whether adaptors are retained in the budding CCV or are left behind at the plasma membrane is still unclear. In the case of epsin, EM images show that at least some epsin is retained in the region of the CCP that will form the new vesicle and hence will be present at disassembly (Edeling *et al.* 2006; Hawryluk *et al.* 2006). Whether concentrations of epsin are high enough to inhibit disassembly in CCVs is not known, nor is it known whether other factors are required to help disrupt epsin inhibition of disassembly. If epsin is present, then it suggests that epsin plays a regulatory role in stabilising cage

structures. If this effect is entirely an *in vitro* phenomenon, then it still tells us something about the mechanism by which epsin interacts with clathrin cages.

9.5.1 Future work: using DLS to further understand clathrin-adaptor interactions in cage assembly

In relation to the assembly work using DLS the original experiments with adaptors must be replicated and expanded from the original conditions. Given the large number of potential variables that can alter the way in which clathrin can assemble, conditions such as clathrin and adaptor concentration and pH should be explored. The assay as used in this investigation was optimised for pH 6.2-6.5 where clathrin is able to assemble. Extending the pH range to 6.6-7.0 would be crucial for monitoring adaptor assembly. This would allow study of the adaptor promotion effect on assembly in a pH not conducive to assembly in the absence of adaptors as used in previous studies on adaptor-clathrin assembly experiments (Goodman *et al.* 1997; Greene *et al.* 2000; Morgan *et al.* 2000; Engvist-Goldstein *et al.* 2001; Scheele *et al.* 2001; Kalthoff *et al.* 2002; Legendre-Guillemin *et al.* 2002; Chen *et al.* 2005). This would be particularly relevant in the case of the epsin clathrin box mutants where the fact that clathrin cages can assemble under these conditions in the absence of an adaptor may be in part obscuring the loss of the epsin's ability to promote assembly.

The assay should also be extended to other adaptor proteins both individually and in combination. Of particular interest are the adaptors AP180 and β arrestin. As discussed at during this thesis, AP180 shows both a very similar structure and function in assembling clathrin cages *in vivo* and *in vitro* (Morgan *et al.* 2000; Kalthoff *et al.* 2002). It would therefore be ideal to compare the effects of this protein to epsin and possibly use a similar suite of mutants to that used for epsin to interrogate its ability to assemble clathrin cages. In the case of β arrestin the interest stems from the observation from gel densitometry that this adaptor is able to bind to but not promote clathrin assembly (Goodman *et al.* 1997). It would be interesting to test this observation using the DLS assay. Auxilin is also able to promote assembly of clathrin and mutations to the clathrin-adaptor binding motifs investigated in the context of disassembly have also been shown to reduce the ability of auxilin to assemble clathrin (Scheele *et al.* 2003). It would also be interesting to extend these observations with the use of DLS.

The investigation of clathrin assembly could be further investigated through the use of recombinant clathrin, both to produce clathrin containing solely CLCa or CLCb but also to produce mutant variants of the full length CHC. The difficulties in expression of recombinant clathrin, in particular the full length CHC, have made the study of clathrin through the use of mutation difficult. In the case of CLC this protein can be expressed in bacterial systems and then recombined with CHC that has been stripped of its native CLCs, therefore allowing the addition of CLC of choice. A more elegant system of expressing both CLC and CHC in insect cells has yielded expression of clathrin containing specific isoforms of the CLC as well as allowing mutations to the CHC that have been used to investigate the role of specific residues implicated in CHC leg interactions (Bocking *et al.* 2014). The recent publication made by Sousa *et al.* used both CHC and CLC expressed in *E.coli* for the first time (Sousa *et al.* 2016). This advance will likely make it considerably easier, quicker and cheaper to express CHC and to conduct site-directed mutagenesis. It would be interesting to investigate both the effects CLCa and CLCb in the assembly of clathrin cages as well as the effects of mutations to the CHC (as per Bocking *et al.* 2014) using DLS.

9.5.2 Future work: further exploring the disassembly of clathrin-adaptor cage complexes

The use of a fluorimeter based light scattering assay has provided a large amount of data presented in this thesis. However, it has a number of disadvantages (see section 5.5.5) and due to these limitations no attempts were made to fit models to the disassembly data. The use of stopped-flow as previously used in previous work in the Smith group at Warwick (Rothnie *et al.* 2011) and more recently by Sousa *et. al.* avoids the limitations as described for the fluorimeter based assay. In particular use of this set-up might reveal subtle effects of adaptors such as Hip1/1R CC and β2 HA that are not observable though the standard assay and would allow the derivation of parameters related to auxilin recruitment that would be important to quantify with the auxilin DPF/DLL mutants. Although a stopped-flow set up was available and was used on a couple of occasions during the course of these investigations the set-up has a large draw back in that it uses a relatively large amount of material compared to the fluorimeter based assay. This was a particular issue in relation to Hsc70 that was difficult to obtain in high yields due to the need for expression using the insect cell-

baculovirus system. Switching to the use of the full length bovine Hsc70 construct, which can be expressed in *E.coli* and has little effect on the ability of Hsc70 to disassemble clathrin cages (Boecking *et al.* 2011; Sousa *et al.* 2016), would make the use of this set up much more viable for regular use in disassembly assays.

The disassembly assay could also be extended to the use of other adaptors such as AP180 and through the use of multiple adaptors as was briefly explored in Chapter 8. Given the similar structure and apparent role of AP180 in comparison to epsin it would be crucial to determine if this adaptor has a similar effect on disassembly. As stated in section 8.8.6 preliminary work subsequent to the completion of lab work for this thesis suggests that AP180 has a similar effect on disassembly to epsin. Conformation of this result would lend support to the observations with epsin and suggest that this effect is common to adaptors that interact with clathrin through the same mechanism. The use of recombinant clathrin cages containing single isoforms of the CLC as well as mutations to interaction sites on the leg and terminal domain of the CHC would also prove invaluable to determining the role of these regions in disassembly. In particular, mutations to the 4 adaptor binding sites on the terminal domain would prove crucial to determining how adaptors such as auxilin and epsin interact with each other and clathrin during disassembly.

Finally, the major test of the observations made during the *in vitro* assays used in this project would be to transfer these mutants to an *in vivo* system. The use of the DPF/DLL motif mutants of auxilin in cells could help to corroborate our results by determining if these mutants reduce the rate of disassembly and clathrin recycling *in vivo* or play other roles in interacting with adaptors. Again the use of epsin clathrin box mutants in cells would help support or disprove the observations of epsin assembly and disassembly effect. Given that epsin appears to be recruited early in the maturation of the CCP (Taylor *et al.* 2011) it is likely that any effects of these mutants would relate to the maturation of the CCP rather than to later effects on disassembly. This would still be of interest, particularly in relation to the observed effects of epsin clathrin box mutants made by Holkar *et al.* (2015) in their *in vitro* assay.

Appendix: GST interactions with clathrin

GST interactions with clathrin cages

The GST tag is used in a number of protein constructs and was retained in a large number of proteins used in disassembly assays. To confirm that GST itself does not affect disassembly GST was incubated with clathrin cages and pelleted by centrifugation at 140,000 x g to determine if non-specific binding occurs (see Figure A1.01) and was incubated with clathrin cage prior to disassembly (see Figure A1.02).

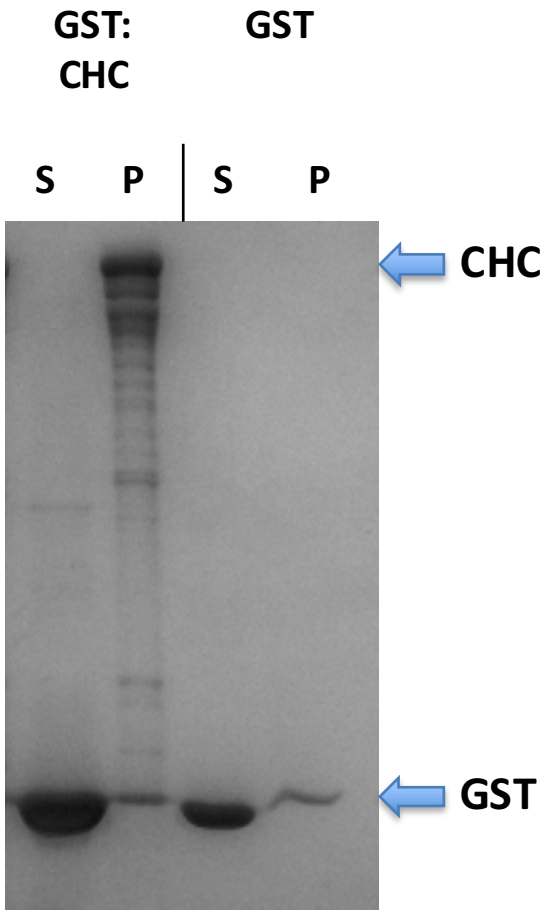

Figure A1.01 Pelleting of GST in the presence of clathrin cages. *Clathrin at 3 μM was incubated with 30 μM of GST for 1 hour before centrifugation at 140,000 x g. GST can be seen in both the pellet (P) and supernatant (S). GST in the absence of clathrin (at 15 μM) was also centrifuged at 140,000 x g. In both cases GST is visible in the pellet suggesting that GST does not interact directly with clathrin but is instead aggregating and hence pelleting from solution.*

Centrifugation of GST both with clathrin and on its own indicates that GST does pellet slightly in polymerisation buffer, suggesting that aggregates have formed (see Figure A1.01). Although a lager amount of protein was incubated with clathrin than in the GST only control the apparent intensity of GST present in the pellet fractions is the same, suggesting that GST does not non-specifically interact with clathrin. This supports the observations that GST-fusion proteins used in these study that interact with clathrin are interacting through direct interaction with clathrin or other adaptors as opposed to non-specific interactions via the GST-tag.

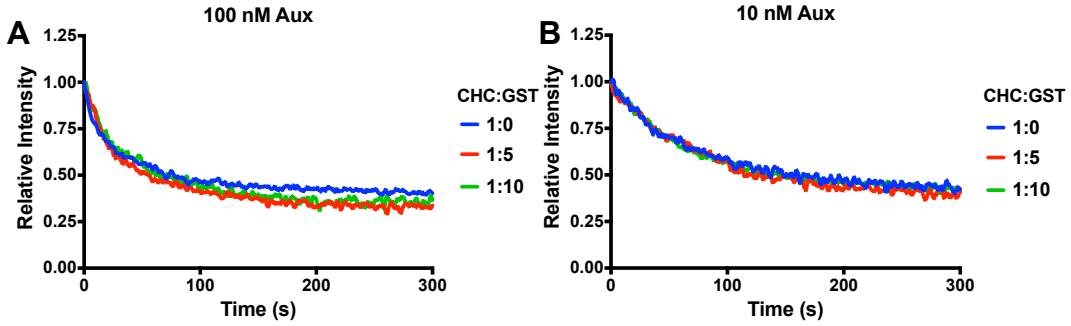

Figure A1.02 Disassembly of clathrin cages in the presence of GST. Clathrin *cages (3 μM) were incubated with a 5 fold or 10 fold excess of GST for 1 hour before disassembly with 1 μM Hsc70, 500 μM ATP and either 100 nM auxilin (A) or 10 nM auxilin (B). Disassembly in the presence of GST with either 10 nM or 100 nM auxilin showed no significant difference in disassembly profiles.*

Disassembly of clathrin cages incubated with 5 or 10-fold excess of GST shows no difference in disassembly profile to the clathrin only control (see Figure A1.02). The fact that GST itself does not appear to interact with clathrin cages and does not alter disassembly suggests that observations made with GST-adaptor fusion proteins are attributable to the adaptor itself and not to the GST affinity tag.

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