Role of clathrin-associated sorting proteins (CLASP) in clathrin assembly on membrane

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Of the degree of

Doctor of Philosophy

By

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CERTIFICATE

Certified that the work incorporated in the thesis entitled “Role of clathrin-associated sorting proteins (CLASPs) in clathrin assembly on membranes”, submitted by Sachin S. Holkar was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Dr. Thomas Pucadyil

(Supervisor)

Date:

DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources that have not been properly cited or from whom proper permission has not been taken when needed.

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Date:

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Synopsis

Role of clathrin-associated sorting proteins (CLASP) in clathrin assembly on membranes

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Introduction:

Eukaryotic cells internalize various receptors (cargo) via clathrin-mediated endocytosis (CME). Cargo molecules have sorting signal at their cytoplasmic side which interacts with specific clathrin-associated sorting protein (CLASP) molecules (*2*) (*1*) . These CLASPs and cargo complexes get clustered on the plasma membrane with other endocytic machinery and simultaneously convert planar membrane into curved clathrin-coated pit (*1*). This pit subsequently forms a narrow neck followed by scission which releases clathrin-coated vesicle in the cytoplasm. CME plays role in different physiological functions including nutrient uptake, virus internalization, cell signaling, retrieval of membrane proteins after exocytosis and regulation of the types and concentrations of membrane proteins at the plasma membrane (*2–6*). It is well studied but exact mechanism of its regulation and precise role of individual CLASPs in CME is not yet clear.

CME process is broadly divided into three main stages namely: initiation, maturation and fission (*1*). Initiation starts with the recruitment of initiation complex at the plasma membrane which marks the site of clathrin-coated pit (*7*) (*8*, *9*) (*10*). This CCP site then starts converting planar bilayer into curved pit with the recruitment of other endocytic proteins at the maturation stage. Different cargo molecules get clustered and sorted at the CCP. Protein sorting and progression of CCP maturation is mainly driven by membrane curvature and interactions between various protein

molecules. Membrane curvature generation is endergonic reaction and requires energy (*11*) (*12*) . Various membrane active endocytic proteins sense and generate membrane curvature which affect the timings and rates of recruitment of these proteins at the CCP. The protein-membrane and protein-protein interactions result in the formation of complex network of cargo, CLASPs, clathrin and other endocytic proteins at the CCP (*13*). Membrane-endocytic protein interaction and protein-protein interactions are exergonic reactions and release energy which is used in the formation of clathrin-coated vesicle, clathrin being the major player involved in this process.

Involvement of multiple protein interactions, membrane curvature and different biophysical factors make it difficult to study clathrin-coated pit formation in cells. Cell free reconstitution is an attractive option to study CCP formation *in vitro* where we can vary individual components and study their roles in CCP formation. Current assay systems are mostly dependent on end point observations and lack the dynamics of the CCP formation *in vitro*. In order to study it, there is a need for an assay system where we can control physical, chemical properties of membrane, composition and concentrations of the protein machinery and membrane curvature.

Clathrin is self-polymerizing protein which can bind and clusters the adaptor proteins. It forms planar clathrin lattice and curved coated structure. Factors deciding planar or curved clathrin assembly are not yet clear. There are multiple CLASP molecules involved in CME and all these CLASPs have clathrin binding motifs. The numbers and types of these clathrin binding motifs varies in these CLASPs. This variation across CLASP may affect the initiation, efficiency and the kinetics of clathrin assembly on membrane. To address this question we have used different CLASP molecules and studied their property of clathrin assembly. Clathrin has multiple interaction sites for different CLASP molecules and acts as hub in CME. These CLASP molecules may get cluster or sort in clathrin-coated structures during clathrin assembly reaction on model membrane system. It is also interesting to study the role of clathrin in CLASP sorting process.

In this study we developed a novel technology to study the role of epsin in clathrin assembly. The membrane curvature sensitivity of epsin regulate the clathrin assembly on the model membrane system. Next we analyzed the role of clathrin binding sites and clathrin disassembling molecules HSC70, auxilin, ATP in clathrin assembly reaction. This new novel model membrane system allowed us to study kinetics of epsin-mediated clathrin assembly which is similar to the clathrin-

coated vesicle formation kinetics in cells. This is the first study where we could measure the rate of clathrin assembly on curved model membrane system.

Epsin, AP2, AP180, Dab2, ARH, β-arrestin and NUMB are members of CLASP family which are used in this study. We have shown the importance of two clathrin binding sites in clathrin assembly reaction. Out of these seven CLASPs molecules, epsin, AP180, Dab2 and ARH were able to assemble clathrin in our assay conditions with different kinetics of clathrin assembly which will help us to understand the precise role of these CLASP family members in clathrin assembly on the membrane. In cells, AP2 is necessary molecule for clathrin-mediated endocytosis and its depletion ceases the clathrin mediated endocytosis but surprisingly its clathrin assembly subunit is not able to assembly clathrin in our system. Presence of the other CLASP family member on the membrane can induce the clathrin assembly as well as can cluster clathrin on the membrane. We have also shown the role of clathrin in different CLASP clustering and their co-clustering.

Results and discussion:

Special localization of epsin regulate the clathrin assembly on Supported Membrane Tubes (SMrT):

Epsin is one of the indispensable molecules in CME. Its knockdown or knockout stops the CME and arrest the CCP maturation (*14–16*) . ENTH domain of epsin shows the curvature dependent membrane binding (*17–20*) and C-terminal tail or full length epsin can able to form clathrin-coated pits on the membrane surface (*21*) . To test its role in clathrin assembly we developed novel model membrane system called Supported Membrane Tubes (SMrT). Exposing these tubes to hypertonic buffer resulted in the formation of regions with high and low curvature which are called as budded SMrT templets (*22*) . ENTH domain of epsin has high binding affinity for phosphatidylinositol 4, 5-bisphosphate (PIP_2). In our Budded SMrT template, purified full length epsin preferentially binds to highly curved membrane compared to low curved membrane which is dependent on DOPIP2. This curvature sensitivity of epsin on budded SMrT templates regulated the clathrin assembly and could assemble clathrin on highly curved membrane tubes compare to low curved membrane buds. The time constant of clathrin assembly on curved membrane was found to be ~ 74s which was similar to the time constant of clathrin-coated vesicle formation in cells. Our finding could be correlated to the CCV formation process in cells and explain the role of epsin clathrin-coated pit formation. Epsin may get recruited at the curved clathrin-coated pit followed by

recruitment and assembly of clathrin on plasma membrane at the maturation phase. There are two clathrin binding motifs (CBMs) (^{257}LMDLAD and ^{480}LVDLD) present in epsin which cooperatively bind to clathrin (*23*) . In our study, we have shown the necessity of these two CBMs for efficient clathrin assembly. Out of these two clathrin binding motifs, the ^{480} LVDLD motif can partially assemble clathrin on SMrT templates. HSC70, auxilin and ATP can disassemble clathrin coat *in vivo* and *in vitro* (*24*, *25*) . These proteins were not able to disassemble epsin-mediated clathrin assembly, but facilitate fast clathrin assembly in our assay system. This is the novel finding and may suggest the new role of these proteins in CCP formation.

In this study we have developed tandem method of protein purification, new simple assay system to measure binding constant between protein and membrane, SMrT template assay system for studying membrane curvature sensitivity of proteins and protein-protein interactions on membrane surface. These technologies could be useful to study other cellular processes.

Role of CLASP molecules in clathrin assembly and clathrin-mediated CLASP co-clustering:

Epsin, AP2, AP180, Dab2, ARH, β-arrestin and NUMB are members of CLASP family (*26*) . We have purified, fluorescently labeled these CLASPs and recruited on the SMrT templates via 6xHis tag (at the N-terminus of CLASP) and DGS-Ni^{2+}NTA lipid interaction. Texas red labeled clathrin was flown on the CLASP coated SMrT templates. Out of these members epsin, AP180, Dab2 and ARH were able to assemble clathrin, whereas β subunit of AP2, β-arrestin and NUMB were unable to assemble clathrin on SMrT templates. Previously we have shown the importance of two clathrin binding motifs in clathrin assembly. Our results show that CLASPs having two (epsin, Dab2, ARH) or more than two clathrin binding sites (AP180) are able to assemble clathrin whereas single clathrin binding motif (β-arrestin) or null clathrin binding motifs (NUMB) CLASPs are not able to assemble clathrin. His-GFP AP2 β subunit has more than two CBMs, still it is unable to assemble clathrin on membrane surface. Our result is contradictory to the previously reported results where AP2 could assembled clathrin on GUV surface or in solution (*27*, *28*) . This may be due to complete washing of unbound AP2 β subunit from solution and may suggest incapability of clathrin assembly by membrane recruited AP2 β subunit.

SMrT templates allowed us to analyze kinetics of clathrin assembly. We analyzed the kymographs of CLASPs-mediated clathrin assembly and got the initiation time (onset time) and time constant of clathrin assembly by epsin, AP180, Dab2 and ARH. The net time constants and onset times for

clathrin assembly on SMrT templates were different in case of epsin, AP180, Dab2, and ARH. We further analyzed the kinetics of individual puncta. The time constants and initiation times of individual clathrin puncta were different. This data shows the differences in kinetic parameters of clathrin assembly mediated by different CLASPs molecules and these variations may be due to varying affinity of these CBMs with clathrin binding sites of N-terminal domain of clathrin (*1*)(*2*).

We classified these CLASPs based on their clathrin assembly capability as CLASP "A" (capable to assemble clathrin) and CLASP "B" (incapable to assemble clathrin). The efficiency of clathrin assembly mediated by CLASP "A" molecules and their clustering ware different acorss these CLASPs. AP180, and Dab2 were efficiently assembled clathrin (higher clathrin intensity in each puncta) as well as get clustered in clathrin foci compared to epsin and ARH. Further, we classified these CLASP "A" members based on their clathrin puncta efficiency as CLASP "A1" (efficiently clustered CLASPs) and CLASP "A2" (inefficient clustered CLASPs).

These CLASPs have different types and numbers of clathrin and AP2 binding motifs which interact with terminal domain of clathrin and appendage domains of AP2 respectively. The presence of Dab2 and AP2 β subunit together on SMrT templates were able to assemble clathrin with AP2 β subunit clustering in clathrin foci. This may be due to direct interaction of AP2 β subunit with clathrin or Dab2. Dab2 was also able to induced clustering of β arrestin in clathrin foci with clathrin assembly.

The kinetic parameters of clathrin assembly mediated by AP180 and Dab2 were different but presence of these CLASPs together on SMrT templates showed their co-clustering.

Clathrin terminal domain has redundancy for CLASPs motifs. There are CLASPs molecules which have single clathrin binding motif but are unable to assemble clathrin like β arrestin, AP2 β subunit. Dab2 induce clustering of these CLASPs get clustered in clathrin assembly. This indicates the role of Dab2 and clathrin in CLASP clustering/sorting during clathrin-coat assembly reaction.

Our study shows the novel facile technique to study CLASPs mediated clathrin assembly reaction. It also shows the differences of clathrin assembly mediated by various CLASP molecules.

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Chapter 1
Introduction

Introduction:

Plasma membrane is the boundary between the living cellular and non-living extracellular environments. It is organized in the form of a lipid bilayer comprising of a hydrophilic surface and a hydrophobic core in which are embedded various transmembrane and peripheral membrane proteins. These proteins play important roles in cell signaling, nutrient uptake, and transport of specific molecules. Vesicular transport manages the transfer of membrane proteins and lipids across different cellular compartments. There are three major vesicular transport pathways in eukaryotic cells based on their coat forming proteins. These are the COPI-, COPII- and clathrin-mediated vesicular transport pathways (reviewed in (*3*)). These coat-forming proteins convert a planar lipid bilayer into a lipid vesicle in presence of other proteins. The COPII pathway is involved in vesicular transport of membrane proteins via the anterograde vesicular transport, from the ER to the golgi apparatus. The retrograde vesicular transport pathway works in the reverse direction and is mediated by the COPI pathway. Clathrin-coated vesicular transport pathways manage sorting and transfer of membrane proteins from the Golgi apparatus and endocytosis at the plasma membrane (Figure 1.1). Clathrin-mediated endocytosis (CME) is the major pathway for internalization of different receptors in cells and is also referred to as receptor-mediated endocytosis. This process is managed by recruiting specialized adaptor molecules and clathrin to the plasma membrane and is followed by generation of a clathrin-coated vesicle. This vesicle is composed of various membrane proteins and other extracellular material. This process plays important role in nutrition uptake, signaling, apoptosis, cell division etc.

1.1 History of Clathrin-Mediated Endocytosis (CME):

Clathrin-mediated endocytosis is one of the most well-studied vesicular transport pathways, using both cell biological, biochemical and biophysical techniques. In 1964 Roth and Porter observed coated vesicular structures in insect oocytes by electron microscopy (*4*). Subsequenctly, these coated structures were isolated from pig brain (*5*, *6*) and were found to be

enriched in 180-190 kDa protein later called clathrin (from latin *clatratus* which means like a lattice) (*7*). In the late 1970s, it was discovered that the low-density lipoprotein receptors (LDLR) are internalization by CME (*8–10*). Clathrin self-assembles at low pH or with adaptor proteins at physiological pH to form empty clathrin cages. This cage assembly reaction was later characterized in numerous studies (*11–15*). How the clathrin cage assembly is linked to endocytosis was understood by the discovery of sorting signals present on receptors such as the LDLR and transferrin (*16*)(*17*). Reconstitution of clathrin-coated pit assembly on isolated patches of the plasma membrane was done in various studies by using cytosol or CCV extract. All these studies were based on electron microscopic observations, site directed mutagenesis and receptor internalization assays. The dynamics of clathrin coated pits and vesicles in cells were later studied at the molecular level after the cloning and expression of GFP-labeled clathrin light chains (*18*). Several previous studies have characterized adaptor protein interactions between receptors and clathrin. The reconstitution of clathrin-coated pit assembly using purified components on a membrane surface was carried out on a lipid monolayer (*19*, *20*). Subsequently, clathrin-coated pits were assembled on giant unilamellar vesicles (GUV) using purified components (*21*, *22*).

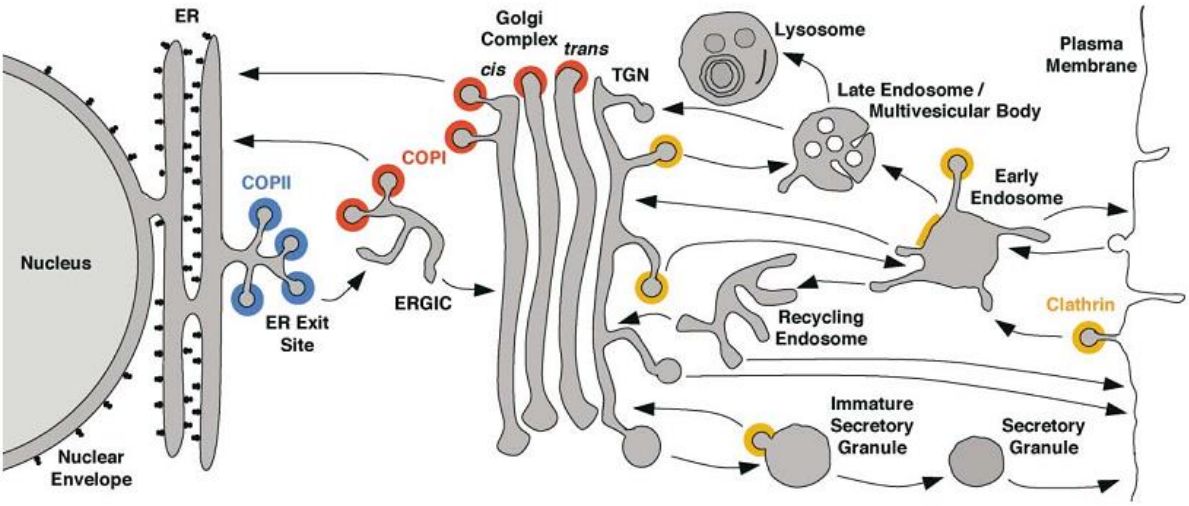


Figure 1.1: Membrane protein trafficking. Blue coat shows COPII-mediated trafficking from ER to the Golgi apparatus. Red coat indicates COPI-mediated trafficking from the Golgi apparatus to the ER and inter cisternal transport in the Golgi apparatus. Orange coat represents clathrin-coated vesicular transport from the Golgi apparatus to the plasma membrane, early and late endosomes. This pathway also transports proteins and membrane in the reverse direction. Figure is reproduced from (*3*)

1.2 Proposed molecular mechanism of clathrin-mediated endocytosis:

CME is comprised of discreet steps. Based on ultrastructural and cell biological observations McMahon and colleagues have proposed a model for CME (figure 1.2) consisting of five stages. These are nucleation, cargo selection, coat assembly, fission and uncoating.

Nucleation: Nucleation is the first step in CME. It marks the site for clathrin-coated pit (CCP) formation in cells. Based on knockdown studies in cells, a set of proteins consisting of FCHO1, 2, EPS15, and intersectin have been proposed to be recruited on the plasma membrane FCHO is an FBAR-domain containing protein that can sense low degrees of membrane curvature. Its knock down inhibits the nucleation of CCP and transferrin internalization in cells (*23*). EPS15 and intersectin are scaffolding proteins that interact and recruit a large number of accessory proteins (*24*, *25*).

Cargo selection: The nucleator proteins have binding sites for the adaptor protein 2 (AP2) and other accessory proteins and recruit these to sites that mark CCPs. AP2 knock down arrests CCP at the nucleation stage (*26*). It has been recently shown that AP2 recruitment by FCHO2 leads to a change in its conformation resulting in the latters cargo and clathrin-binding sites becoming open (*27*). The appendage domains of AP2 have binding sites for other adaptor proteins (*28–31*). These adaptor proteins get recruited with their cargo molecules and cluster cargo-adaptor complexes at the CCP site. Few adaptor molecules are presumed to generate membrane and during this stage clathrin starts recruiting at the site of CCP.

Clathrin-coat assembly: AP2 with other adaptor proteins increases the number of clathrin binding sites at the CCP. This results in the recruitment of clathrin which can stabilize adaptor complexes, membrane curvature and pack all proteins in its cage (*32*). It has been shown that membrane effector proteins like FCHO, epsin, EPS15 get displaced towards the edge of the CCP where they may be able to bend membrane effectively and convert a shallow clathrin-coated pit into an invaginated curved clathrin-coated vesicle (*33*, *34*).

Vesicle scission: Membrane curvature-sensitive BAR-domain proteins like amphiphysin, endophilin, SNX9 get recruited at the site of CCPs (*35–37*). These proteins have SH3 domain which can interact with the proline-rich domain (PRD) of dynamin. This results in the recruitment of the mechanochemical GTPase dynamin (*38*), which assembles at the neck of CCPs and mediates membrane scission (*39*, *40*). Dynamin depletion or inhibition of its activity can arrest CCPs.

Depletion of BAR-domain proteins also prevent dynamin recruitment at the neck of CCP and results in arrested CCPs (*41*).

Vesicle uncoating and clathrin coat component recycling: Vesicle scission is followed by uncoating of the clathrin coat. Coat uncoating is an ATP-dependent process, which is driven by the 70 kDa heat shock cognate protein (Hsc70) and the J-domain family protein auxilin or cyclin-G dependent kinase (GAK) (*42–46*). Auxilin is a neuron-specific protein whereas GAK is ubiquitously expressed. They have an N-terminal PTEN like membrane binding domain and C-terminal clathrin- and Hsc70-binding (J domain) regions. It binds to the clathrin heavy chain and AP2 in the coat as well as Hsc70 (*47*, *48*). Hsc70 comprises of nucleotide and substrate binding domains. The C-terminal region of clathrin heavy has a QLMLT motif which binds to the substrate-binding site of Hsc70 and causes coat disassembly (*49*, *50*). Auxilin and Hsc70 get recruited to and destabilize the clathrin cage (*51*). This process is ATP dependent and disassembles clathrin-coated vesicles *in vivo* and *in vitro*. Bound Hsc70 with clathrin is dissociated by the 100kDa nucleotide exchange factor protein Hsp110 and then it gets recycled for new cycle of clathrin-coated vesicle formation (*52*, *53*).

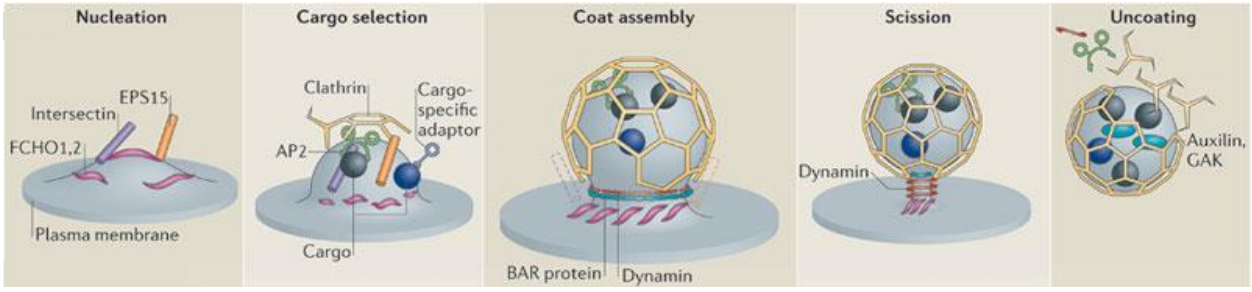


Figure 1.2: The proposed molecular mechanism of clathrin-mediated endocytosis. Nucleation: FCHO1, 2, EPS15, Intersectin recruit at the PIP2-rich plasma membrane and form nucleation complexes. Cargo selection: AP2 gets recruited at the site of CCP and then recruits other CLASP-cargo complexes. AP2 acts as a hub in this stage. Coat assembly: AP2 and other CLASPs recruit clathrin, which forms the coat. Clathrin-coated pit starts invaginating during this stage. Membrane curvature and protein complexes are stabilized by clathrin. Other BAR-domain containing proteins start recruiting at the neck of CCPs. Scission: BAR-domain proteins recruit dynamin (GTPase) at the neck of CCP followed by vesicle scission and release clathrin-coated vesicle. Uncoating: HSC 70, auxilin get recruited to clathrin-coated vesicles and disassemble the cage by using ATP. These components get recycled for new cycle of CME. (Figure reproduced from (*150*))

1.3 Components involved in CME:

Clathrin-mediated endocytosis comprises of various micro reactions involving different functional modules. The major components in CME are clathrin, adaptor proteins, plasma membrane and membrane proteins (cargo).

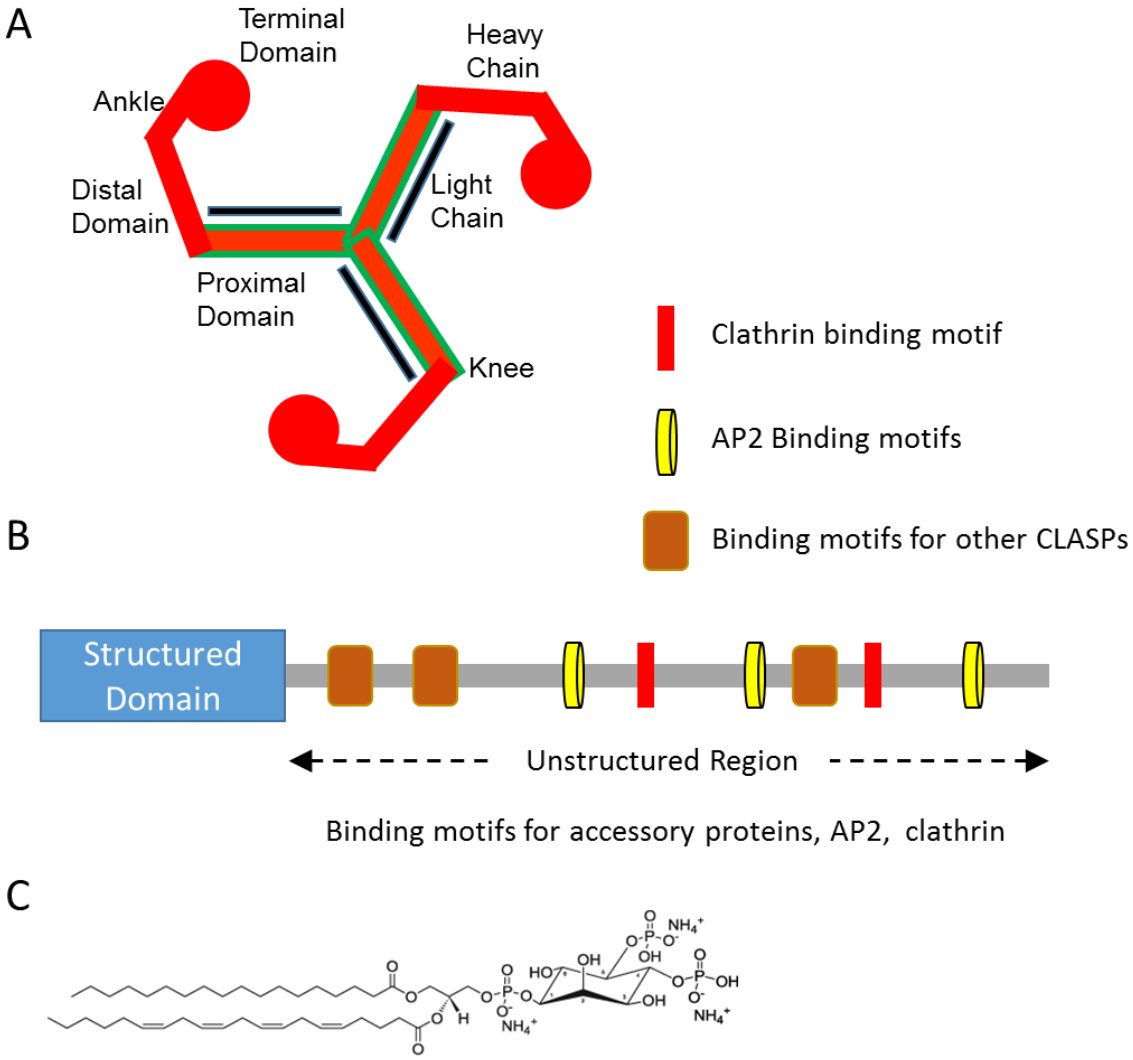


Figure 1.3: Components involved in CME. A) Structure of clathrin triskelia which is composed of 3 heavy and 3 light chains. Each heavy chain has an N-terminal domain, ankle region, distal and proximal domains, trimerization domain and a short unstructured region at the C-terminal tail. B) General structure of clathrin-associated sorting proteins (CLASPs). An N-terminal structured domain is followed by a C-terminal unstructured region, which has binding motifs for AP2, clathrin and other endocytic proteins. C) Structure of phosphatidylinositol 4, 5-bisphosphate (PIP2) which is one of the important lipids in plasma membrane. It binds to most of CLASPs and recruits proteins to initiate clathrin-mediated endocytosis.

1.3.1 Clathrin:

Clathrin is a trimmer of three heavy chains (180-190 kDa each) and three tightly bound light chains (*54*)(25-27 kDa each) that together form a triskelion (figure 1.3A) (*12*, *14*). Each heavy chain of clathrin consist of an N-terminal domain (NTD) (1-390), a linker region (391-493), a distal leg segment (494-1073) and a proximal leg segment (1074-1522) having 42 alpha helical zig-zags with approximately 30 amino acids in each helix and unstructured c-terminal trimerization/vertex domain (1523-1675) (*55–57*). Three heavy chains of triskelia are held together by the trimerization domain. Based on EM (*12*, *58*) and X-ray diffraction studies, dimensions and structure of clathrin triskelia have been determined. Each triskelia is approximately 47 nm in length and 2 nm in thickness. Clathrin has pucker in its structure (*59*). The distance between the two N-terminal domains is approximately 40 nm and the height of vertex from the NTD plane is around 22 nm. The clathrin NTD has a globular structure and consist of WD40 repeats. This NTD binds to CLASPs and recruits clathrin to the membrane. Clathrin can assemble into cages at low pH (*14*) or in the presence of adaptor proteins (*60–62*) or, alone at high concentration, at physiological pH (*63*). The proximal and distal domains of each heavy chain consist of alpha helical repeats with key histidine residues which causes clathrin to self-assembly at low pH (*51*). These domains interact with each other and stabilize the clathrin assembly. The minimal unit for clathrin self-assembly consist of the trimerization, proximal and distal domains (*12*, *64*).

Clathrin get polymerized and forms planar clathrin lattice or clathrin-coated vesicles (figure 1.4 B, C, D). The size and shape of clathrin-coated vesicles varies in cells. It ranges from 0.1 to 1 μm. This size is dependent on the way of clathrin polymerizes and forms lattice on the membrane surface. Clathrin can assemble as hexagonal, planar or curved lattice with pentagons. The incorporation of pentagons in hexagons converts the planar lattice into curved clathrin coats (figure 1.4 E). Thus, the relative proportion of hexagon and pentagonal lattices controls the size and shape of CCVs (*11*, *61*).

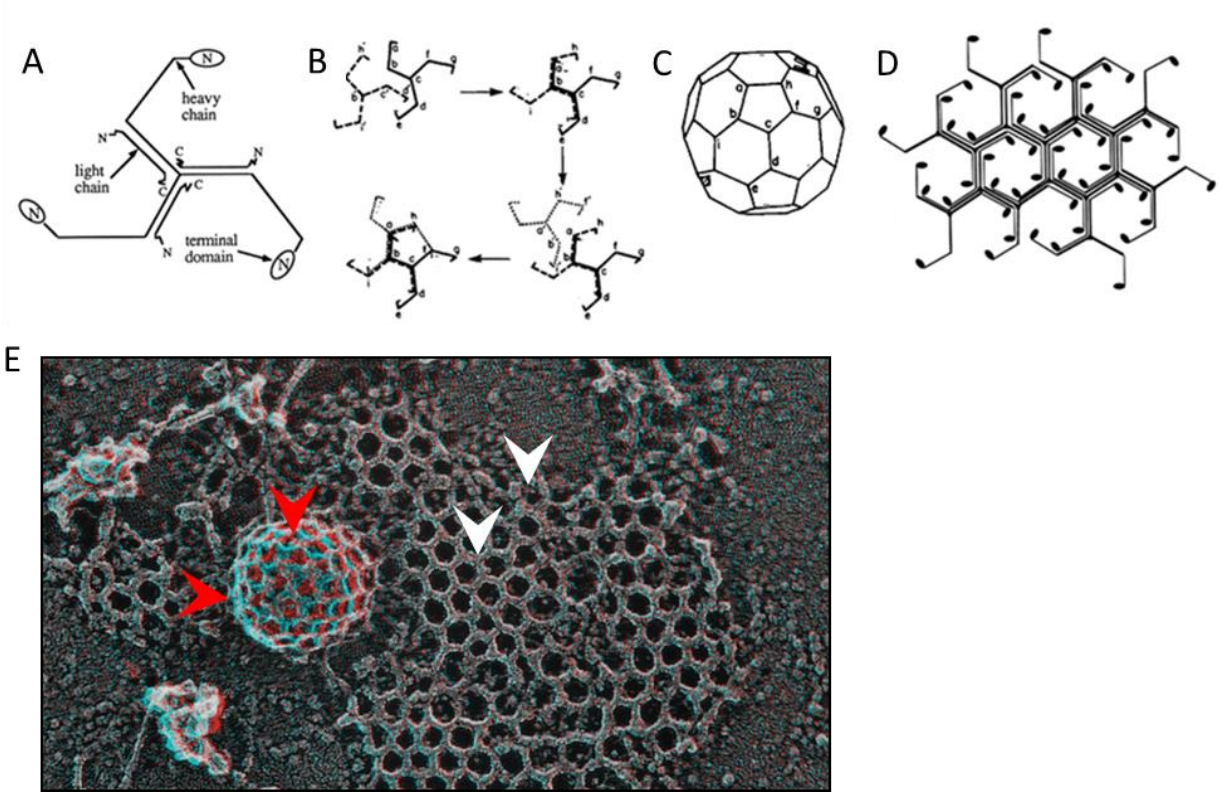


Figure 1.4: Clathrin assembles as planar and curved lattices. A) Model of a clathrin triskelion and its assembly which forms clathrin vesicles or flat lattices. Figure modified from (*61*) and (*11*). B) An EM image of clathrin assembly as planar (white arrowheads) and a curved clathrin coat (red arrowheads). Image reproduced from (*212*).

1.3.2 Clathrin-associated adaptor proteins:

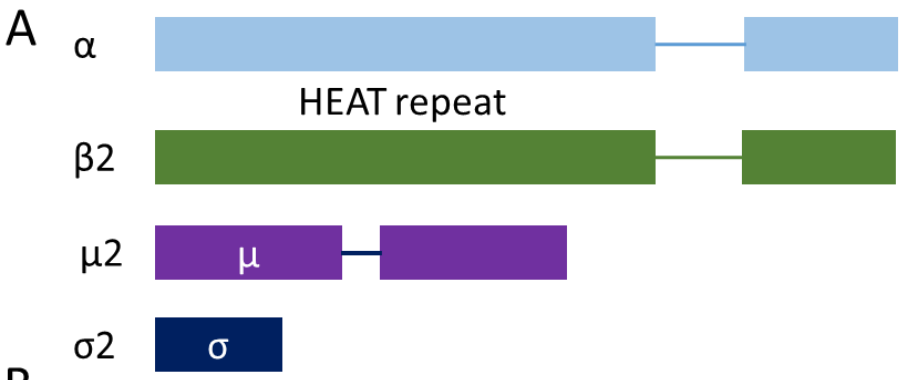
The proteins that interact with clathrin, AP2, accessory proteins, membrane or cargo are collectively called clathrin-associated sorting proteins (CLASPs) (*65*). These proteins interact with different cargo molecules and/or membrane lipids. The general structure of these proteins consist of an N-terminal structured domain/domains for membrane binding or protein interactions and a C-terminal unstructured region with motifs for AP2 and clathrin-binding (figure 1.3 B). These motifs have low affinity for their binding partners but their numbers vary in different adaptor molecules. The multiple interacting motifs in the unstructured region render them accessible for binding interacting partners and avoid steric hindrance. Multiple CLASPs are recruited at different times and with different kinetics to CCPs (*66*).

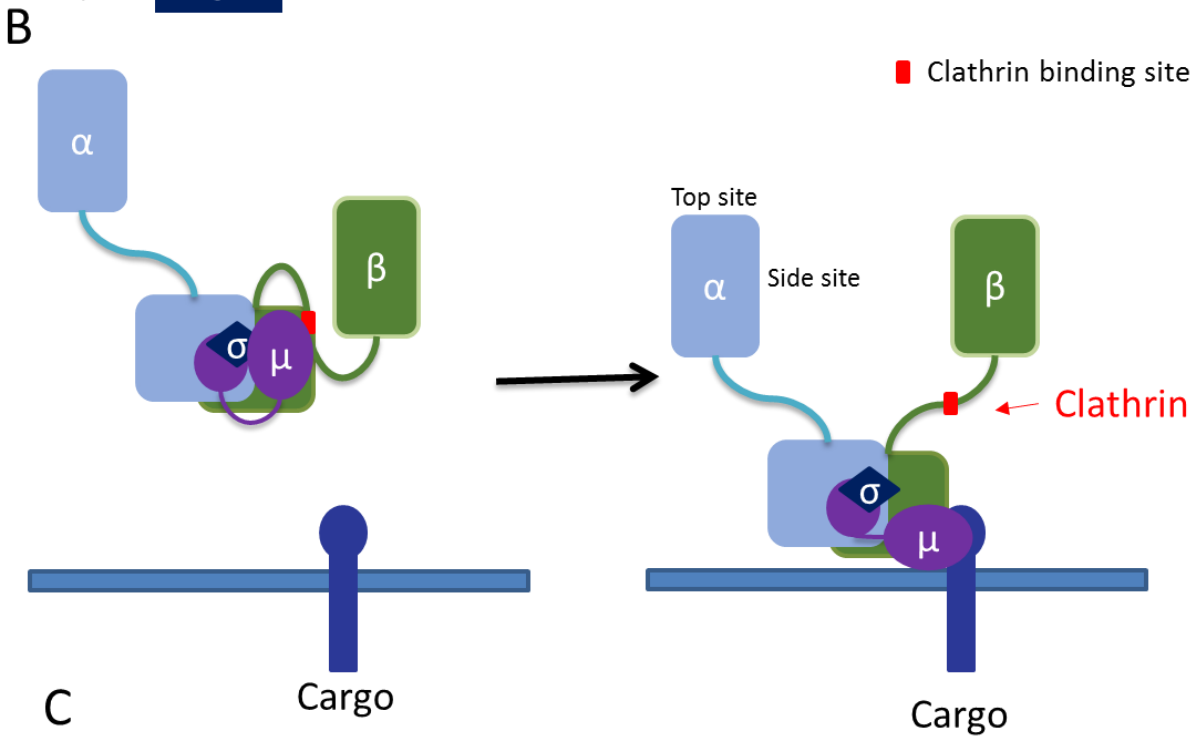
1.3.2.a Adaptor Protein 2 (AP2): The AP2 is the second most abundant protein in clathrin-coated vesicle after clathrin (*67*). It represents a central hub in CME. AP2 knock down leads to cessation

of CME in cells (*68, 69*). It consists of four subunits: 107 kDa alpha (α), 108 kDa beta (β), 50 kDa mu (μ) and 17 kDa sigma (σ) (figure 1.5A). The heterotetrameric AP2 has a core trunk region and 2 free appendage domains. The trunk region binds PIP_2 at two different sites. The N-terminal residues (21-80) of the α subunit (*70*, *71*) and the μ2 surface (*72*) (figure 1.3A) interacts with PIP_2 via basic residues. AP2 can also interact with different cargo molecules having a YxxΦ motif, which interacts with the μ subunit (*17*, *73*, *74*) and the dileucine D/ExxLL motif, which interacts with the β subunit (*74*). Binding of AP2 to the membrane and cargo is regulated by the ARK family kinase adaptor-associated kinase 1 (AAK1) (*75*, *76*) and knock down of AAK1 inhibits transferrin receptor internalization in cells (*77*, *78*). The AAK1 interacts with the alpha subunit of AP2 and phosphorylates the linker region between two domains of μ2 subunit at the Thr156 residue. This phosphorylation causes a large conformational change in the μ subunit of AP2 (figure 1.5B) (*79*). This large-scale conformational change opens one domain of the μ subunit and exposes binding sites for cargo molecule and membrane. This new open state of AP2 has higher affinity for the membrane and cargo. AP2 can simultaneously bind to two kinds of sorting motifs. Recent studies have shown that a clathrin-binding site, present in the linker region of the AP2 β subunit, is not accessible and is concealed between the μ and α subunits. This clathrin binding site gets exposed in the open conformation of AP2 (figure 1.5B) (*80*). This indicates that membrane recruitment, cargo recognition and clathrin binding are allosterically regulated.

FCHO is considered as a one of the nucleator proteins in CME. It has a binding site for AP2. FCHO2 and SGIP1 interaction with AP2 also results in the latter's conversion from a closed to an open conformation (*27*) (figure 1.5B).

Figure 1.5: Domain organization and its interactions with AP2 subunits A) α and β2 subunits have N terminal HEAT repeats followed by a linker region and an appendage domain. The μ2 subunit has N terminal and C terminal domains, which are connected via short linker region. The σ2 subunit has a single domain. B) Heterotetrameric structure of AP2 complex. It has a closed and an open conformation. Cargo and clathrin -binding sites are not accessible in the closed conformation but upon its conversion to an open conformation by AAK kinase or FCHO2 binding exposes the cargo-binding site (in the μ2 subunit) and the clathrin-binding site (in the linker region of AP2 β2 subunit) on the membrane. C) Crystal structure of AP2 α and β2 appendage domains. Each domain has two different binding sites for AP2 binding motifs. One at the top and other is at the side. Table shows the AP2 binding motifs and their binding sites on the AP2 appendage domain. (Panel (C) is reproduced from (*82*).





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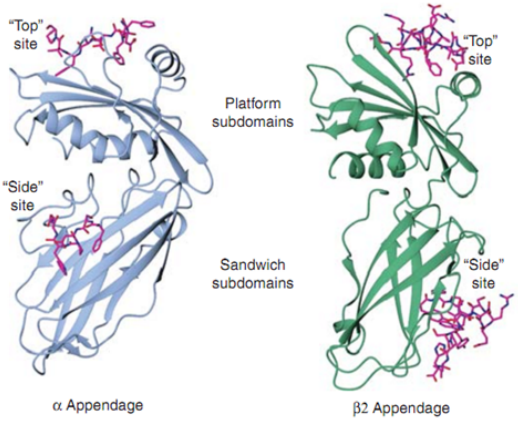

site	Consensus motifs for binding
α Top	Dx[FW], FxDxF
α side	[FW]xx[FW]
β Top	$[DE]_n$xFxx][FL]xxxR
β Side	[FL]xx(G0[FL]xDF

6

α and β appendage domains of AP2 have binding sites for different CLASP and accessory proteins (*28–31*). Each domain has two different binding sites for different binding motifs. Top site is referred as C-terminal side or side towards the clathrin and side site is horizontal side in the appendage domains. Top side of alpha appendage domain interact with Dx[FW] or FxDxF motifs and side site interacts with [FW]xx[FW] (figure 1.5C) (*81*). Beta appendage domain has top site for binding to [DE]nxFxx[FL]xxxR and side site for [FL]xx(G)[FL]xDF motifs (figure 1.5C) (*31*). These domains have low affinity for their binding motifs but number of binding sites vary in different CLASPs. These interactions recruit other clasps at the time of CCP formation. Proteins at the early stage (nucleators) have multiple binding sites for AP2 and increases local AP2 binding sites after formation of nucleation complex in cells. (*82, 83*) (*84*). This results in the recruitment of multiple copies of AP2 at the CCP site followed by recruitment of other CLASP molecules. Clathrin knockdown cells depicts the AP2-mediated protein clusters on the plasma membrane. This may suggest the role of AP2 in cargo sorting process.

1.3.2.b EPS15 interacting protein (Epsin): Epsin was discovered in 1998 as an EPS15 protein interacting. It acts as membrane curvature sensor and inducer, adaptor for ubiquitinated cargo, efficient clathrin and AP2 recruiting protein. It get recruited at the maturation phase and acts as checkpoint agent in CCV formation (*85*). It has epsin N-terminal homology (ENTH) domain and C-terminal unstructured region (figure 1.6A). It has high affinity for PIP_2 and can sense curved membrane (*86, 87*). It also has amphipathic H_0 helix which is formed after binding to PIP_2 in lipid bilayer and partially inserts into the membrane (figure 1.6A). This insertion is responsible for epsin's membrane curvature sensitivity and membrane bending property (*20, 88*) . It has been suggested that this insertion also plays role in membrane fission activity (*20, 88*).

Epsin depletion by different approaches such as its antibody injection or RNAi (knock-down) or knockout resulted in the accumulation of multi-headed clathrin-coated structures (*88*) or shallow membrane buds (*89, 90*). Epsin has three isoforms in mouse and their knockout cells show accumulation of early and mid-phase CCPs (*90*). In partial reconstitution of clathrin coated pits on plasma membrane of unroofed cells, epsin localizes with clathrin on membrane tubes formed by cytosol (*90*). Also epsin localizes at the rim of the CCP in cells (*33*). This shows epsin's role at the neck (rim of half spherical CCP) of CCP and its involvement in it's the progression into complete spherical shape CCV.

Unstructured C-terminal region of epsin (*91*) has binding sites for ubiquitin, clathrin, AP2 and EPS15 (*92*). The number of these binding sites are not conserved across the different organisms as well as varies with different isoforms in the same species. Human epsin 1 has three ubiquitin binding sites which interact with polyubiquitin and internalizes ubiquitinated cargo molecules for e.g. EGFR (*93*, *94*). It also has two clathrin binding sites which co-operatively bind to clathrin (*95*) (*91*). DPW motif of epsin has weak affinity for AP2 but presence of multiple motifs result in high net affinity of epsin with AP2. Epsin can simultaneously bind to multiple molecules of AP2. It can polymerize clathrin on lipid monolayer and forms bud like structure (*20*). His tag unstructured C-terminal region of epsin recruited via DGS-Ni^{2+}NTA lipid on giant unilamellar vesicle (GUV) bilayer is capable to form CCPs (*21*). Full length epsin recruited via PIP_2 is also able to form CCPs on GUV surface (*22*, *96*).

1.3.2.c Clathrin Assembly Lymphoid Myeloid leukemia (CALM) and Adaptor Protein 180 (AP180): CALM and AP180 are enriched in clathrin-coated vesicles. These proteins have AP180 amino-terminal homology (ANTH) domain C-terminal unstructured tail (figure 1.6B). ANTH domain of CALM and AP180 have high similarity in mammals (*19*). ANTH domain binds to PIP_2 and has dissociation constant (Kd) 0.3 um (*97*) .

AP180 was discovered in 1986 and was fallaciously considered as clathrin based on its migration on SDS-PAGE (*98, 99*). AP180 is expressed in neurons and has binding specificity for PIP_2. Its C-terminal region is highly unstructured and flexible. It anonymously runs on SDS PAGE (around 180 kDa)(*91*). It also has binding motifs for clathrin, AP2 and other accessory factors. There are more than ten clathrin binding motifs present in the C-terminal region of AP180. Each motif has low affinity for clathrin terminal domain, but high number of these motifs results in higher avidity effect for clathrin (*1*, *64*, *100, 101*). This high avidity, high number of clathrin binding motifs and flexibility of C-terminal region are responsible for clathrin binding and formation of empty clathrin coats *in vitro*. Clathrin coats formed by AP180 have narrow size distribution (*98*). Overexpression of full length AP180 or its C-terminal region in cells inhibit clathrin-mediated endocytosis (*19*). Deletion of AP180 homolog in drosophila or its knock down in rat hippocampal neurons increases the size of clathrin-coated vesicles and number of synaptic vesicles and also transmission (*102, 103*). Its simultaneous binding to PIP_2 and clathrin results in

the formation of clathrin lattice or clathrin coated pit in presence of AP2 on lipid monolayer (*19*). It can form empty clathrin coats or clathrin coated vesicles on GUV surface (*22*) (*21*).

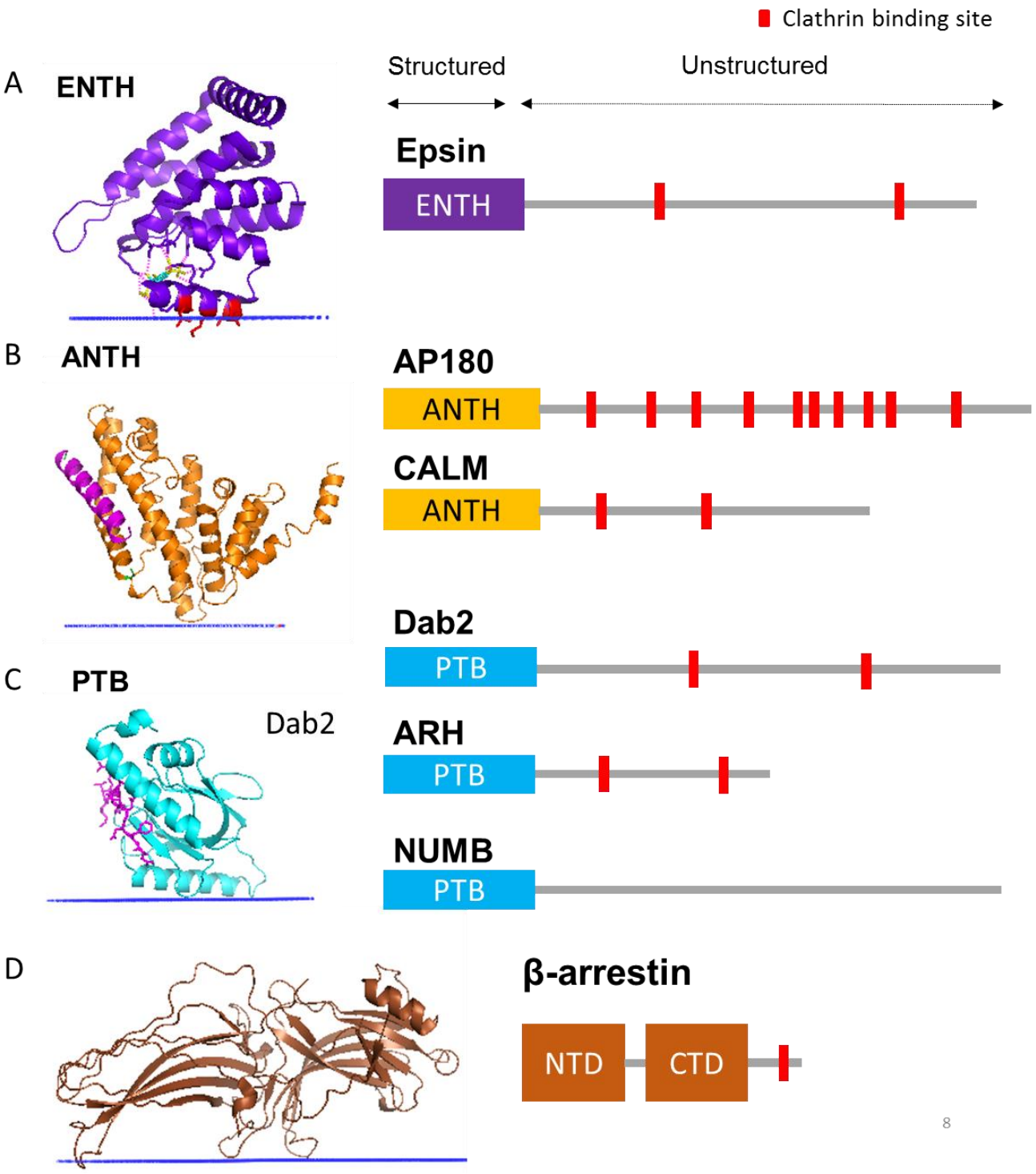

Figure 1.6: Crystal structure of membrane binding domain and domain architecture of bona fide CLASP molecules A) Crystal structure of epsin ENTH domain. Ho helix formed after its interaction with lipid molecule. Red residues in the Ho helix get inserted into membrane and can cause membrane bending. Full length epsin has ENTH domain at the N-terminus and unstructured C terminal tail which has binding sites for AP2, Clathrin, and EPS15 proteins. B) Crystal structure of ANTH domain of CALM with VAMP 8 binding domain (magenta color) PDB: 3ZYM. Domain organization of AP180 and CALM. Each protein has N-terminal ANTH domain followed by unstructured C-terminal region with clathrin and AP2 binding sites. C) Crystal structure of PTB domain of DAB2 with NPXY peptide (magenta) present in LDLR cargo molecules. Domain architecture of Dab2, ARH, NUMB which have N-terminal PTB domain followed by C-terminal region with AP2 and/or clathrin binding motifs. D) Crystal structure of β-arrestin which has N and C domains. C domain has clathrin binding motif which is allosterically regulated by GPCR and membrane binding of β-arrestin. Blue line in each structure is indicated as membrane and its coordinates are imported from http://opm.phar.umich.edu/. Structures are superimposed and modified from PDB files: ENTH domain (1H0A), ANTH domain (1HFA and 3ZYM), PTB domain (1M7E and 1NU2), β-arrestin (1SUJ).

CALM is an isoform of AP180 which expresses ubiquitously in cells and is involved in various leukemias (*104*) as well as in iron homeostasis (*105*). Its depletion causes irregular shape and size of CCPs in HeLa cells (*106*) but overexpression inhibits of CME (*107*). Its ANTH domain has binding sites for VAMP proteins and plays role in their internalization (figure 1.6B) (*108, 109*). The affinities are different for VAMP2, VAMP3 and VAMP8 and show different efficiency of VAMP internalization (*110*). Short C-terminal region of CALM has binding motifs for clathrin, α subunit of AP2. AP2 knockdown reduces its recruitment to the plasma membrane, whereas clathrin knockdown does not. It indicates CALM recruitment before clathrin on the plasma membrane. It does not affect the epsin, EPS15 and AP2 recruitment to CCPs (*106*). AP180 and CALM plays role in regulating size and shape of clathrin-coated vesicles in cells.

1.3.2.d Disabled 2 (Dab2), Autosomal Recessive Hypercholesterolemia (ARH), NUMB: These are phosphotyrosine binding domain (PTB) containing CLASPs. N-terminal PTB domain binds to plasma membrane and cargo molecules followed by unstructured C-terminal region (figure 1.6C). Dab2 is 80kDa protein having PTB domain followed by C-terminal unstructured region. PTB domain of Dab2 specifically recognize FxNPxY motif containing cargo (figure 1.6C) for e.g. LDLR, LRPs , β-integrins, and the β-amyloid precursor protein (APP) (*111–113*). C-terminal region of Dab2 has binding sites for clathrin, AP2, FCHO, myosin VI, Grb2 (*114–117*) etc. Overexpression of Dab2 and cargo increases the size of CCPs and also affect the distribution of AP2 on the cell surface (*118*). Dab2 knockdown reduces LDLR internalization rate whereas knockdown of ARH with Dab2 stops the LDLR internalization but does not affect transferrin internalization (*113, 114*). Clathrin box 1 (LVDLN) and W box (PWPYP) are two different

clathrin binding sites present in human Dab2. It also has three AP2 binding sites, five NPF motifs and three proline rich sequences. There are two isoforms of Dab2, p96 (full length) and p67 in human cells (*119*). P67 isoform lacks central clathrin binding region, two AP2 binding regions and two NPF motifs. It has been suggested that Dab2 is sufficient to form clathrin-coated vesicle independent of AP2 (*112*). Dab2 is an essential molecule in yeast for CME. Dab2 plays role in different signaling pathways for e.g. the mitogen-activated protein kinase (MAPK) signaling (*120*), Wnt signaling pathway cascade (*121*). It also plays role in embryonic development. Its knock out in mouse embryo die before gastrulation phase (*122*).

Autosomal recessive hypercholesterolemia disease is caused by mutations in a gene that encodes a phosphotyrosine binding (PTB)-domain protein called ARH (*123*). arh-/- mice are normal and fertile but hypercholesterolemic and sensitive to dietary cholesterol. Clearance of 125 I-labeled LDL is reduced in arh-/- mice (*124*). It has been suggested that nitrosylation of ARH is required for LDLR internalization (*125*). ARH is not able to internalize LDLR by its own but it requires AP2. ARH binds to AP2 and clathrin (*126*, *127*) and has affinity for clathrin ~ 40nM.

Numb also has PTB domain like Dab2 and ARH (figure 1.6C). It binds to ear domain of α subunit of AP2 (*128*). It does not have clathrin binding site but internalizes cargo through CME. It has AP2 binding sites which recruit NUMB-cargo complex to CCP and internalize its cargo molecules (*129*). Recently it has been shown that NUMB interacts with Niemann- Pick C1-Like 1 (NPC1L1) cargo and internalizes that through CME (*130*). It also plays role in Notch signaling as well as differentiation of various cells. It plays role in embryonic development (*131–133*).

1.3.2.e Arrestin: Arrestins play major role in GPCR signaling and their internalization in cells (*134*). There are four families of mammalian arrestins, rod and cone cells have visual arrestins (arrestin-1 and arrestin-4) respectively, which quench phototransduction. There is also ubiquitously expressing nonvisual arrestins (β-arrestin1 and β-arrestin2, a.k.a. arrestin-2 and arrestin-3). All arrestins have similar structural organization. β arrestin is one of the important arrestins and consist of N and C-domains (figure 1.6D) which are connected by loops and short α helix. G-protein-coupled receptors (GPCRs) get activated after phosphorylation at their cytosolic site (*135*). This phosphorylated GPCR is recognized by β arrestin. Membrane recruited β-arrestin forms homo or heterodimerize with large conformational change in its structure (*136*, *137*). This

conformation change leads to exposure of endocytic protein binding motifs and leads to internalization of activated GPCRs (*137–139*).

There are two variants of β arrestins present in mammalian cells. β arrestin 1 has two clathrin binding motifs (LIELD and LLGDC) whereas its splice variant has only one clathrin binding site (LLGDL) (*140, 141*). It also has AP2 binding motifs (*142, 143*). These clathrin and AP2 binding motifs are essential for arrestin mediated internalization of GPCRs (*141, 142*). PIP_2 binds to β arrestin at two different sites and plays important role in arrestin mediated endocytosis. It gets homo or heterodimerize which is dependent on binging of PIP_2 (*144, 145*). Post translational modification of β arrestin also plays role in arrestin-mediated endocytosis. Arrestin-2 get S-nitrosylated at cys-410 and enhances its recruitment at CCPs leads to increase in the rate of GPCR internalization (*146*). Ser-412 phosphorylation inhibit arrrestin interaction with endocytic machinery (*147*). Sumoylation at lys400 facilitate GPCRs internalization (*148*). Ubiquitnation of arrestin plays role in arrestin-mediated endocytosis of GPCRs (*149*) .

1.3.2.f Other proteins involved in clathrin-mediated endocytosis: There are multiple proteins involved in CME. These proteins have different functions like membrane bending, cargo sorting, membrane fission, recruitment of other proteins etc. (table 1.1). These proteins get recruited at different timings and different rates during CCV formation (*66*). EPS15, intersectin, FCHO1, 2 are supposed to be nucleator proteins and mark clathrin coated pit site on the plasma membrane. EPS15 can act as adaptor for ubiquitinated proteins. It has multiple AP2 binding sites and also has EH domains which interact with epsin. FCHO 1,2 are F-BAR domain containing proteins and is able to generate initial membrane curvature on the plasma membrane. It also has μ like domain which interact with other CLASP molecules during CCP formation. It's binding to beta appendage domain of AP2 results in conversion of AP2 to open conformation. Intersectin is a scaffolding protein and links to various components of the clathrin machinery. Amphiphysin, SNX 9 are bar domain containing proteins and have interaction sites for clathrin, AP2 and dynamin. Dynamin is a GTPase and causes scission of clathrin-coated vesicle. Auxilin/GAK and HSC 70 play role in disassembly of clathrin-coated vesicles in cells. Molecules like NECAP, Stonin, and HRB act as adaptor molecules for different membrane proteins and interact with AP2.

Table 1.1: Components involved in CME and their roles (Revived in (*150*))		
Sr. No.	**Protein**	**Role in CME**
1	Clahtrin	Self-polymerize and form clathrin basket on the vesicle. Stabilizes membrane curvature and protein-protein complexes in the CCV, acts as hub during CME.
2	AP2	Hetero-tetrameric protein, Acts as central hum in CME, binds and recruit other CLASPs and accessory proteins in CCVs.
3	FCHO	Acts as nucleator. Binds to AP2 and converts it into open state, generate membrane curvature.
4	EPS 15, Intersectin	Acts as nucleator, scaffolding protein and recruits other endocytic proteins.
5	Epsin	Acts at the maturation phase. Generate membrane curvature and binds to ubiqutinated cargo molecules.
6	AP180/ CALM	Regulates size and shape of CCV. CALM plays role in VAMP internalization.
7	Bar domain proteins	Amphiphysin, SNX9 bind to the membrane, AP2 and clathrin, recruit dynamin and play role in vesicle scission.
8	HSC 70, Auxilin	Play role in vesicle un-coating and recycling of clathrin coat machinery.
9	Cargo specific adaptor molecules	Recruit different cargo molecules in the CCP and may play role in clathrin-coat assembly.
10	Kinases	Phosporylates AP2 and other proteins involved in CME.
11	Phosphatases	Recruited by AP2 or intersectin and degrade PIP_2.

Kinases and phosphatases also play role in CME. Kinases like AAK 1 and CVAK104 phosphorylate AP2 and DYRK1A phosphorylates various proteins in CME. Phosphatases like SHIP2 and OCRL involve in the maturation of CCP whereas synaptojanin plays role in disassembly reaction. It has been suggested the role of actin in the CME. Proteins like HIP1, cortactin act as nucleator for actin and play role in membrane remodeling.

1.4 Plasma Membrane and Cargo molecules:

Plasma membrane: The plasma membrane acts as a platform for CME. It composes many lipid molecules like phosphatidylcholine, phosphatidylserine, cholesterol, sphingomyelin, phosphatidylinositol 4, 5-bisphosphate (PIP_2). Of these lipid molecules, PIP_2 plays a major role in recruiting proteins necessary for clathrin-coated pits (CCPs) formation (figure 1.3C). It can do so by interacting with different domains for e.g., ANTH present on AP180, ENTH present on epsin, and PTB domain present on the adaptor proteins Dab2, ARH, and NUMB etc (*151*). In addition, several accessory proteins such as amphiphysin and the large GTPase dynamin contain the plectstrin and phox homology domains that bind PIP_2 and as a result are recruited to CCPs. This lipid mainly plays role in early stages of CME.

Phosphatidylinositol 4-phosphate 5-kinase (PIP5K) generates PIP_2 at the plasma membrane. Its depletion by RNAi inhibits CME whereas overexpression increases the number of clathrin-coated pits at the plasma membrane (*152–154*). PIP_2 levels can also be reduced by incubating cells with butanol or ionomycine. Cells incubated with 1 butanol results in the generation of phosphatidylalcohols instead of phosphotidylacid (PA) by Phospholipase D (PLD). This lowers the activation of PIP5K subsequently leads to low amount of PIP_2 in cells (*152*, *155*). 1 butanol treatment decrease the transferrin uptake and inhibition of new clathrin-coated sites on the plasma membrane (*155*, *156*). Cholesterol, PS also plays role in CME. Cholesterol depletion results in reduction of transferrin uptake in cells (*157*).

Membrane proteins (Cargo): Cargo molecules are sorted into CCPs and then internalized in cells. Cargo internalization is mainly classified into two different classes; ligand-induced internalization (*158*, *159*) and constitutive internalization (*160*, *161*). Ligand-induced internalization is dependent on ligand binding to the receptor (for e.g. EGF receptor). Ligand-receptor complex induces phosphorylation of residues in the cytoplasmic domain of the receptor which triggers its internalization (*162*). On the other hand, constitutive internalization is

independent of ligand binding. Uptake of nutrients by the transferrin receptor (TrfR) and low density lipoprotein receptor (LDLR) are examples of constitutive internalization (*160*, *161*). These receptors have sorting motifs in their cytoplasmic tails, which can interact with specific adaptor proteins followed by clathrin-mediated receptor internalization.

There are different factors that affect receptor internalization in cells. These include characteristics of the sorting motifs present on receptors, bulk receptor concentration in the plasma membrane, receptor oligomerization/clustering, and sequences present in the transmembrane domains of receptors (figure 1.7). Transferrin and LDL receptors have been extensively studied as representative cargo molecules in the CME (*65*). Trowbridge and colleagues in 1990 showed that mutating the transferrin receptor sorting motif YTRF to ATRF inhibit its internalization (*163*). Also, mutating the LDL receptor sorting motif NPTY to APTY inhibits its internalization (*164*). These small sorting motifs can interact with adaptor proteins and thereby get concentrated in CCPs.

Trowbridge and colleagues (1993) added an extra copy of the YTRF sorting motif at different positions in the transferrin receptor cytoplasmic tail. Addition of an extra YTRF motif in a membrane proximal position led to a 2-fold increase in the rate of transferrin receptor internalization whereas addition in a membrane distal position did not show any increase (*165*). These data show that the rate of transferrin receptor internalization is not only dependent on the number but is also dependent on the position of the sorting motif. Allen and Schmid (2010) showed that transferrin receptor clustering on the plasma membrane can increase the rates of initiation of CCPs (*166*).

Transmembrane domains in membrane proteins have been shown to affect protein internalization through CME (figure 1.4). Casson et al. (2010) showed that for the same receptor, a shorter hydrophobic transmembrane domain causes efficient internalization while a longer hydrophobic transmembrane domain reduces internalization efficiency (*167*). This type of sorting may be due to hydrophobic mismatch between transmembrane domain length and plasma membrane thickness. Membrane proteins with smaller transmembrane domains are generally localized in the endoplasmic reticulum and Golgi apparatus whereas longer transmembrane domains cause membrane proteins to localize mainly to the plasma membrane (*168*). Due to a match between transmembrane domains of proteins and hydrophobic thickness of membrane bilayer, proteins that are retained in the plasma membrane tend to have a longer transmembrane

domain. Thus, this apparently passive mode of protein may in fact be due to efficient routing of membrane proteins with shorter transmembrane domains to the ER and Golgi membranes through CME. In addition to the above factors, insertion of polar residues in the transmembrane domain has also been reported to reduce internalization of the transferrin receptor (*169*)

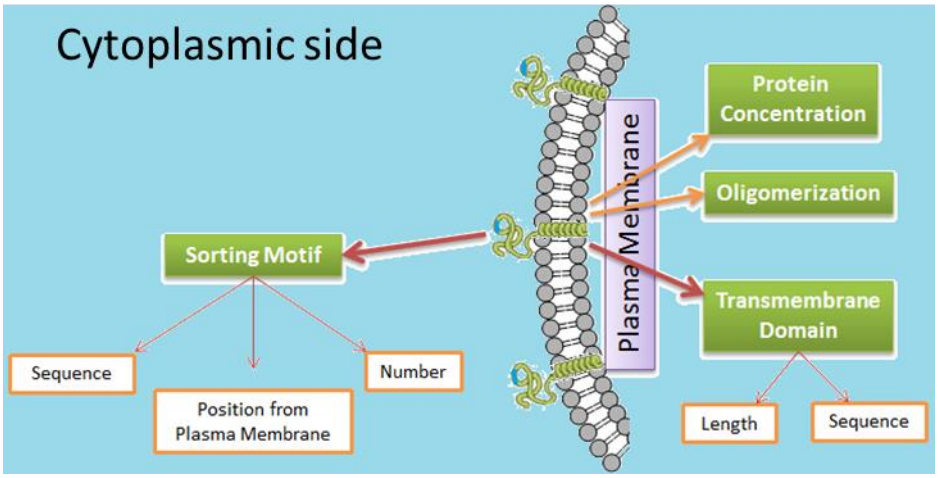

Figure 1.7: Factors affecting membrane protein internalization and sorting. Sorting signal binds to CLASP and then CLASP-cargo complex sorts into CCV. Cargo-CLASP complex formation is dependent on sequence, position from plasma membrane and number of sorting motifs. Cargo concentration and their oligomerization are also additional factors affecting cargo-CLASP complex formation. Transmembrane domain of cargo plays role in protein sorting during CME.

Overexpression of transferrin receptors leads to a saturation in its internalization rate but does not affect rates of LDLR or EGFR internalization (*170*, *171*). This would mean that different receptors are either internalized by the formation of specialized CCVs or that a common pathway that allows for a defined stoichiometry of adaptor proteins that recognize different receptors. Overexpression of proteins which contain tyrosine- or dileucine-based sorting motifs affect transferrin receptor internalization suggesting that the latter of the two possibilities is true since the both these sorting motifs are recognized by a common adaptor AP2.

However, AP2 knockdown in cells dramatically reduces transferrin receptor internalization with a concomitant reduction in the number of CCVs but LDLR does get internalized albeit with less efficiency suggesting that internalization of other receptors may still occur even upon drastic perturbations to CME (*68*, *69*). Interestingly, Dab2 acts as an adaptor for LDLR and has been

suggested to form coated pits independently of AP2 (*112*). Interestingly, Dab2 overexpression disrupts transferrin clustering as well as AP2 recruitment on plasma membrane (*111*).

Table 1.2: Different cargo molecules and their adaptor proteins. (Table is reproduced from (*65*))

Sorting Signal Type	**Corresponding Cargo**	**Recognition Protein or Domain of CLASPs**
YXXϕ	Transferrin receptor,	μ2 subunit of AP2
[DE]XXXL[LI]	CD4, LIMP2, CD3gamma	α and σ-subunit of AP2
II	K^+ channel Kir2,3	AP2
[FY]XNPX[YF]	LDL receptor, LRP1	PTB domain of ARH, DAB2 and
Phosphate group	GPCR	β -arrestin 1 and β- arrestin 2
Ubiquitin	EGFR	UIM of epsin and EPS 15
Acidic cluster	Furin, CD-M6PR	AP2 ?

1.5 Protein hubs in Clathrin-Mediated endocytosis: Dictionary meaning of Hub is the effective center of an activity, region, or network. There are multiple proteins involved in CME. These proteins interact with their partner component like lipid, cargo, CLASP, accessory proteins, clathrin (figure 1.8A). Proteins having multiple interactions in the clathrin-mediated endocytosis are called as hubs (figure 1.8B) (*84*). There are different proteins which act as hubs during different stages of CME. AP2 and clathrin are two important proteins which have maximum interactions with endocytic machinery. So these proteins are considered as major hub in CME.

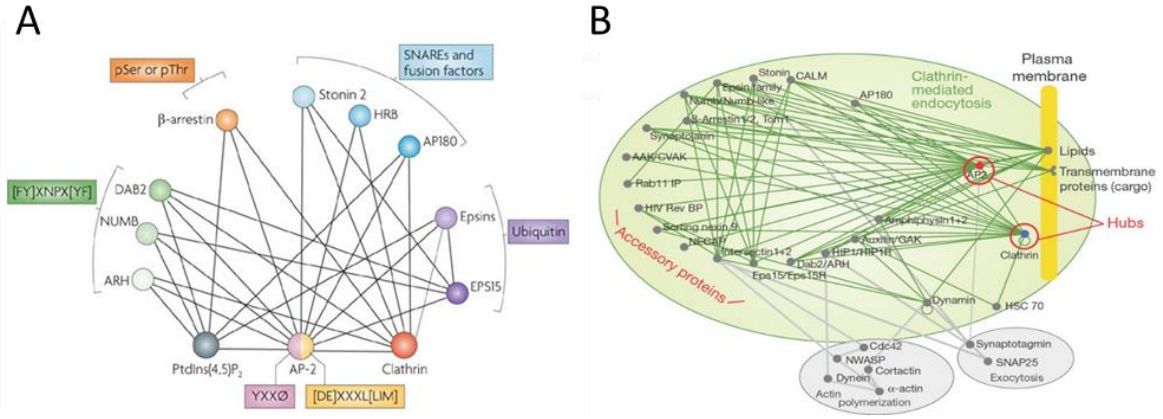

Figure 1.8: Interactomes of CME A) interactions of various CLASPs with their cargo molecules and Ptdins(4,5)P_2, AP2 and clathrin. B) Interactome of all major components in CME and their interactions with lipid cargo, AP2 and clathrin.

Table 1.3: Clathrin binding motifs and their binding sites on the clathrin terminal domain (Table is reproduced from (*172*))

Clathrin Box	Clathrin binding motif	Binding site at TD	Adaptor	Clathrin binding motif
TD site 1: clathrin box motif	LØXØ[DE]	TD groove between blades 1 and 2	Amphiphysin	LLDLD
			β-arrestin 1	LIEFE
			AP-2 β2 subunit	LLNLD
TD site 2: W-box motif	PWXXW	Top of β-propeller	Amphiphysin	PWDLW
			SNX9	PWSAW
TD site 3: β-arrestin 1L site	[LI][LI]GXL	TD groove between blades 4 and 5	β-arrestin 1L	LLGDL
			AP-2 β2 subunit	LLGDL
TD site 4	Unknown	TD exposed surface of blade 7	Unknown	Not available

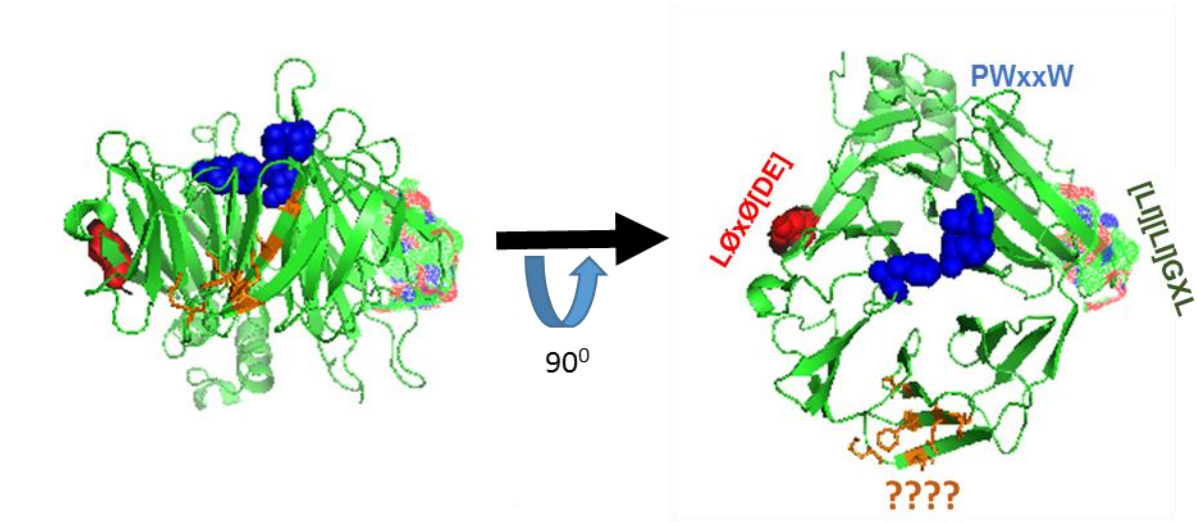

Figure 1.9: Crystal structure of clathrin N-terminal domain with four binding sites for clathrin binding motifs (CBM). LØXØ[DE] motif binds to clathrin site 1 (red), PWXXW motif binds to clathrin site 2 or W box (blue) , [LI][LI]GXL motif binds to clathrin site 3 (light green), the binding motif for clathrin site 4 is not known yet. Figure is modified from PBD file 1C9I.

1.6 Clathrin-mediated endocytosis and clathrin coat assembly:

CLASP molecules recruit clathrin on the membrane and forms planar or curved stable matrix of clathrin. This matrix stabilizes membrane curvature, and holds protein complexes underneath the assembled clathrin. This process starts with low binding affinity interactions which convert into high avidity interactions and finally formation of stable interconnected stable matrix of proteins (*84*). Clathrin is one of the important protein components in the CME which forms the clathrin coat and packs all machinery into its basket.

Clathrin heavy chain has N-terminal domain which has four binding sites for clathrin binding motifs. NTD has globular structure and classified under WD40 family of proteins. It has seven blades of beta sheets. The four binding sites are present in tetrahedral manner and result in accessibility of these sites for all clathrin binding motifs. There are four types of clathrin binding motifs present in various adaptor proteins (figure 1.9) (*172*, *173*). These clathrin binding motifs are specific for their sites and may have different binding affinities for clathrin terminal domain. Recently it has shown that LΦXΦ(DE)v and (LI)(LI)GXL motifs can bind to clathrin terminal domain in 3:1 stoichiometric ratio (*1*). This shows degeneracy of clathrin terminal domain binding to clathrin binding motifs. These interaction are weak and have binding affinity ~250uM (*1*).

Mutation in any three clathrin binding motif sites does not affect the clathrin mediated endocytosis in cells but mutation in all four binding sites stops CME (*173*). Clathrin is trimer and each triskelia consist of three clathrin heavy chains so there are three clathrin terminal domains present in each clathrin molecule. Also there are multiple clathrin binding motifs present in different CLASP molecules (table 3) (*174–176*). These multiple binding sites and motifs lead to increase in high avidity interactions between CLASP and clathrin terminal domain.

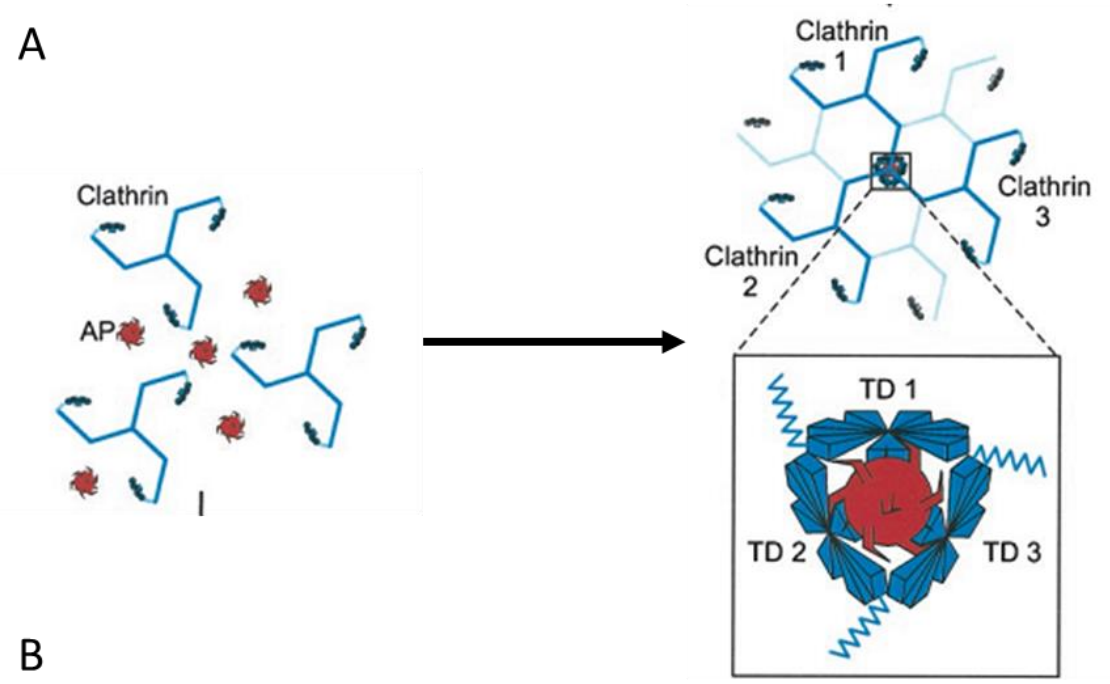

Figure 1.10: Proposed molecular mechanism for CLASP mediated clathrin assembly. A) Multiple clathrin terminal domain binding sites (shown as red spikes) are present in the adaptor protein. These binding motifs bind to sites present on the clathrin terminal domain and cross link the clathrin molecules. This cross linking leads to the formation of clathrin polymer in solution. Figure is reproduced from (*181*). B) Unstructured region of epsin or AP180 (CLASPs) have multiple binding sites for clathrin. These sites recruit clathrin and assemble it on the membrane. There may be different factors playing role in clathrin assembly. These are unstrucuered region of CLASPs C-terminal region, type and number of binding motifs in the CLASPs etc. Figure reproduced from (*91*).

Formation of clathrin-coated vesicle is an endergonic reaction and requires $500K_bT$ energy to bend membrane (*177*). This energy is provided by exergonic reactions of membrane protein and protein-protein interactions. Lifetime of CLASP on the membrane also plays role in the CCP formation energetics. Clathrin-CLASP and clathrin-clathrin interactions are the crucial interactions which releases energy during CCP formation. This energy could be used in the CCP formation process.

There are multiple CLASP molecules interact with their specific cargo molecules and cluster them in the clathrin-coated pit (figure 1.8A). The CLASP-cargo, CLASP-AP2 and CLASP-clathrin interactions are important factors deciding the efficiency/extent and kinetics of cargo clustering/sorting in the clathrin-coated pit. Clathrin-mediated endocytosis is well studied process but the exact role of these interactions in the CCP formation is not clear yet.

1.7 Clathrin planar lattice Vs clathrin coated pits

Clathrin can assemble and forms hexagonal or pentagonal or heptagonal lattice (*11*, *61*, *178*). Clathrin lattice with hexagons is planar but incorporating pentagons in the hexagonal lattice can results in the curved coated structure like soccer boll (figure 1.4). This structure is called as clathrin coated pit (CCP) (*11*, *61*, *178*). This hexagons to pentagons ratio decide the size of clathrin-coated pit. Clathrin triskelia has pucker structure and may affect the curvature of clathrin assembly. Clathrin light chain also plays role in clathrin assembly (*179*). It has been suggested that clathrin light chain affects the stiffness of the clathrin triskelia which further affects the curvature sensitivity of the clathrin on the surface (*180*). CLASPs recruit and assemble clathrin but which factors exactly determine the hexagons to pentagons ratio and curvature of the clathrin assembly is not yet clear. AP180/ CALM knockdown affect the size and shape of CCV and suggested their

role in maintaining size and shape of CCP in cells (*106*). Exact mechanism of their role in clathrin assembly is not yet clear. CCP formation is not only dependent on clathrin recruitment but also dependent on other factors. There are multiple factors which decide clathrin assembly reaction. These may be the numbers and types of binding motifs, distance between clathrin binding motifs present in the C-terminal region of CLASP molecules. Flexibility or structure of the C-terminal region of CLASP molecule may play important role in the formation of clathrin-coated structures (*91*). There are different models proposed for clathrin assembly. "Fishing line of baited hooks" (clathrin cross linking) model suggests role of CLASP molecules as a cross linker between clathrin molecules which lead to clathrin assembly (figure 1.10A) (*181*). There is difference between clathrin assembly in solution and its assembly on membrane surface. Clathrin assembly may be efficient if clathrin binding motifs are away from the membrane and located in highly unstructured region of CLASP molecule.

1.8 Assays for clathrin assembly reaction:

1.8.1 Semisynthetic reconstitution of clathrin-coated pits: Clathrin can assemble in the presence of adaptor proteins at physiological pH. Its terminal domain has binding site for various adaptor proteins. Clathrin can assembled on the striped clathrin coated vesicles (*15*). Anderson et al. had used plasma membrane and added cytosol or clathrin-coated vesicle extract and observed clathrin assembly as clathrin-coated pits on the plasma membrane (figure 1.11) (*182*, *183*). Plasma membrane washed with high salt buffer was not able to assemble clathrin-coated pits, this may be due to removal of peripheral membrane proteins required for clathrin assembly. Wu et al had used unroofed cells and added cytosol to test transferrin receptor clustering by using fluorescence microscopy (*184*). Recently new technique was developed to study clathrin coated structures on the unroofed cells by using electron microscopy and fluorescence microscopy (*185*). This technique gives high special resolution and it is possible to get high special resolution of different adaptor proteins like epsin in the clathrin-coated pits. These systems gives high special information

but involvement of large number of molecules in CME process it is difficult to know the precise role of individual molecules.

1.8.2 In solution clathrin assembly reaction: Clathrin is self-polymerizing protein. It can assemble at low pH and forms empty coat in solution. Pierse et al had used sedimentation assay

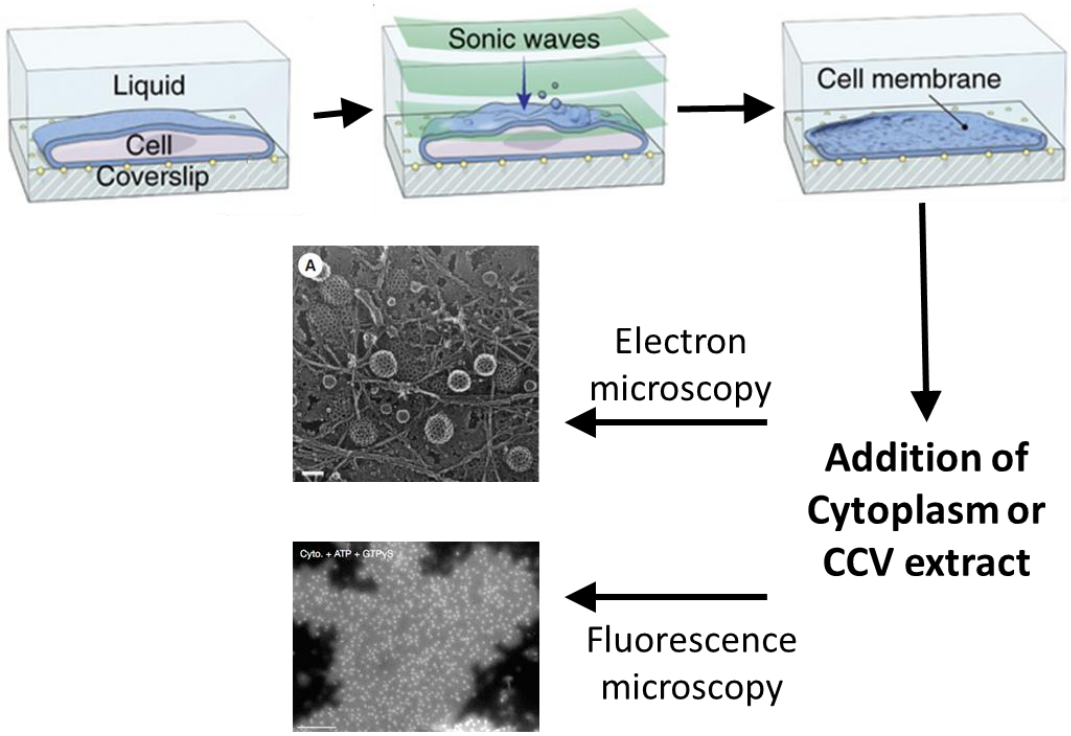

Figure 1.11: Clathrin assembly on unroofed cells. Adhered cells were sonicated and removed layer of dorsal plasma membrane. These adherent membrane was called as unroofed cells. Unroofed cells were used for reconstitution by using cytosol or clathrin-coated vesicle extract and observed under electron microscope (*182*, *183*) (*185*) or fluorescence microscope (*184*). Image is modified from (*185*) (*184*).

where they spun down clathrin solution at low pH and pelleted cages at high g speed (*11*). In 1983 zaremba and keen shown the clathrin assembly in presence of AP proteins in solution at low and physiological pH (*61*, *186*). Morgon et al shown clathrin assembly by using purified CLASP fragment in solution. This study shown the importance of multiple clathrin binding sites in clathrin coat formation process (*181*). When clathrin is incubated at physiological pH, it can self-assemble in solution. This self-assembly is dependent on the concentration of clathrin in solution. The concentration above which clathrin forms coats is called critical concentration for clathrin assembly (*63*). Clathrin incubation with AP proteins at physiological pH leads to formation of empty cages. These cages can be centrifuge and sediment at high g spin or can be separated based

on their sedimentation quotient in isocratic gradient (*186*). These sedimented cages were observed under electron microscope or also ran on SDS PAGE electrophoresis (figure 1.12). This assay was used to study clathrin assembly in solution and factors affecting clathrin coat formation in solution, but clathrin-coated pit forms on the platform of planar plasma membrane. Limitations of this assay are: absence of planar bilayer, difficulty in studying real time clathrin assembly reaction.

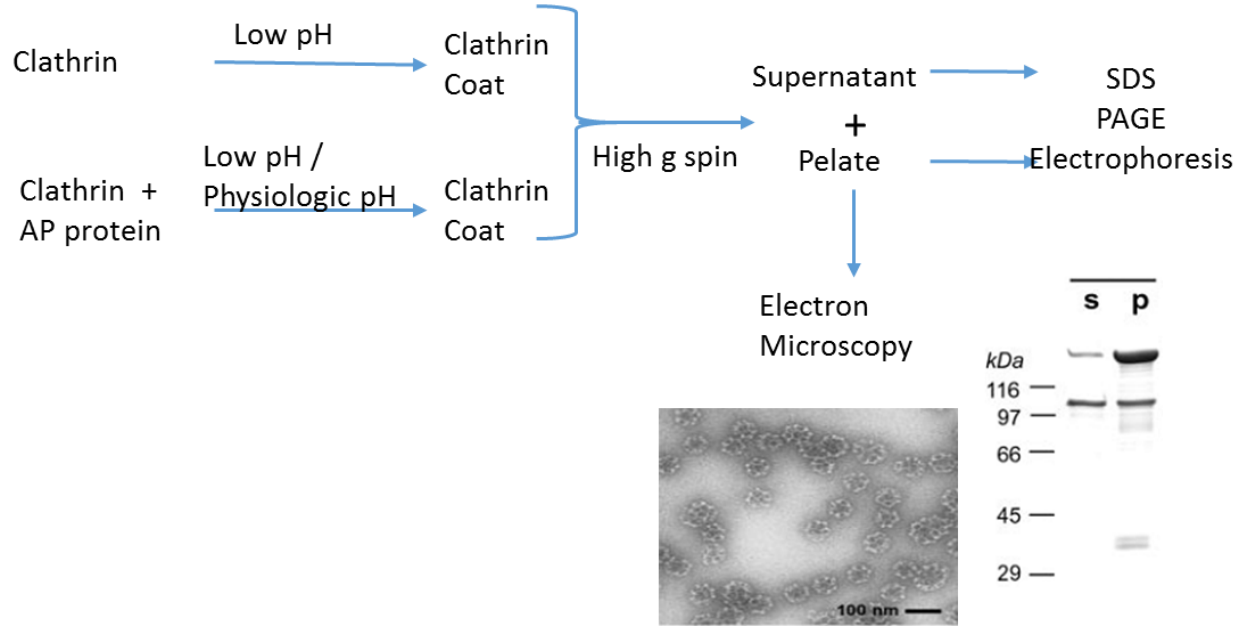

Figure 1.12: In solution clathrin assembly. Purified clathrin incubated without or with adaptor proteins at low or physiological pH respectively. Empty clathrin coats were formed in solution which were sedimented at high g spin. Supernatant and pellet were tested on SDS page (*181*) and pelleted clathrin coats were observed under electron microscope (*61*, *186*).

1.8.3 Clathrin assembly on lipid monolayer: In 2002 ford et al. invented new assay system where they used specialized chamber and made lipid monolayer (figure 1.13) (*19*, *20*). This monolayer was incubated with purified CLASP and clathrin. After incubation, monolayer with clathrin assembly was transferred to the EM grid and imaged under electron microscope. This assay shown the formation of clathrin coat on lipid monolayer by AP180, AP2, Epsin. (*19*, *20*) Epsin is shown to be membrane curvature generator and can form clathrin-coated pit like structures. This assay system allowed to study clathrin assembly on planar lipid monolayer but is not good mimic of lipid bilayer. To form clathrin-coated pit, the membrane needs to be bend which is an endergonic reaction. So the energy required to bend lipid monolayer is not the same as energy required to bend

lipid bilayer. This assay is a tedious and it is not possible to study real time clathrin assembly. These are the major limitations of given assay system.

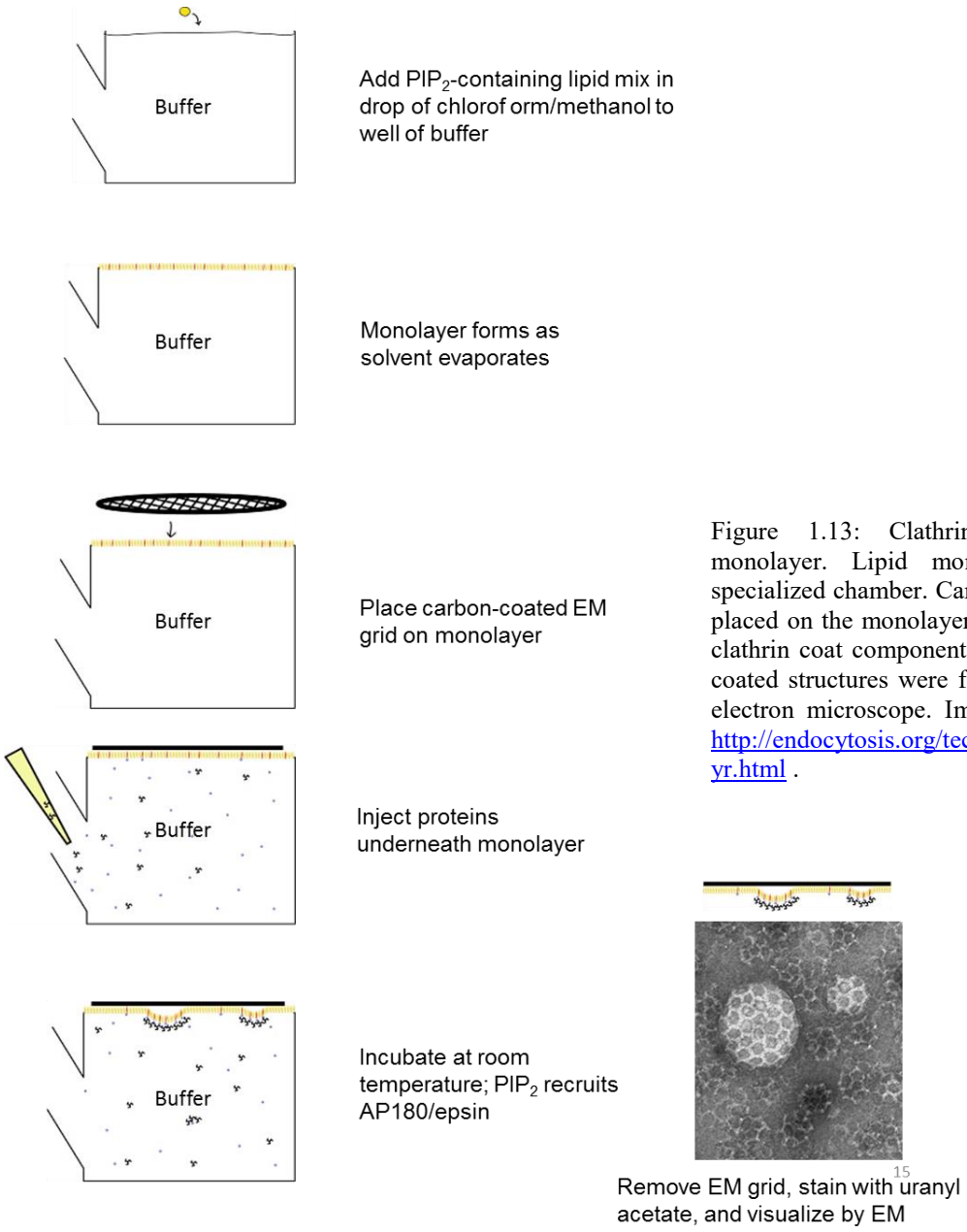

Figure 1.13: Clathrin assembly on lipid monolayer. Lipid monolayer was made in specialized chamber. Carbon coated EM grid was placed on the monolayer followed by addition of clathrin coat components in the buffer. Clathrin-coated structures were fixed and observed under electron microscope. Image is reproduced from http://endocytosis.org/techniqs/monolayr/monolayr.html .

1.8.4 Clathrin assembly on lipid bilayer: clathrin coated machinery incubated with giant unilamellar vesicles (GUVs) can form clathrin-coated pits on GUV surface (figure 1.14). In 1998 takei et al. used cytosol and CCV extract to reconstitute coat structures on protein free GUVs (*187*). (Dannhauser and Ungewickell had used minimal machinery to form clathrin-coated

structure on GUV surface (*21*). They recruited his tagged AP180/ epsin on GUV surface by using DGS- DGS-Ni^{2+}NTA lipids and added clathrin. This reaction then observed under electron microscope and seen clathrin-coated structures. Recently M. saleem et al had shown the clathrin coated structures on GUVs by using florescence microscopy and shown the role of membrane tension in clathrin-coated structure formation on membrane surface (*22*). Clathrin assembly is efficient on the curved surface and affected by presence of clathrin light chain (*180*). So the clathrin assembly reaction on membrane is complex reaction and can be interfered with different parameters like membrane curvature, membrane elasticity, type of CLASP molecule used, concentration of clathrin used in the assay. Assay systems used until now were nice but are dependent on end point analysis.

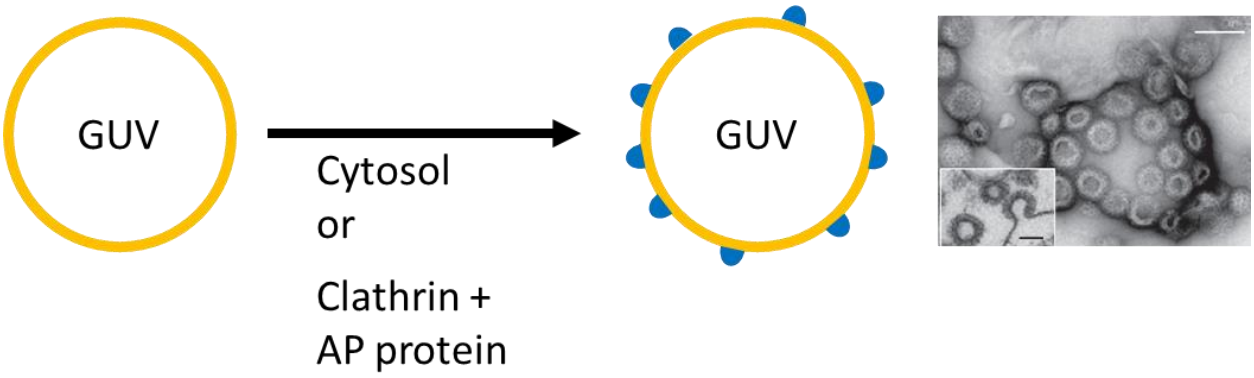

Figure 1.14: Schematic of clathrin assembly on lipid bilayer. GUVs were made and incubated with cytosol or purified adaptor proteins with clathrin. Clathrin-coated pits were formed on GUV surface and observed under electron microscope (*21*) or fluorescence microscope (*22*).

1.9 Motivation of the Dissertation:

Clathrin-mediated endocytosis consists of multiple sub processes (*150*). These stages are driven by protein-protein and protein membrane interactions. Proteins-protein interactions are weak and have low affinity (*84*). Membrane and protein interactions are dependent on the presence of cargo and lipids as well as the presence of specific interacting proteins, and membrane curvature. These factors decide the timing of specific protein recruitment and its recruitment kinetics at the CCP. The cage forming protein clathrin stabilizes protein-protein complexes and membrane curvature in the CCP. Cage components have the property to interact with different CLASPs, AP2 and accessory factors (*65*). This cage forming protein get polymerized and forms a clathrin coat. The lifetime of clathrin coat assembly, its size and the the composition of clathrin-

coated vesicles vary widely in cells. Due to complexity of CME, it is difficult to understand the role of individual molecules and factors affecting on these parameters in cells. Cell-free reconstitution is an attractive option to study CCP formation since it is possible to uncover the roles of the individual components in this complex process. Current assay systems mostly dependent on end point observations (as described above) and are lacking in insights into the dynamics of the CCP formation.

Clathrin is a self-polymerizing protein, which can bind and cluster the adaptor proteins. It forms planar clathrin lattices as well as curved-coated structures. What factors decide if clathrin adopts either of these structures is not yet clear? There are multiple CLASP molecules involved in CME and all these CLASPs have clathrin-binding motifs. The numbers and types of these clathrin binding motifs vary in CLASPs. This variation across CLASPs may affect the initiation, efficiency and the kinetics of clathrin assembly on membrane. To address this question we have used different CLASP molecules and studied their property in clathrin assembly reaction. Clathrin has multiple interaction sites for different CLASP molecules and acts as hub in CME. These CLASP molecules may get cluster or sort in clathrin-coated structures during clathrin assembly reaction on model membrane system.

Chapter 2
Experimental Methods

2.1 Experimental Methods for Chapter 3

2.1.1 Protein expression and purification: Rat epsin1 and GST-auxilin547-910 were kind gifts from Ernst Ungewickell. ENTH domain (2-156), epsin1 (L6W) and clathrin-binding site mutants (CBS1: 257LMDLAD to 257AAAAA and CBS2: 480LVDLD to 480 AAAAA) were generated by site-directed mutagenesis. Bovine HSC70 (Hsc70.RSET.FL.wt(NarI)) was a gift from David McKay (Addgene plasmid # 12532, (*188*)). Except GST-auxilin547-910, all bacterial expressed constructs had 6xHis tag at N-terminus and strep tag at C terminus. In brief, proteins were expressed in BL21 (DE3) in auto-induction media (Formedium, UK) at 18^{o}C for 30hrs and culture spun down at 3000g for 10 min at 4 ^{o}C. Bacterial pellet was suspended in HBS buffer (20mM HEPES pH 7.4 +150mM NaCl) supplemented with protease inhibitor cocktail (Roche) and then sonicated to lyse cells. Cell lysate was spun down at 20,000g for 20 min at 4^{o}C. Supernatant containing epsin was incubated with Co-talon resin (Thermo Scientific) for 1hr at 4^{o}C. Flow through was removed and beads were washed in PD10 (Biored) column with 50 mL of HBS. Protein was eluted in Buffer A (20mM HEPES, 150mM NaCl, 300 mM Imidazole). Fractions ware pooled and passed through StrepTrap HP column (GE Lifesciences). Column was washed with 10CV of HBS buffer and protein eluted by 2.5mM d-Desthiobiotin (sigma D1411) in HBS buffer. GST-auxilin(547-910) was purified using glutathione sepharose beads (GE Lifesciences). Protein fractions were dialyzed overnight against HKS (20mM HEPES pH 7.4 +150mM KCl) buffer. Final 10% glycerol was added in dialyzed protein solution. Small aliquots of purified proteins were stored in -80 ^{o}C.

2.1.2 Clathrin-coated vesicle preparation: CCVs were isolate by using protocol described in (*189*). In brief, goat brain (110 g approx.) was cleaned in cold PBS to remove meninges and blood vessels. The tissue was chopped and mixed with an equal volume of 1X MES buffer (100 mM MES, 1mM EGTA, 0.5mM $MgCl_2$, pH 6.8). This mixture was homogenized using a waring

blender. Homogenate was centrifuged at 17,700 g for 30 min at 4^0C. The supernatant containing coated vesicles was pelleted at 70,000g for 60 min at 4^0C. The pellet was dispersed in 1XMES buffer by using a dounce homogenizer and transferred to gradual cylinder. Equal volume of 12.5% w/v ficoll-400 (sigma) and 12.5%w/v sucrose solution was added and mixed thoroughly by slowly inverting cylinder. Mixture was spun down at 41,400 g for 40 min at 4^0C. The supernatant containing CCVs was transferred into graduated cylinder and diluted 5 folds with 1X MES buffer + 0.1mM PMSF. This diluted CCV solution was then spun down at 85195 g for 60 min at 4^0C to pellet down CCVs.

2.1.3 Clathrin purification: Small volume of disassembly buffer (10mM Tris-Cl pH 8.0+1mM DTT+ 1mM PMSF) was added to CCV pellet and dispersed pellet by daunce homogenizer (around 20 to 30 strokes). This homogenate was incubated at RT for 2-3 hrs and spun down at 100,000 g for 1 hr at RT. Supernatant containing clathrin-coated vesicle extract was dialyzed overnight against Buffer A (25mM Tris-Cl, 75mM NaCl). Next day, homogenate was spun down at 100000g for 1 hr and supernatant passed through anion exchange Q sepharose column (GE Lifesciences). Column was washed with 10 CV of buffer A and protein eluted by gradient of buffer B (25mM Tris-Cl+1M NaCl). Fractions containing clathrin were pooled. Final 30% ammonium sulfate was added in pooled fractions (for clathrin salting out) and incubated for 1-2 hrs at 4^0C. Clathrin suspension was spun down at 20000g for 30 min at 4^oC. Pellet was re-suspended in 10mM tris-Cl buffer with 10%glycerol. Flash frozen the samples and stored at -80^0C.

2.1.4 Chemical labeling of proteins:

Purified epsin and clathrin was labeled with 10-fold molar excess of thiol-reactive Alexa488 and Texas Red C2 maleimide dyes (Invitrogen), respectively for 1 hr. at RT. Reaction was quenched with DTT. Free excess dye was removed from labeled epsin by using SM2 Bio beads (Biorad) at RT and from labeled clathrin by assembling it into empty coats. Labeled clathrin was dialyzed against assembly buffer (100mM MES pH 6.4+5mM MgCl2+0.5mM EGTA) for overnight at 4^0C to form empty coats. These coats were pelleted at 100,000g for 1 hr. at RT. Pellet was re-suspended in 10 mM Tris-Cl pH8.0 buffer and dialyzed against 10mM tris-Cl pH 8.0 buffer. Free dye was checked on 10% SDS-PAGE electrophoresis.

2.1.5 PEGylation of glass coverslips : Glass coverslips were passivated by using protocol described in (*190*). In brief, glass coverslips were treated with 3M NaOH for 5 min and then

extensively rinsed with water. Coverslips were treated with piranha solution (60% of conc. H2SO4 + 40% of 30% H2O2) for 1 hr. at RT. After this treatment, these coverslips were rinsed with water and dried on the heating block set at 90 °C. These dried coverslips were transferred to a clean petri dish and few drops (just enough to cover all the coverslips) of 3-glycidyloxypropyltrimethoxysilane (Sigma) were poured with a glass pasteur pipette. This reaction was kept in vacuum for 5 hrs with intermittent swirling. After silanization, coverslips were rinsed with acetone (Sigma) and dried. These dried coverslips were added in the molten PEG 8000 and kept for 48 hrs at 90°C. Excess molten PEG from the beaker was removed and washed with hot water. Coverslips were stored in dry condition.

2.1.6 Preparation of Supported membrane tubes (SMrT templates): Lipid mix was made in chloroform with 0.5 mol% of the fluorescent DiD (Invitrogen) lipid probe and stored at -80 °C. PEG 8000-treated coverslip was cleaned with 1% SDS, water, methanol and then MQ water then Wiped with a tissue paper. 1-5 nmol lipid mix was spread on the coverslip and dried under high vacuum for 5 min. A ~35 µl flow cell (Bioptechs) was assembled with 0.1 mm silicon spacer between PEGylated coverslip and ITO coated slide. 1% w/v BSA (Sigma) solution in HKS buffer was flown in the chamber and incubated for 10 min to form large vesicles. Excess HKS buffer was flown in the chamber which extruded vesicles. These vesicles were converted into supported membrane tubes (SMrT).

2.1.7 Clathrin assembly reactions on SMrT templates: SMrT templates were made in HKS buffer as described above. Lipid mix used for SMrT templates preparation were PC:PS:DOPIP$_2$:DiD (79:15:5:1 mol %) or PC:PS:NiNIT DiD (79:15:5:1mol %). SMrT templates were equilibrated in filtered and degassed assay buffer [HKS, 1% BSA + oxygen scavenger cocktail of 0.2 mg/ml glucose oxidase (Sigma, G-2133), 0.035 mg/ml catalase (Sigma, C-40), 4.5 mg/ml glucose]. 200 µl of 200nM Alexa 488 labeled epsin was passed on SMrT templates and incubated for 10 minutes. Unbound epsin was washed with 600 µl of assay buffer. Texas Red labeled Clathrin, freshly diluted in assay buffer or buffer with 1 mM Mg2+ containing HSC70 (1 µM), GST-auxilin547-910 (1 µM) and ATP (1 mM). All reactions were carried out at 25 °C.

2.1.8 Field emission scanning electron microscopy (FESEM): Clathrin or streptavidin coated SMrT templates were fixed by using 3% w/v glutaraldehyde (200uL) for 10 min and washed with PBS. Membrane was solubilized with 1% SDS solution and washed with excess MQ water. Fixed protein sample was dehydrated by sequentially passing 10%, 20%, 40%, 60%, 80% and 100% ethanol. There were no gross changes in protein distribution on membrane tubes which was confirmed by fluorescence imaging. Chamber was disassembled carefully. Coverslip was taken out and dried in vacuum desiccator for overnight. Next day, sample was gold coated by using a Q150T Turbo-Pumped Sputter Coater (Quorum Technologies) and imaged on an Ultra plus Field Emission Scanning Electron Microscope (Zeiss) using a 1.9 kV electron beam and secondary electron detector. Images were analyzed by using Fiji.

2.1.9 Liposome Preparation: Specific lipid mixture was aliquoted in glass tube and dried under stream of N_2. Residual chloroform was evaporated in centri-VAP at 50^0C for 1 hr. Final 1mM lipid mixture was made by MilliQ and hydrated at 50^0C for 1hr. Hydrated lipid mixture was sonicated for 10 min at 30% amplitude (2 sec on 3 sec off pulse). Sonicated small uni-lamellar vesicles were spun down at 100000g for 20 min at RT. Supernatant SUVs were stored at 4^{o}C for a month.

2.1.10 Membrane binding assays: Small volume chambers were prepared by attaching clipped eppendorf heads to PEGylated coverslips by using a silicone adhesive (Dow Corning). SUPER templates were prepared as described earlier (22) by using liposomes, and were added to solutions containing increasing concentrations of fluorescently-labeled proteins, incubated for 10 min and imaged.

2.1.11 Fluorescence microscopy: Epi-fluorescence imaging was carried out on an Olympus IX71 inverted microscope. All images were acquired through ×100 oil (NA1.4) objective. A LED light source (Thor Labs) was used for fluorescence imaging. Single-band pass filters (Semrock) were used with excitation/emission wavelength band pass of 482 ± 35 nm/536 ± 40 nm for Alexa488, 562 ± 40 nm/624 ± 40 nm for Texas Red and 628 ± 40 nm/692 ± 40 nm for DiD probes. Images were acquired by using Evolve 512 EMCCD camera (Photometrics) with exposure time 100msec, Gain 1X, multiplication Gain 100. Metamorph version 7.7.9.0 (Molecular Devices) was used to control image acquisition.

2.1.12 Image analysis: All images, time-lapse movies were analyzed by Fiji (*191*) and nonlinear regression analyzed by Graphpad Prism. Dead time of the flow cell was estimated in situ for each

experiment by calculating the onset of fluorescent clathrin into the microscope field using a plateau followed by one-phase exponential rise function. Frames before the calculated onset were removed from time-lapse sequences. Background-corrected kymographs were generated from lines placed across the entire length of the membrane tube. Pixel intensity versus time data for all pixels on a kymograph were exported and fitted to a plateau followed by one-phase exponential rise function. Mean time constants were plotted from fits with an $R2 \geq 0.8$, which sorted-out artifacts caused by microscope focus drifts and/or flow-induced lateral movement of foci. Contiguous events were sorted out based on considering the fit with the smallest onset, which reduced oversampling of data caused by clathrin assembly extending across multiple pixels.

2.2 Experimental Methods for Chapter 4:

2.2.1 Protein expression and purification:

Rat epsin1 (uniprot ID: O88339), Mouse Dab2 (uniprot ID: P98078), Human ARH (uniprot ID: Q5SW96), Mouse AP180 (uniprot ID: Q61548), human β2-arrestin (uniprot ID: P32121), human NUMB (uniprot ID: P49757) and human AP2 β subunit (584-951) (uniprot ID: P63010) were cloned in HTS vector and purified described in section 1.

2.2.2 Image analysis: All images, time-lapse movies were analyzed by Fiji (*191*) and nonlinear regression analyzed by Graphpad Prism. Kymographs were generated as described in chapter 1. Kinetics of each clathrin assembly from kymograph was analyzed by using imageJ plugin and macro (Intensityextractor) as described in (figure 1). Pointfromkymograph plugin (*192*) was used to extract (x,y) co-ordinates of maximum intensity pixels in each trajectory and saved in a folder. Macro (Intensityextractor) was used to extract intensity values from kymograph by using saved (x,y) co-ordinates. The intensity values were fitted in plateau followed by one phase association equation and extracted onset time and time constant for clathrin assembly.

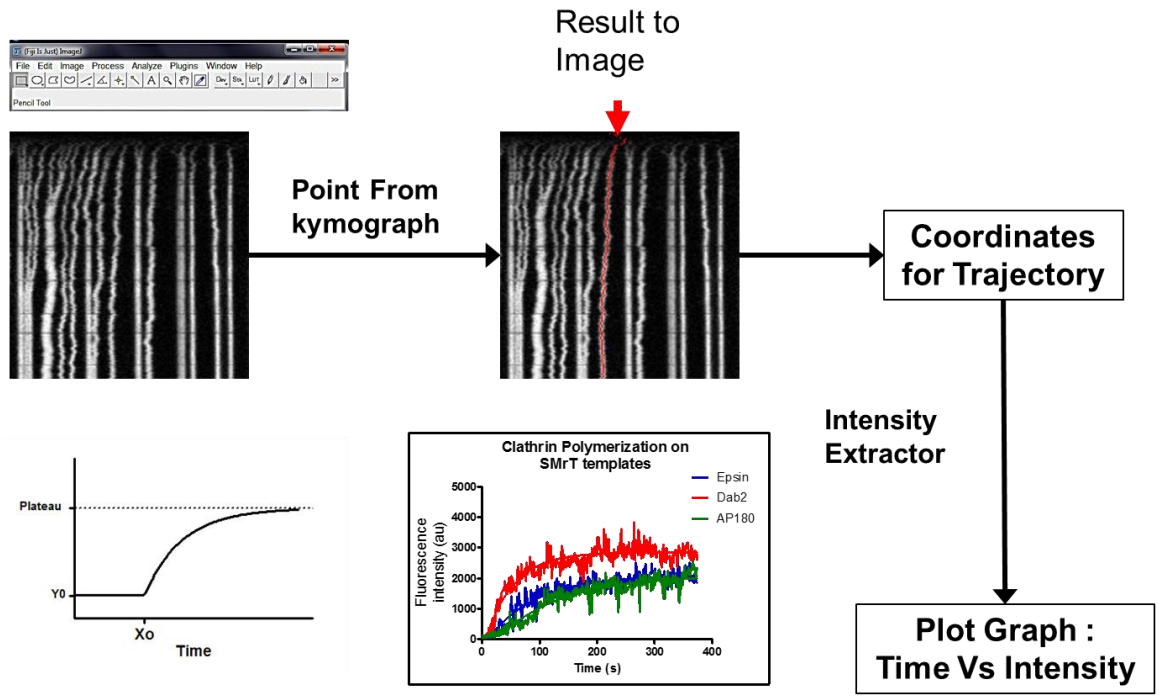

Figure 2.1: kymograph processing to measure the rate of single clathrin-coated puncta. Pointfromkymograph plugin (*192*) was used to extract coordinates of maximum intensity pixels in each trajectory. Macro Intensityextractor extract the intensity values from the kymograph. These values were plotted and analyzed in Graphpad prism.

Code for Intensity extractor Macro

Choose the file to Open : You have to choose x,y coordinates file which is generated by pointfromkymograph plugin.

Result of Reslice of Stream1.tif : Choose the kymograph window which need to analyze.

Macro.ijm

```
//Intensity Extractor
//This macro is usefull to get Intensity profile
//from given coordinates
pathfile=File.openDialog("Choose the file to Open:");
filestring=File.openAsString(pathfile);
rows=split(filestring, "\n");
x=newArray(rows.length);
y=newArray(rows.length);
for(i=0; i<rows.length; i++){
```

```
columns=split(rows[i],"\t");
x[i]=parseInt(columns[1]);
y[i]=parseInt(columns[0]);
selectWindow("Result of Reslice of Stream1.tif");
run("Point Tool...", "mark=0 auto-measure add label selection=yellow");
makePoint(x[i], y[i]);
roiManager("Add");}
run("Select All");
run("Set Measurements...", "mean redirect=None decimal=1");
roiManager("Measure");
String.copyResults();
waitForUser;
roiManager("Delete");
run("Close");
```

Chapter 3
Developing technology to study epsin-mediated clathrin-coat assembly

Summery:

Epsin is indispensable molecule for clathrin-mediated endocytosis (CME). It can sense membrane curvature as well as assemble clathrin but its precise function in not yet clear in CME. Clathrin is self-assembly protein which forms coat on membrane mediated by epsin or AP180. We have developed a novel assay system which allow us to study epsin's curvature sensitivity and its role in clathrin assembly. Budded supported membrane tubes (SMrT) display planar and highly curved membrane surface. Epsin preferentially binds to highly curved membrane tubes and assemble clathrin in the presence of phosphatidylinositol-4, 5-bisphosphate. This shows the role of epsin's curvature sensitivity in clathrin assembly. Clathrin foci grow in intensity with a typical time constant of ~75 s, similar to the time scales for coated pit formation seen in cells.

3.1 Background:

Clathrin-mediated endocytosis (CME) internalizes various cargo molecules in cells. Epsin is one of the important molecules in CME. It plays role in membrane remodeling (*20*, *88*) as well as internalization of ubiquitinated cargo (*93*, *94*). It also has binding motifs for important endocytic proteins like AP2, clathrin, EPS15 (*92*) (*95*) (*91*). Its depletion by RNAi or antibody injection or knockout results in the arrested clathrin coated pits in cells (*89*, *90*) and affect transferrin internalization (*88*). It acts as checkpoint protein during CME (*85*).

Epsin has N-terminal ENTH domain and C-terminal unstructured region (*91*). Amphipathic Ho helix of ENTH domain inserts in the membrane and makes protein membrane curvature sensor or generator (*86*, *87*) (*20*, *88*) (*193*). In partial reconstitution of clathrin-coated structures on unroofed cells, epsin localizes with clathrin on the membrane tubes formed by cytosol (*90*). Epsin and other proteins (EPS15 and FCHO) get excluded from clathrin coated vesicle and found to be at the rim of the CCP. It has been proposed that these proteins effectively bend membrane and convert half spherical clathrin coated pit to fully curved spherical vesicle (*33*).

Epsin can polymerize clathrin on lipid monolayer (*20*) or on GUV bilayer (*21*, *22*) and forms clathrin coated pits. These *in vitro* biochemical studies were dependent on the end point analysis which lack the information about dynamics of the clathrin-coated pit formation. To study clathrin polymerization kinetics by using fluorescence microscopy, we had used GUVs as model membrane systems. CCPs were moving on the GUV surface which made it difficult to study real time CCP formation. Also soluble epsin and clathrin were polymerized in solution which decreased the effective concentration of soluble clathrin in solution. We used SMrT template as a new model membrane system in the flow chamber where we could flow in and out proteins. This system is a good mimic of dimple structure of clathrin coated pit (It has curved membrane tube). Protein-protein complexes remain in the focus on membrane surface, which makes it easy for real time imaging of clathrin assembly. In this study, we tested role of epsin 1 (hereafter referred to as epsin) in clathrin assembly and assembly dependence on the membrane curvature by using novel model membrane system.

3.2 Results:

3.2.1 Membrane binding affinity of epsin and membrane tubulation:

Epsin has unstructured C-terminal region which is prone to degradation. This makes it difficult to purify recombinant protein from bacteria. We have used tandem affinity purification method to purify full length epsin. His tag followed by strep tag purification removed all partially degraded epsin molecules and gave highly purified epsin (figure 3.1A, B). ENTH domain of epsin has binding specificity and high affinity for PIP_2 (*20*, *97*, *194*, *195*). We have used SUPER template binding assay for measuring affinity between protein and lipid. This fluorescence microscopy based assay requires purified fluorescently labeled protein, SUPER templates as a model membrane system (*196*) and the passivated glass chamber to avoid protein sticking to the glass surface. We used this assay system to measure affinity between ENTH domain and PIP_2 which was 37.0±7.6 nM. This affinity was similar with its reported binding affinity for PIP_2 (*20*, *97*, *194*, *195*), but full length epsin had 10 fold higher affinity for PIP_2 which was 5.1±0.6 nM (figure 3.1 C, D).

ENTH domain of epsin is membrane active and able to bend membrane. SUPER template has excess reservoir and has been used earlier to study membrane tubulation property of dynamin and BAR domain containing proteins (*197*, *198*). 200 nM of full length epsin was able to tubulate

SUPER templates (figure 3.1E) as shown earlier (*20*, *97*, *194*, *195*),but was not showing any sign of membrane fission.

3.2.2 Membrane curvature sensitivity of Epsin:

Binding of ENTH domain to the membrane is curvature sensitive and has high binding preference for curved membrane compared to planar membrane. To test curvature sensitivity of full length epsin, we have developed budded supported membrane tubes (budded SMrT) as a new model membrane system. Budded SMrT templates were made by flowing buffer in the chamber containing dried lipid which resulted in the supported membrane tubes (SMrT). These tubes were exposed to high osmotic shock to get budded SMrT templates as described in experimental section (see schematic, figure 3.2A, B). This system has highly curved membrane tubes with low curved membrane buds in the same chamber. The diameter of these tubes and buds were measured by using scanning electron microscopy (FESEM). Membrane tubes and bulges composed of DOPC:DOPS:Biotin Cap PE (84:15:5 mol%) were bound to streptavidin followed by fixing as described in experimental section. Membrane tubes and buds were indicating diameters of 63.3 ± 29.2 nm (mean ± SD, N = 180 profiles on 17 tubes) and 425.0 ± 106.0 nm (mean ± SD, N = 34 buds) respectively (figure 3.2C).

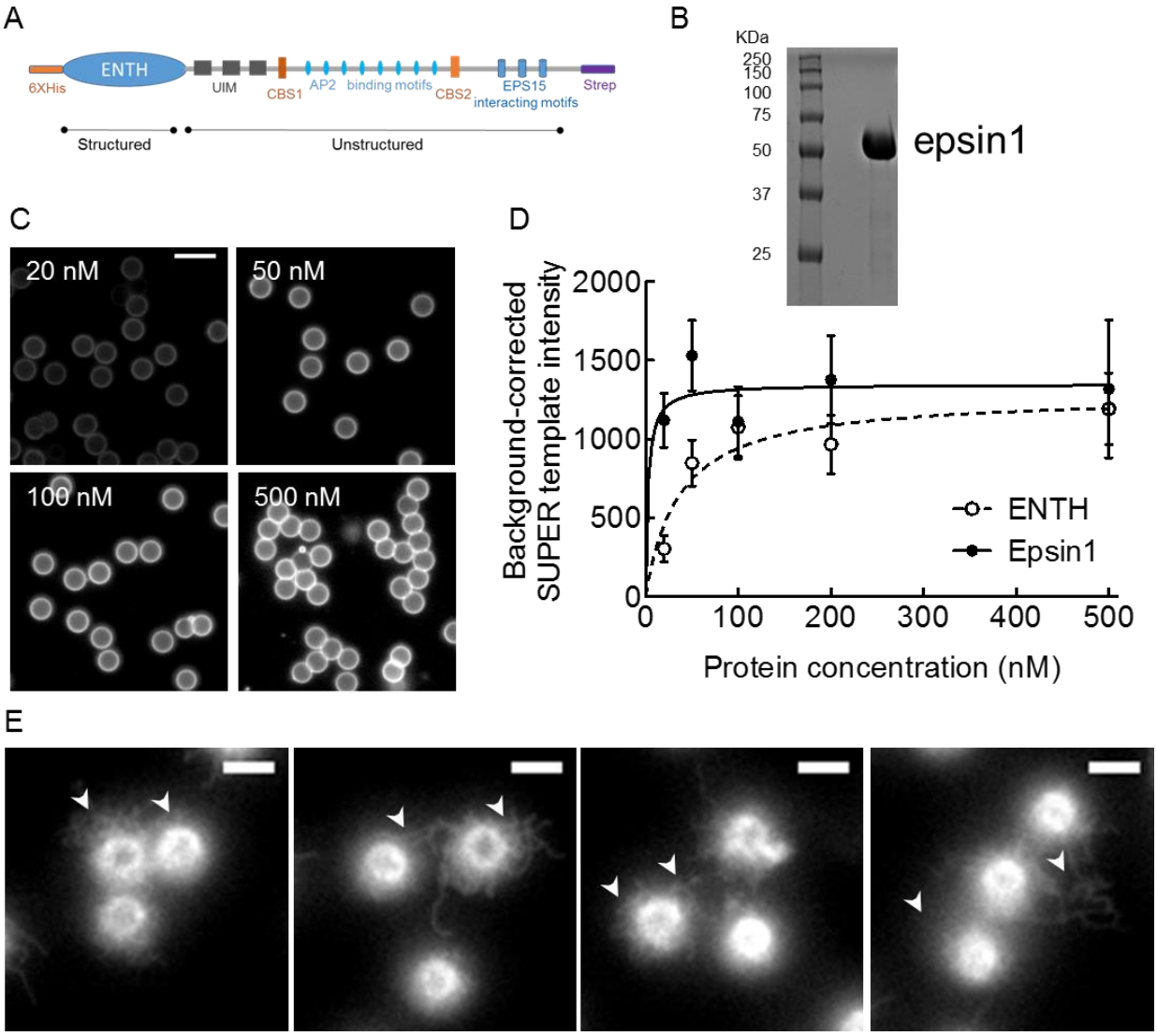

Figure 3.1. Membrane binding and tubulation by epsin. (A) Domain organization of epsin1. (B) SDS-PAGE of purified epsin1 protein used in the study. (C) Fluorescence micrographs of SUPER templates incubated with increasing concentrations of Alexa488 maleimide labeled epsin1. Scale bar = 10 µm. (D) Background-corrected fluorescence associated with SUPER templates of Alexa488 maleimide-labeled epsin1 (closed circles) and ENTH domain (open circles) fitted to a one-site binding equation. (E) Panel showing various fields of tubulated (black arrowheads) SUPER templates upon incubation with 200 nM epsin1. Images are inverted in contrast for clarity. Scale bars = 5 µm. (Figure is reproduced from (*96*))

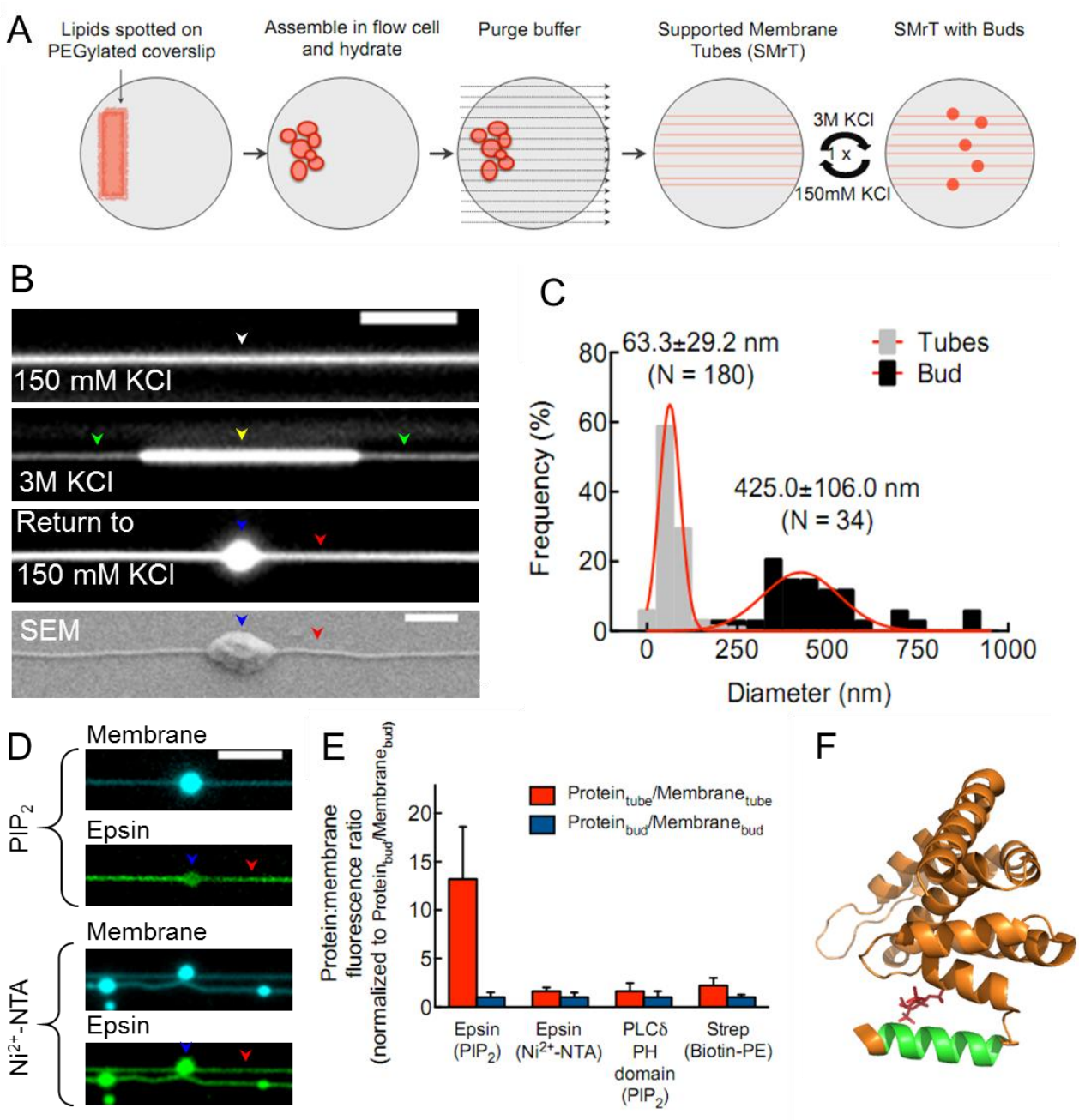

Figure 3.2: Epsin-induced clathrin assembly is controlled by membrane curvature. (A) Schematic of generation of budded supported membrane tubes (SMrT). (B) Scanning electron micrograph of streptavidin-bound, fixed and gold-coated budded SMrT templates. Scale bar = 500 nm. (C) Size distribution of tube and bud diameters. (D) Fluorescence micrographs of epsin distribution on budded SMrT templates recruited via PIP2 or DGS NTA (Ni2+). Red and blue arrowheads mark the tube and bud, respectively. (E) Protein density on membrane tubes (red) and buds (blue) (F) Structure of the epsin1 ENTH domain [PDB code: 1H0A, (*20*)] with the H0 amphipathic helix shown in green. (Figure is reproduced from (*96*))

To test curvature sensitivity of epsin1, we passed alexa 488 labeled epsin on budded SMrT templates composed of DOPC:DOPS:DOPIP$_2$:DiD (84.5:15:5:0.5 mol%) and imaged after 10 minutes. Membrane buds were diffraction limited. Fluorescence intensities of diffraction limited objects are proportional to surface area (*199*). Based on this assumption we analyzed membrane curvature sensitivity of epsin and compared ratios of protein to membrane on tubes Vs buds. Budded SMrT templates composed of DOPIP$_2$ were showing 13 fold higher protein to membrane ratio on the membrane tubes compared to buds ($ratio_{tubes}$ = 13.20 ± 5.4, mean ± SD, N = 17, $ratio_{bud}$ = 1.0 ± 0.5, mean ± SD, N = 15, P < 0.0001, Student's t-test) (figure 3.2D, E). This indicates the higher epsin recruitment to the curved membrane tubes compared to membrane buds. To test uniform distribution of PIP$_2$ on the membrane, we had used mEGFP-PLC δ PH domain which has specificity for PIP$_2$ and supposed to be membrane curvature insensitive. Protein to membrane ratio was similar on membrane tubes and buds ($ratio_{tubes}$ = 1.6 ± 0.8, mean ± SD, N = 19, $ratio_{bud}$ = 1.0 ± 0.6, mean ± SD, N = 17). This indicated the uniform distribution of DOPIP$_2$ in membrane tube and bud. Residues in the H0 helix of epsin bind to PIP$_2$ which induces its folding on the membrane surface (figure 3.2F). Absence of PIP$_2$ in the membrane should not form Ho helix of the epsin and expected to be membrane curvature insensitive. 6xHis tag Epsin recruited via DGS-Ni2+NTA lipid [DOPC:DOPS:DGS-Ni^{2+}NTA:DiD (84.5:15:5:0.5 mol%)] was membrane curvature insensitive in our assay ($ratio_{tubes}$ = 1.6 ± 0.4, mean ± SD, N = 20, $ratio_{bud}$ = 1.0 ± 0.5, mean ± SD, N = 20). Streptavidin binding to biotinylated lipid is curvature insensitive (*200*). In our assay system this strepatavidin binding to biotinylated lipid was also membrane curvature insensitive ($ratio_{tubes}$ = 2.2 ± 0.8, mean ± SD, N = 20, $ratio_{bud}$ = 1.0 ± 0.5, mean ± SD, N = 20) (figure 3.2E). This data shows higher preference of epsin binding to the curved membrane compared to planar membrane in presence of PIP$_2$, which is consistent with the earlier observations (*86*, *87*).

3.2.3 Clathrin assembly on budded SMrT templates:

Budded SMrT templates composed of PIP$_2$ or DGS-Ni2+NTA were incubated with epsin followed by washing of unbound free epsin from the solution. These budded SMrT templates were then incubated with 40 nM of Txred labeled clathrin. Clathrin was assembled on the membrane. PIP$_2$ containing budded SMrT templates were showing clathrin assembly on the curved membrane tubes, whereas on DGS-Ni2+NTA containing budded SMrT templates were uniformly coated with

clathrin (figure 3.3A, B). This pattern of clathrin assembly was similar to the epsin binding pattern on the budded SMrT templates.

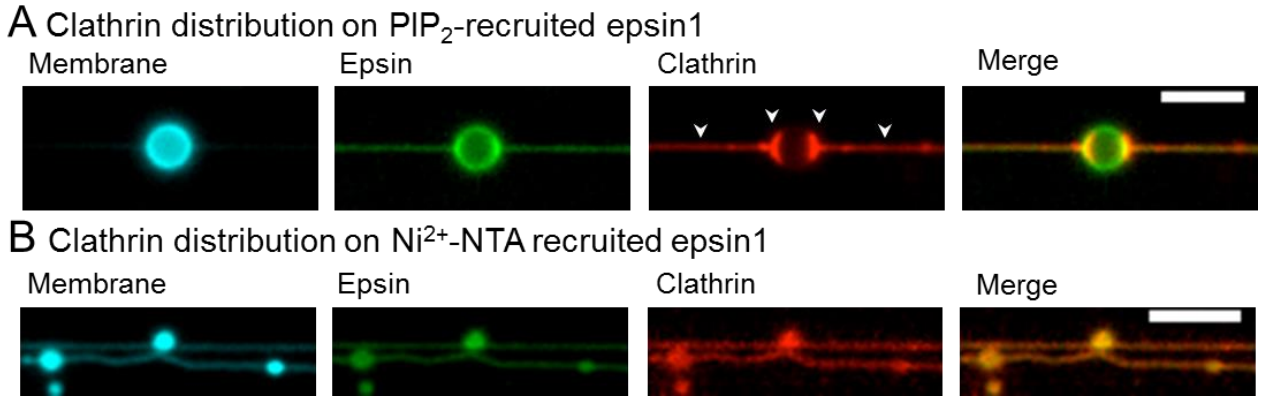

Figure 3.3: Clathrin distribution on membrane tubes coated with epsin recruited via PIP2 (A) or DGS NTA (Ni2+) (B). Scale bars = 5 μm. (Figure is reproduced from (*96*))

3.2.4 Novel assay system for clathrin assembly:

40nM of Texas red labeled clathrin assembled as foci on epsin coated SMrT tubes with epsin clustering (figure 3.4 A, B). These fluorescently labeled clathrin foci were diffraction limited. To get high special resolution, we had done FESEM imaging of these clathrin coated tubes. These tubes showed budded clathrin assembly as compared to cross-linked streptavidin coated membrane tubes (figure 3.4C). This elliptical/spherical shaped assembly showed intrinsic curvature of assembled clathrin on a membrane tubes. It was difficult to transfer these clathrin-coated structures on EM greed so we could not able to get high resolution transmission electron microscopy data of these clathrin coated structures.

3.2.5 Effect of Epsin on membrane tubes:

Epsin has N terminal ENTH domain which binds to PIP_2 and forms H_0 helix. It inserts in the membrane and causes membrane bending or fission (*20*, *195*). L6W mutation in ENTH domain enhance the effect of Ho helix insertion and promote membrane fission (*20*, *88*). In our assay, WT and L6W epsin did not show membrane constriction and fission. Clustered epsin (WT and L6W epsin) in clathrin foci was also not showing membrane constriction compared to dynamin (bona fide membrane constricting protein).

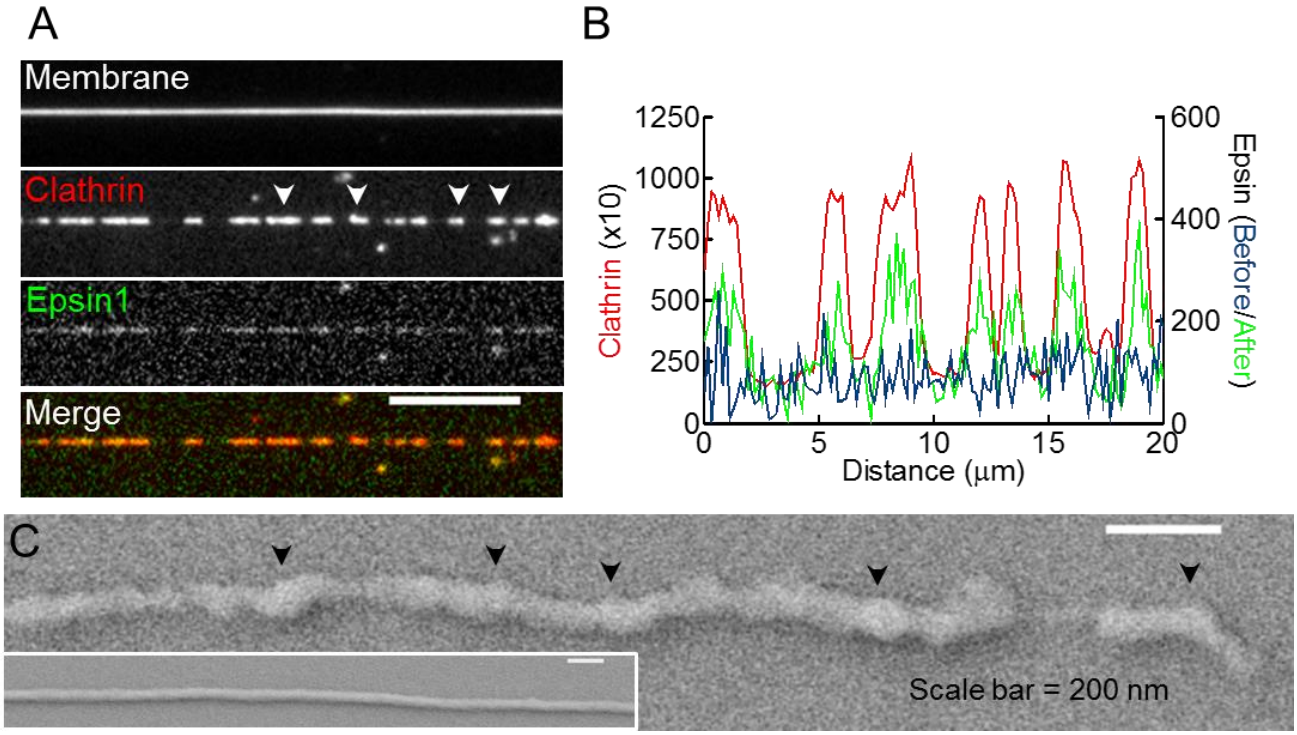

Figure 3.4: Specific interactions between the clathrin binding sites on epsin with clathrin are required for clathrin assembly. (A) Representative fluorescence micrographs of clathrin and epsin distribution on PIP2-containing SMrT templates. White arrowheads mark sites of assembled clathrin. Scale bar = 10 µm. (B) Fluorescence profiles of epsin distribution on PIP2-containing tubes before (blue) and after (green) 10 min incubation with clathrin (red). (C) Scanning electron micrograph of epsin-induced clathrin assemblies on SMrT templates. Black arrowheads mark regions showing pucker in the clathrin coat. Scale bar = 400 nm. (Figure is reproduced from (*96*))

3.2.6 Importance of clathrin binding motifs for the clathrin assembly:

Epsin C terminal tail has two clathrin binding motifs (CBMs) (^{257}LMDLAD and ^{480}LVDLD) (*95*) (*91*). These both CBMs bind cooperatively to the clathrin and assemble it on SMrT templates. We counted the number of clathrin foci per 100 µm of membrane tube to quantify the efficiency of clathrin assembly. Epsin recruited SMrT templates were showing ~ 38 clathrin coated puncta /100 µm (38 ± 9 mean ± SD, N = 38). CBMs were mutated into alanine and assayed for clathrin assembly to test the important clathrin binding motifs as shown in (figure 3.5A). CBM1 mutant (8 ± 7 mean ± SD, N = 17) and CBM2 mutant (3 ± 3 mean ± SD, N = 22) were not showing efficient clathrin assembly on SMrT templates compared to WT epsin (figure 3.5 B). Presence of CBM1 and CBM 2 mutants together on SMrT templates showed little increase in the number of

clathrin-coated puncta (13 ± 7 mean ± SD, N = 32). CBM null mutant showed very less or nonspecific clathrin binding to SMrT templates (4 ± 4 mean ± SD, N = 26).

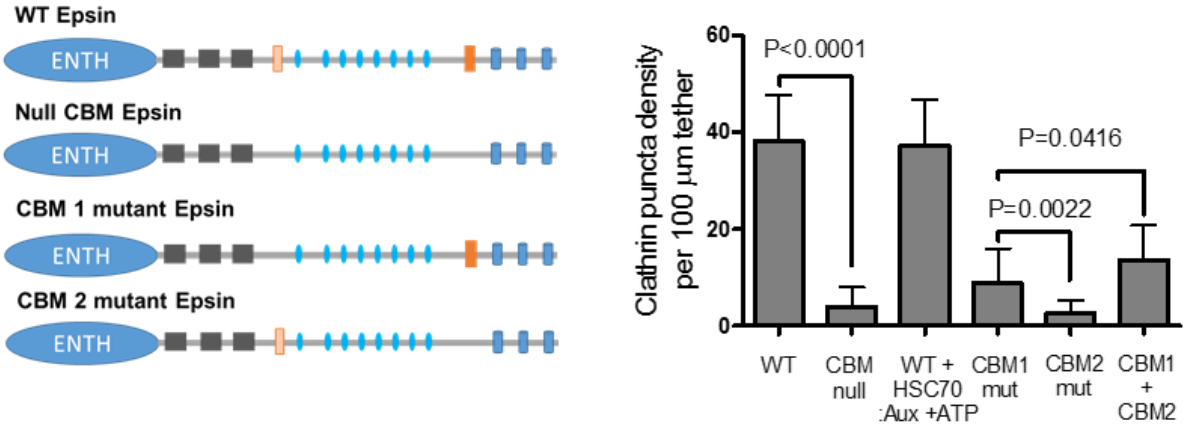

Figure 3.5: Effect of Clathrin binding motifs in clathrin assembly: A) Schematics of epsin mutants. B) Clathrin foci density on PIP2-containing tubes with epsin and its mutants. (Figure is reproduced from (*96*))

3.2.7 Dynamics of CCP Formation:

It is possible to study real time clathrin assembly reaction by using SMrT template assay system. This allowed us to determine clathrin assembly rate with high temporal resolution. As soon as clathrin entered in the chamber, it started binding and polymerizing on epsin coated SMrT templates (figure 3.6A). This pattern of clathrin assembly was similar to the CCP formation pattern in cells i.e. initiation followed by polymerization. These clathrin assemblies were dynamic and moving on the membrane tubes (figure 3.6B). In the Initiation phase few of these clathrin polymers moving on the template and merged to form larger clathrin polymer (figure 3.6C). We had plotted and analyzed kymograph to get kinetic parameters of clathrin assembly (Figure 3.6 D, E). Our single pixel analysis picked stable clathrin puncta. Each polymerization event fitted in the Plateau followed by one phase association equation. This analysis gave us time constant (τ) of clathrin assembly which was approximately ~74 s (lower 95% CI = 63 s, upper 95% CI = 86 s, N = 118) (figure 3.7B). This time constant is similar to the time constant of clathrin-coated vesicle formation in cells (*201*, *202*).

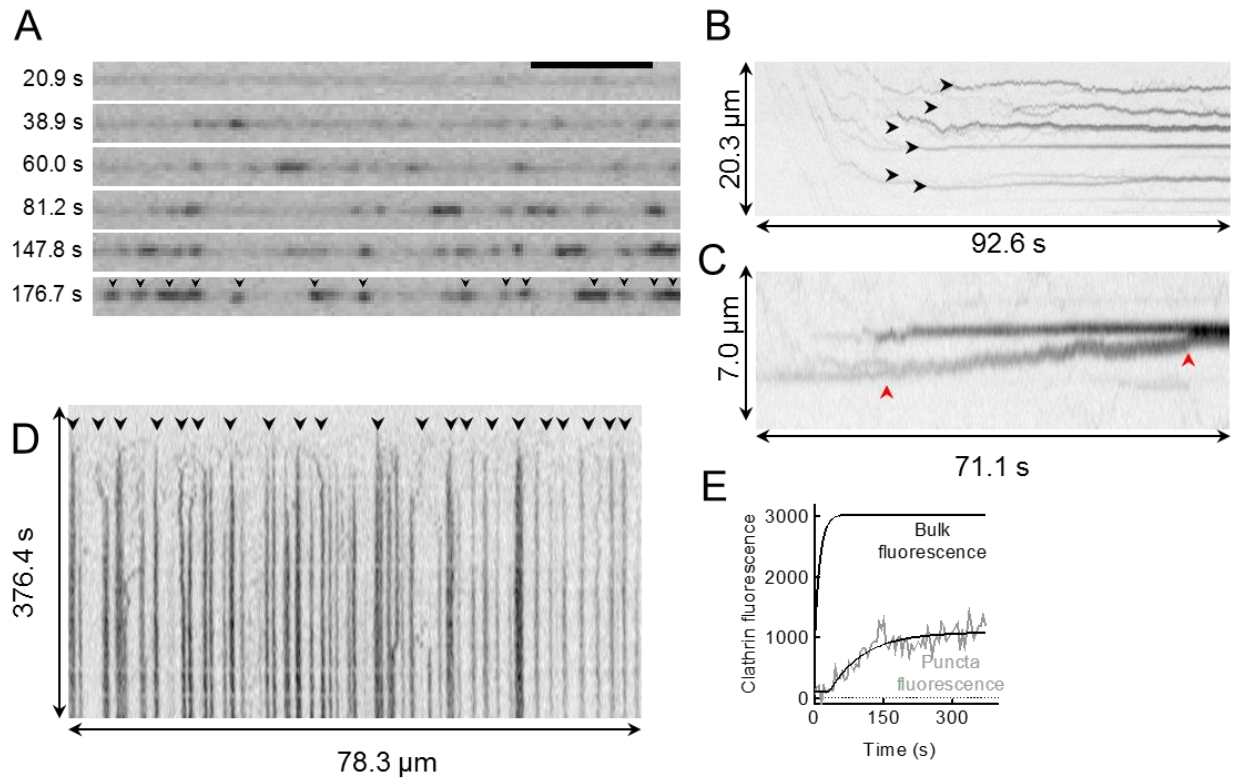

Figure 3.6: Kinetics of epsin-induced clathrin assembly. (A) Frames from a time-lapse movie on epsin-coated tubes showing the formation of fluorescent clathrin foci (black arrowheads) (see Movie 3). Kymographs generated from clathrin assembly reactions imaged at high temporal resolution (100 ms/frame) showing growth (black arrowheads) (B) and merging (red arrowheads) (C) of clathrin foci. (D) Kymographs from low temporal resolution (5 s/frame) imaging of the entire assembly reaction showing nucleation and growth of numerous clathrin foci (black arrowheads). (E) Plot showing single pixel fluorescence traces at a clathrin focus (gray) and bulk clathrin fluorescence in solution

3.2.8 Role of HSC 70, Auxilin and ATP in clathrin assembly:

HSC 70, auxilin and ATP can disassemble clathrin coat *in vivo* and *in vitro* (Ungewickell et al. 1995) (*203*, *204*). To test effect of HSC70, Auxilin and ATP on clathrin assembly, we added these proteins with clathrin on epsin coated SMrT templates, which were neither disassemble nor prevent clathrin assembly. The number of CCPs/100µm on the tube were not significantly different from WT epsin mediated clathrin assembly (figure 3.7A). These disassembly proteins decreased the clathrin assembly time constant as shown in ~28 s ($N = 26$, $P < 0.0001$, Mann-Whitney t-Test) (figure 3.7B).

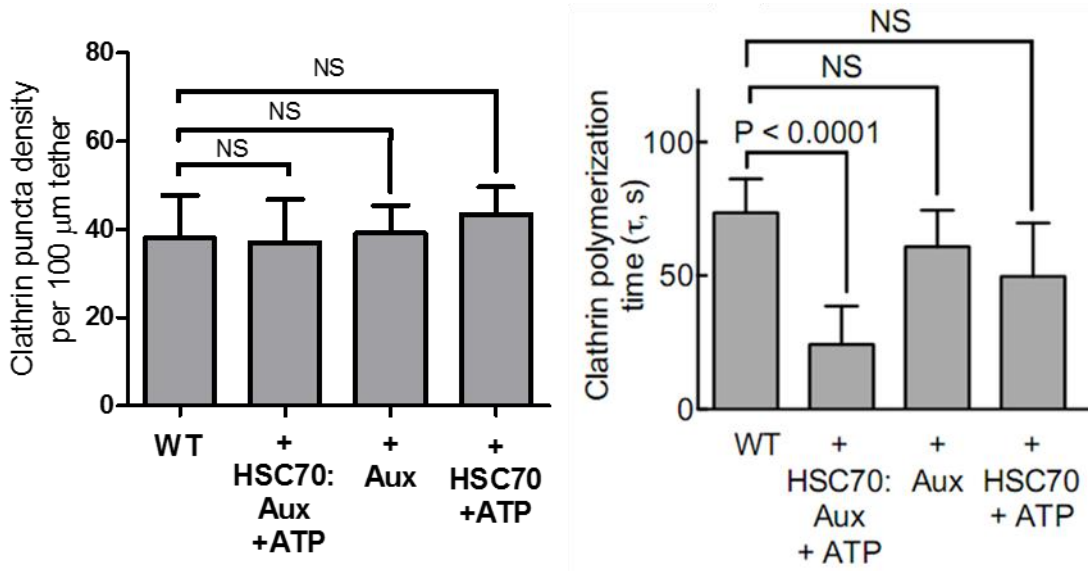

Figure 3.7: Role of HSC70, auxilin and ATP in clathrin assembly: A) Puncta density in absence and presence of uncoating machinery B) Time constant of clathrin assembly in presence and absence of uncoating machinery. (Figure is reproduced from (*96*))

3.3 Discussion:

In this study we have used a novel assay system which mimics the intermediate stage of CCP formation. Tandem affinity purification method allowed us to rapidly purify proteins with high purity. SUPER template binding assay is a simple system to measure protein affinity for membrane. SMrT templates with and without buds are simple, fast and high throughput assay systems for studying protein membrane curvature sensitivity and protein-protein interaction on membrane surface.

Epsin is one of the indispensable molecule in clathrin-mediated endocytosis. It is curvature sensitive and is supposed to recruit at CCP during its maturation phase. Recently it has been shown the elasticity of membrane plays role in clathrin-coated pit formation on GUV surface (*22*). Curved surface of polystyrene beads coated with epsin facilitates the clathrin assembly (*180*). Our assay system indicates the decisive role of membrane curvature in epsin-mediated CCP formation. Epsin's curvature sensitivity recruit it on the highly curved membrane followed by rapid clathrin assembly on membrane surface.

HSC70 and Auxiline disassemble clathrin coats in presence of ATP but these proteins could not able to disassemble epsin-mediated clathrin assembly on SMrT templets. Previous results suggested that these proteins reduced clathrin-coated pits on GUVs (*21*). In our assay condition, presence of these proteins could destabilize the clathrin-clathrin interactions and results in the less merging of clathrin foci as well as increased in the rate of assembly.

In our assay system epsin is not showing membrane constriction or fission which may support the role of actin in the progression of CCP in cells (*90*). Also there may be role of other proteins like AP2, other CLASPs and bar domain proteins in the maturation of the CCP into CCV.

Chapter 4

CLASP-mediated clathrin assembly and role of clathrin assembly in CLASPs sorting

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Summery:

Clathrin-associated sorting proteins (CLASPs) are an evolutionarily conserved set of adaptors that internalizes membrane proteins via the AP2-dependent, clathrin-mediated endocytic pathway. CLASPs recognize sorting motifs on specific cargoes; bind phosphoinositide lipids, clathrin and the heterotetrameric adaptor AP2. CLASPs like epsin and AP180 can assemble clathrin on membrane whereas the role of other CLASPs in clathrin assembly as well as precise role in cargo-CLASPs sorting is not clear yet. Using a novel real-time fluorescence microscopy-based assay that allows for a high throughput analysis of clathrin assembly, we analyzed kinetics of clathrin assembly and consequent adaptor clustering for 7 different CLASPs and compared them to that seen for a constitutively active AP2 mimic. Also we studied the role of clathrin inn CLASPs co-clustering.

4.1 Background:

Clathrin-mediated endocytosis internalizes various membrane proteins (cargo) in the cells which have internalization/ sorting signal at their cytoplasmic region. Specific clathrin-associated sorting proteins (CLASPs) recognize these sorting signal and forms CLASP-cargo complex at the plasma membrane(*65, 150, 205*). These CLASPs have membrane (PIP_2), AP2 and clathrin binding sites. These multi-interaction sites on CLASPs are responsible for recruitment of CLASP-cargo complexes in the clathrin-coated pit (CCP) or these complexes can form CCP in presence of other endocytic proteins. Epsin, AP2, β-arrestin interact with ubiquitinylated cargo, YxxΦ / (DE)xxxLL(I) motifs of cargo and G protein couples receptors (GPCRs) respectively (*94, 206*)(*207*) (*134, 208*) . Dab2, ARH, NUMB recognize FxNPxY motif containing cargo (*111–113, 123, 127*). AP180 is a neuronal isoform of CALM (recognize VAMP) and plays role in synaptic vesicle recycling (*102, 103*). These CLASPs have different numbers and types of AP2, clathrin binding motifs which may affect the efficiency of clathrin assembly by these CLASPs and their recruitment at the CCPs (*65*). AP2 and Epsin depletion result in arrested clathrin-coated pits at the

early and late stages of CCP formation respectively (*88*, *90*) (*68*, *69*). AP180, Dab2, ARH, β-arrestin, NUMB depletion affect internalization of their specific cargo molecules but do not show devastating effect on clathrin-mediated endocytosis (*102*) (*114*, *123*, *209*). Overexpression of Dab2 and its cargo increases the size of clathrin-coated pits and change the distribution of AP2 in BSC1 cells (*118*). Large number of these CLASPs in cells make it difficult to understand their precise role in clathrin assembly and factors determining sorting of CLASP-cargo complex during CCP formation. Minimal reconstitution of clathrin coat assembly is one of the approach to study the capability of CLASPs in clathrin assembly. A set of membrane remodeling proteins recruited at the early stage of the CCP formation which were responsible for early membrane curvature followed by recruitment of other CLASPs molecules and clathrin at the CCP (*150*). Curved membrane facilitate the clathrin recruitment on the membrane mediated by CLASPs molecules (*180*). Generation of curved membrane may be rate limiting step during CCP formation. Current biochemical assays on planar GUV surface studied clathrin coat formation by epsin and AP180 (*21*, *22*, *96*). These assays relied on the end-point electron microscopic analysis of clathrin assembly reaction on planar bilayer. In our previous study, we had used curved membrane tubes as a model membrane system (*96*). In this study we have done comparative analysis of different CLASP family members in clathrin assembly reaction. We further studied the role of clathrin in CLASP clustering and sorting during clathrin assembly reaction.

4.2 Results:

4.2.1 Experimental design and clathrin assembly reaction of CLASPs:

CLASPs have membrane binding N-terminal structured domain and unstructured C-terminal tail (figure 4.1A) (*210*). The unstructured region of the CLASP molecules are prone to degradation which makes it difficult to purify recombinant full length proteins from bacteria. We have used a tandem affinity purification method for the purification of full length CLASPs. N-terminal 6xHis tag followed by C-terminal strep tag affinity purification remove all truncated proteins and give pure full length CLASP molecule. These highly purified proteins were fluorescently labeled (figure 4.1B). Previously we have used SMrT templates to study epsin-mediated clathrin assembly. We recruited epsin on membrane tubes via ENTH- PIP_2 interaction or N-terminus 6xHis- DGS-Ni^{2+}NTA interaction and assembled clathrin. As there was not

significant difference between clathrin assemblies mediated by PIP_2 or DGS-Ni^{2+}NTA recruited epsin.

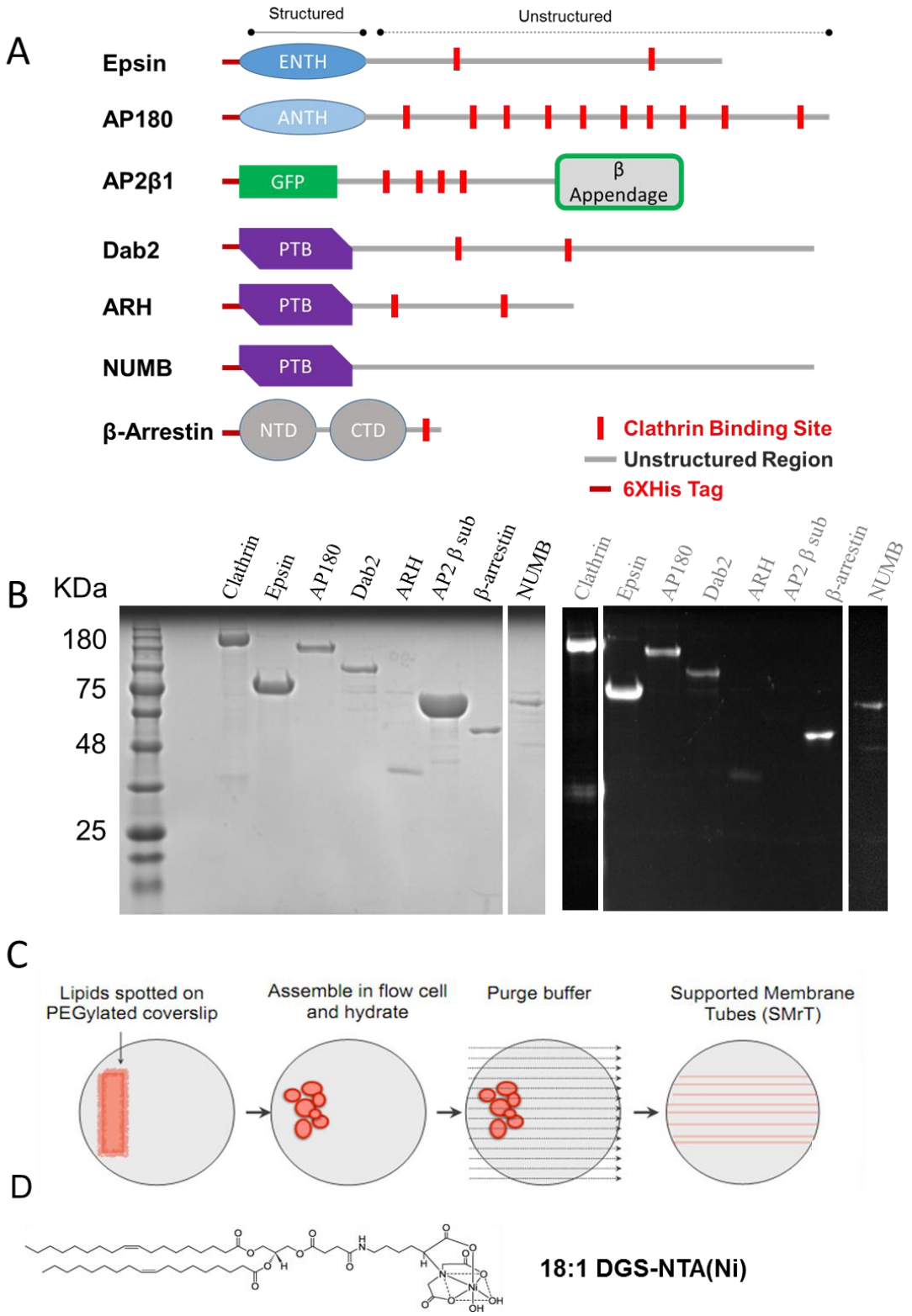

Figure 4.1: Design of clathrin-associated sorting proteins constructs and experimental strategy. A) Domain organization of the CLASP molecules. B) SDS-PAGE of coomasie blue stained and fluorescently labeled, affinity purified CLASPs molecules used in this study C) Schematic of SMrT templates preparation D) Structure of 18:1 DGS-NTA (Ni) lipid used to bind CLASP molecules on the membrane surface.

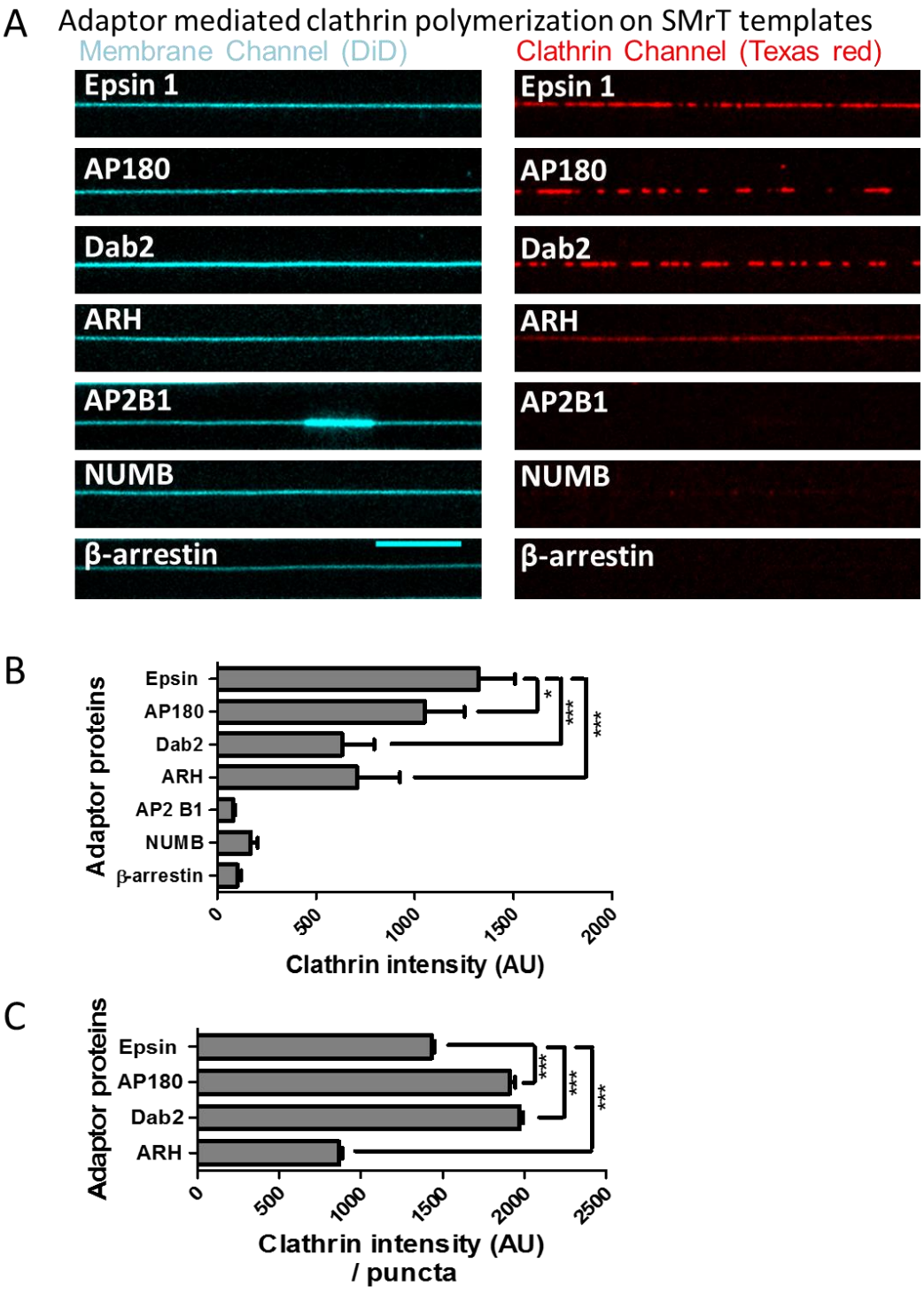

Figure 4.2: CLASP-mediated clathrin assembly on SMrT templates. A) Florescence microscopic images of clathrin assembly on CLASP coated SMrT templates in membrane channel (cyan) and clathrin channel (red). Scale bar = 10μ . B) Fluorescence intensity of clathrin along the SMrT tube. C) Fluorescence intensity of clathrin in each clathrin foci.

Table 4.1: CLASPs interaction with clathrin terminal domain (NTD)			
	Protein	Clathrin binding motif (CBS)	Clathrin terminal domain site (NTD)
1	Epsin	LMDLAD	TD SITE 1
		LVDLD	
2	AP180	DLL	
		DLF	
3	Dab2	LVDLN	TD SITE 1
		PWPYP	W BOX
4	ARH	LLDLE	TD SITE 1
		DLF	
5	GFP AP2 β1	DLL, DLLNLD, DLL, DLF	TD SITE 1
			TDSITE 1
6	β Arrestin	LIEFD	TD SITE 1
7	NUMB		

We recruited different CLASP molecules by using 5 mol % DGS-Ni^{2+}NTA lipid (figure 4.1D) to maintain similar densities of these CLASPs on membrane surface and studied clathrin assembly. This strategy allowed us to compare clathrin assembly mediated by different CLASP molecules.

4.2.2 Clathrin polymerization potential of CLASPs:

SMrT templates can be used to study dynamics of epsin-mediated clathrin assembly reaction (figure 4.1C). CLASPs family has other members which play role in internalization of their specific cargo molecules in cells (*65*). We have used epsin, AP180, Dab2, ARH, β -arrrestin, NUMB CLASPs for our clathrin assembly reaction. AP2 is a hetero-tetrameric CLASP molecule which has clathrin binding sites in the linker region of β subunit. His tag GST fusion with linker and appendage domain of AP2 β subunit shows clathrin assembly on GUV surface (*80*). For our comparative clathrin assembly study, we included AP2 β subunit with 6xHis mEGFP tag at N-terminal tail. CLASPs-mediated clathrin assembly was scored by taking mean intensity of clathrin along SMrT templates and compared across CLASPs (figure 4.2B). Intensities of clathrin along the membrane tubes were listed in table 4.2. Epsin, AP180, Dab2 and ARH were able to assemble

clathrin whereas GFP-AP2 β subunit, β-arrestin, NUMB were not able to assemble clathrin on SMrT templates in our assay (figure 4.2 A, B). This bulk clathrin assembly analysis gives the average intensity of clathrin along the membrane tube, but is unable to give information about the pattern of clathrin assembly, intensity of clathrin in each clathrin foci.

Table 4.2: Mean clathrin intensity along the SMrT tube

	Epsin	AP180	Dab2	ARH	AP2 β1	NUMB	β-arrestin
Geometric mean	1325	1051	633	708.2	80.83	170.8	104.5
Lower 95% CI	1164	881.8	503.6	542.7	71.66	142.8	90.73
Upper 95% CI	1507	1252	795.6	924.2	91.18	204.3	120.5
Number of values	22	18	42	13	20	9	15

Table 4.3: Clathrin intensity per clathrin foci

	Epsin	AP180	Dab2	ARH
Geometric mean	1434	1912	1971	867.5
Lower 95% CI	1417	1882	1950	850.6
Upper 95% CI	1451	1942	1992	884.8
Number of	634	558	1202	306

The pattern of clathrin assemblies are different across epsin, AP180, Dab2 and ARH. Epsin and ARH were showing uniform clathrin assembly along the SMrT templates whereas AP180 and Dab2 were appeared as punctate distribution of clathrin. These differences in clathrin assembly may be due to differences in number of clathrin molecules per clathrin assembly (foci). To test this we measured clathrin intensity in each clathrin foci as describe in the experimental procedure. This analysis showed highest intensity of clathrin in AP180 and Dab2 mediated clathrin assembly (table 4.2) (figure 4.2C).

4.2.3 Dynamics of clathrin polymerization by different CLASPs: We monitored kinetics of clathrin assembly by different CLASPs on SMrT templates for 4 minutes. Clathrin assembly assay on SMrT templates allowed us to get time constants (τ) and initiation/onset time of clathrin assembly reaction mediated by different CLASPs molecules. The pattern of CLASPs mediated clathrin assembly was either uniform or punctate (figure 4.3A). We used two different methods to analyze clathrin assembly kinetics. Bulk clathrin assembly analysis method represents the rate of

clathrin recruitment and assembly along the SMrT templates. The time constants and initiation time for bulk clathrin assembly by epsin, Dab2, AP180 and ARH are listed in the table 4.4 and table 4.5 respectively (figure 4.3B, C). There was significant difference of clathrin assembly time constants across these CLASP molecules. This bulk analysis gave us information about the clathrin recruitment on membrane tubes which can be affected by the pattern of clathrin assembly (either uniform or punctate assembly).

Table 4.4 : Bulk clathrin assembly time constant

	Epsin	AP180	Dab2	ARH
Geometric mean	79.8	79.36	52.02	201.4
Lower 95% CI	72.85	71.88	47.91	154.4
Upper 95% CI	87.41	87.63	56.49	262.8
Number of values	14	7	12	9

Table 4.5 : Bulk clathrin assembly onset time

	Epsin	AP180	Dab2	ARH
Geometric mean	21.84	16.87	2.901	20.98
Lower 95% CI	20.51	14.9	2.135	17.94
Upper 95% CI	23.26	19.09	3.942	24.53
Number of values	14	7	12	11

To get precise time constants and initiation time for clathrin assembly in each puncta we generated kymographs (figure 4.3D). Different trajectories were selected and analyzed by using "pointfromkymograph" plugin in the ImageJ software (*192*). This method of analysis is semi-automated and selects maximum intensity points in each trajectory. This analysis gave us precise time constants for clathrin assembly in different clathrin foci. The time constants and initiation time for clathrin assembly are listed in table 4.6 and table 4.7 respectively (figure 4.3E, F). There is significant difference in onset of clathrin assembly mediated by Dab2, Epsin and AP180. ARH kymographs are showing uniform clathrin assembly along the tether so it was difficult to analyze these kymographs by using this method.

Table 4.6 :Time constant of clathrin assembly in puncta

	Epsin	AP180	Dab2
Geometric mean	68.65	45.57	41.89
Lower 95% CI	59.08	38.48	37.8
Upper 95% CI	79.77	53.97	46.41
Number of values	50	70	46

Table 4.7 : Clathrin assembly onset time of each puncta

	Epsin	AP180	Dab2
Geometric mean	23.54	35.27	9.544
Lower 95% CI	20.7	31.93	7.941
Upper 95% CI	26.77	38.95	11.47
Number of values	50	70	46

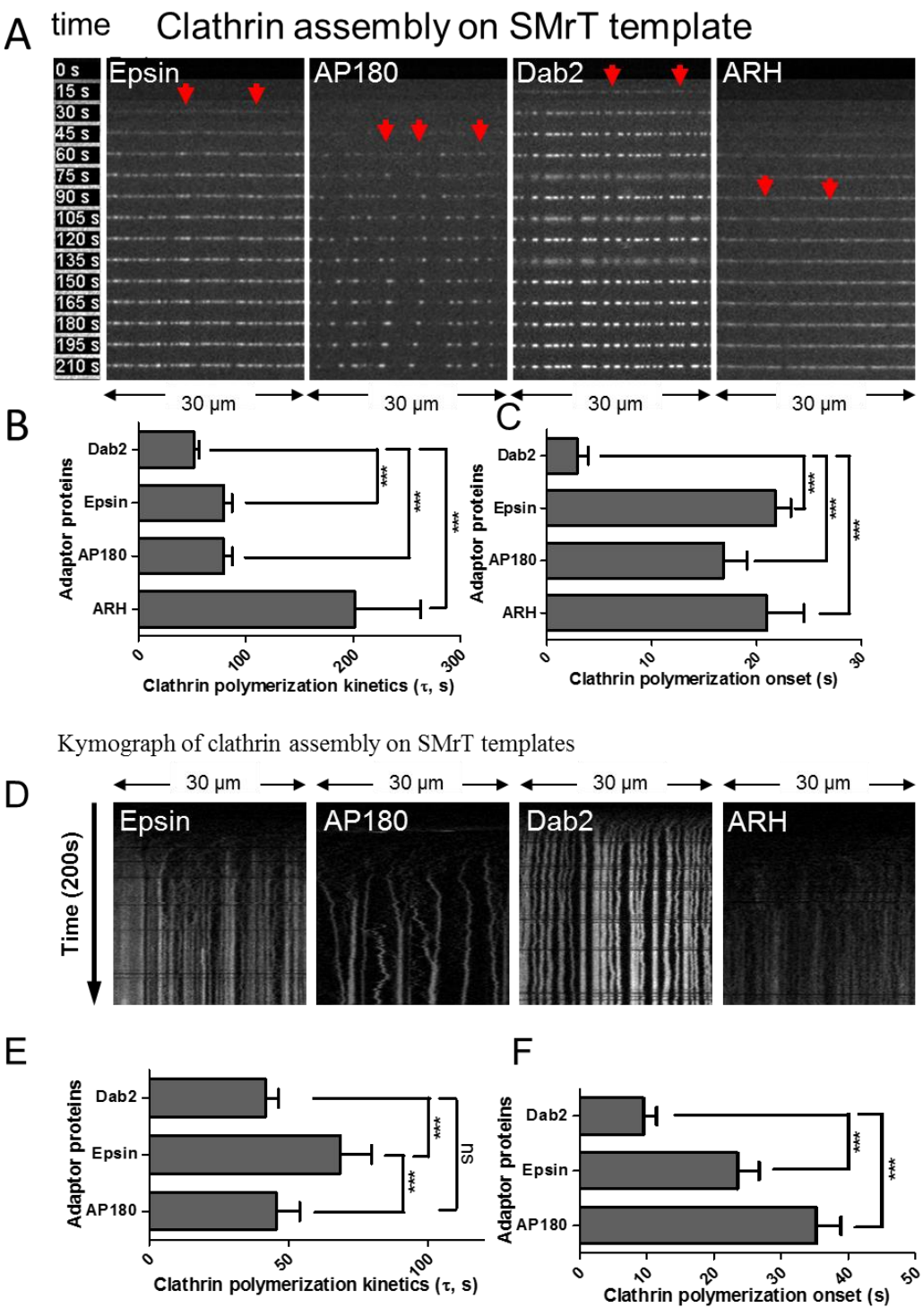

Figure 4.3: Kinetics of clathrin assembly on SMrT templates. A) Frames from time series images of clathrin assembly on Epsin, AP180, Dab2, ARH coated SMrT templates. Red arrow indicates the initiation of clathrin assembly on SMrT templates. B) Bulk clathrin assembly time constant along the SMrT templates. C) Bulk clathrin assembly onset time along the SMrT templates. D) Kymographs of clathrin assembly on the SMrT templates coated with CLASPs. E) Time constant of clathrin assembly in a single puncta on the SMrT templates. F) Onset time of clathrin assembly in a single puncta on the SMrT templates.

4.2.4 CLASPs clustering with clathrin assembly and their classification: CLASPs interact with clathrin and assemble it on the membrane. These CLASP molecules get clustered during clathrin assembly. AP180 and Dab2 were showing their clustering in the clathrin foci whereas epsin and ARH did not show an efficient clustering with clathrin assembly (figure 4.4 A,B,C,D).

We have classified CLASPs based on their clathrin assembly ability on SMrT templates (figure 4.4E). Epsin, AP180, Dab2, ARH are sufficient for clathrin assembly and are classified as "CLASP A" family. These "CLASP A" molecules can be further classified based on their clustering during clathrin assembly reaction. AP180 and Dab2 get clustered during clathrin assembly reaction and are further classified as "CLASP A1". Epsin and ARH show uniform clathrin assembly and it is difficult to see their clustering so we classified them as "CLASP A2". AP2 β subunit, β-arrestin, NUMB are CLASPs, which are unable to assemble clathrin and are classified as CLASP "B" family proteins.

4.2.5 "CLASP A1" induced clustering of "CLASP B" proteins during clathrin assembly on SMrT templates. AP2 β subunit and β-arrestin have clathrin binding motif/motifs at their C-terminal region but are not able to assemble clathrin. Hence these molecules are unable to get clustered on SMrT templates. We wanted to test, if "CLASP A1" and "CLASP B" family proteins present together on SMrT templates then "can clathrin assembly clusters "CLASP B" family protein in the clathrin foci?" To test this we recruited unlabeled Dab2 (CLASP "A") and Alexa 488 labeled β-arrestin (CLASP "B") on SMrT templates. Dab2 was sufficient for clathrin assembly and assembled clathrin on SMrT templates with its clustering. Alexa 488 labeled β-arrestin (CLASP "B") was not able to assemble clathrin as well as unable to cluster. But Alexa 488 labeled β-arrestin (CLASP "B") was clustered with Dab2 in the clathrin foci (Figure 4.5). This explains

the role of clathrin in CLASPs clustering during CCP formation. Similarly AP2 β subunit get clustered in presence of Dab2 during clathrin assembly reaction (figure 4.6).

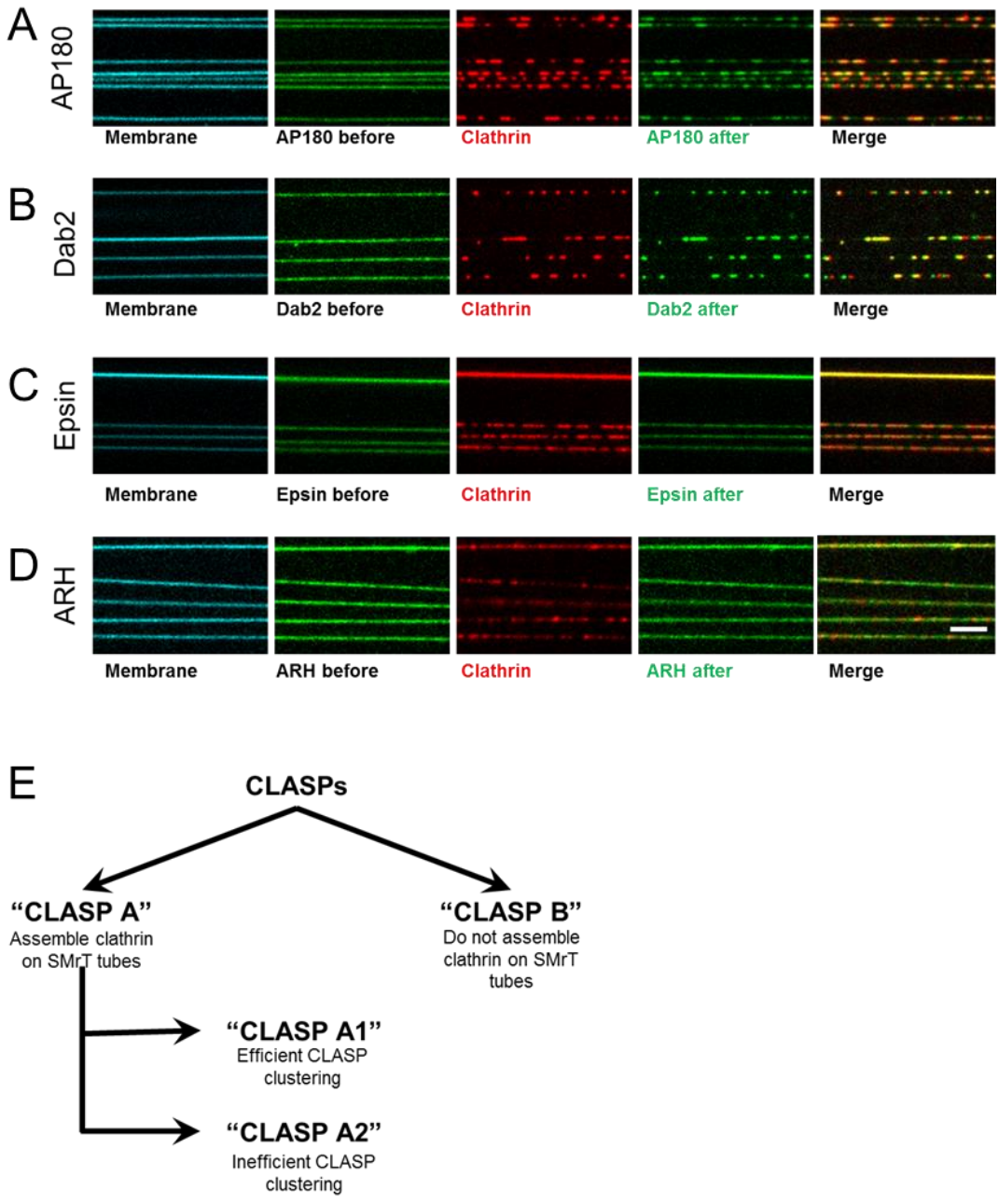

Figure 4.4: CLASP clustering with clathrin assembly: A, B) AP180, Dab2 clusters with clathrin assembly. C, D) Epsin and ARH inefficient clustering with clathrin assembly. Scale bar = 5µ. E) CLASP classification based on their clathrin assembly and their clustering property.

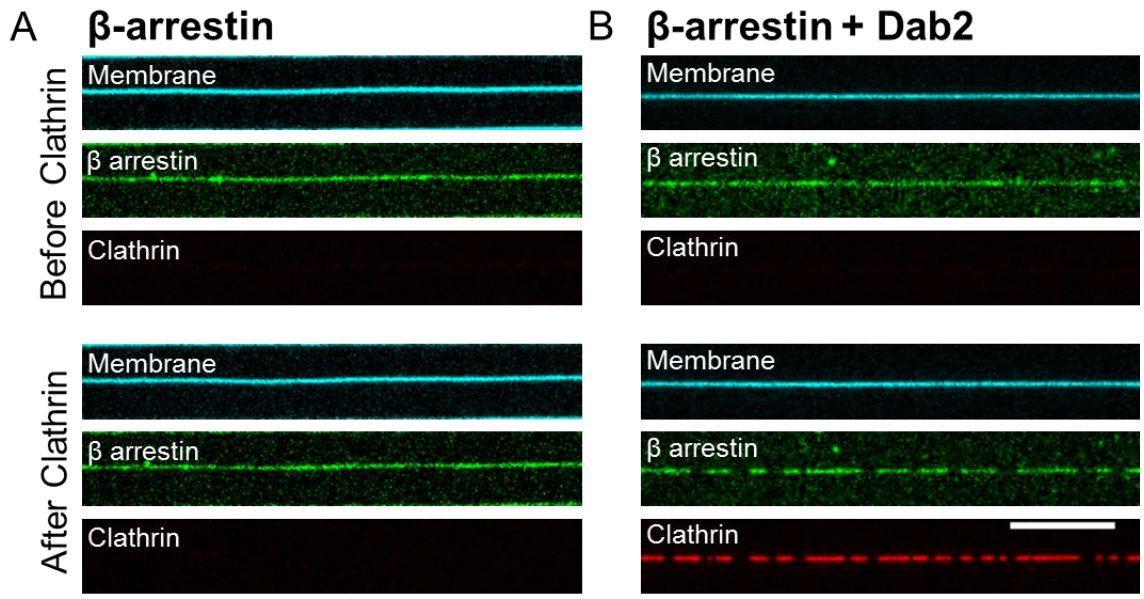

Figure 4.5: β-arrestin clustering in presence of Dab2 and clathrin. A) Uniform localization of β-arrestin after clathrin addition on β-arrestin coated SMrT tube. B) Clathrin assembly with β-arrestin clustering on β-arrestin and Dab2 coated SMrT templates. Scale bar = 10μ.

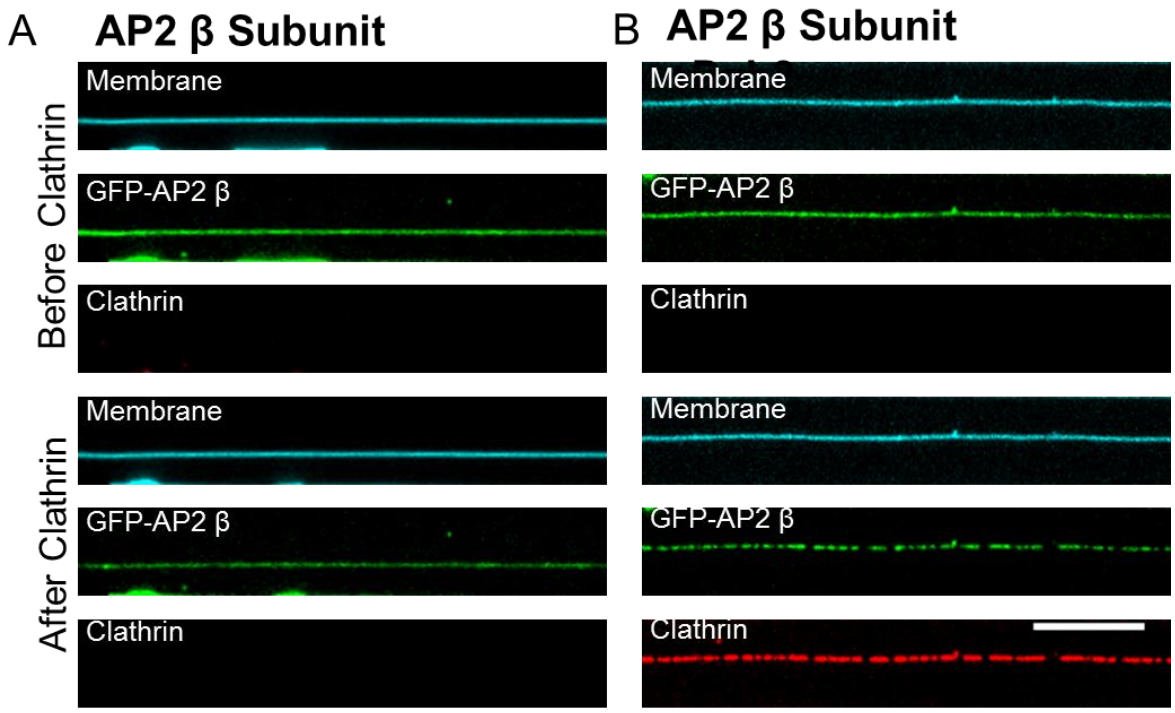

Figure 4.6: AP2 β subunit clustering in presence of clathrin and Dab2. A) Uniform localization of GFP-AP2 β subunit after clathrin addition on GFP-AP2 β coated SMrT tube. B) Clathrin assembly and GFP-AP2 β clustering on GFP-AP2 β subunit and Dab2 coated SMrT templates. Scale bar = 10μ.

4.2.6 CLASPs co-clustering during clathrin assembly reaction: Onset of Dab2 mediated clathrin assembly is lower than AP180 mediated clathrin assembly (figure 4.3A). So we recruited two different CLASPs together on SMrT templates and asked question "whether two different "CLASP A" molecules having different clathrin assembly onset times get differentially sorted or co-sorted during clathrin assembly?" SMrT templates coated with Alexa 488 labeled AP180 and Texas red labeled Dab2 were showing co-clustering during unlabeled clathrin assembly (Figure 4.7).

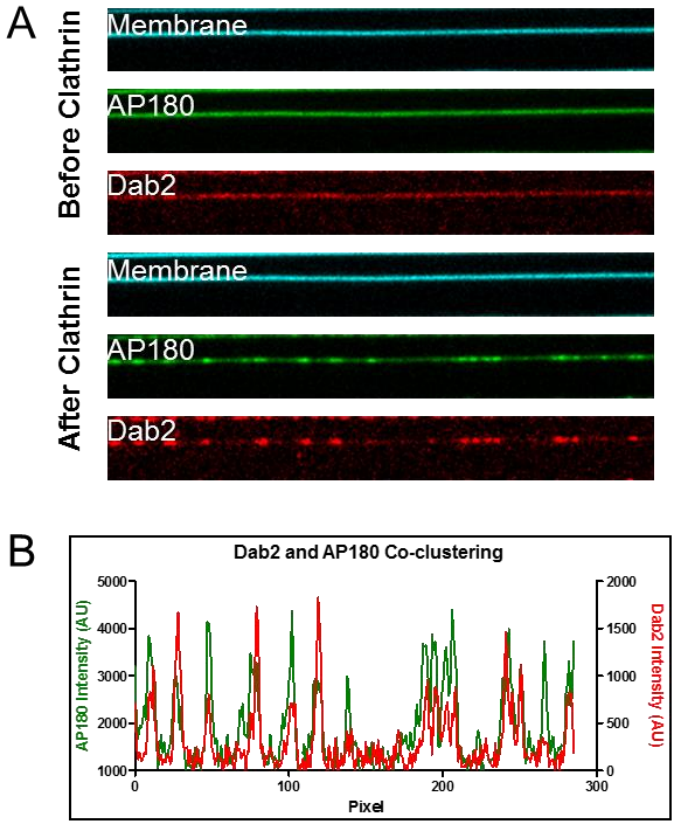

Figure 4.7: AP180 and Dab2 co-clustering after clathrin assembly on SMrT templates. Scale bar = 10µ. B) Intensity profile of AP180 and Dab2 on SMrT templates after clathrin assembly.

4.3 Discussion: CLASPs molecules have low affinity for their cargo or lipid molecules. This dynamic interactions of clasp with cargo and membrane decide the lifetime and densities of CLASP molecules on plasma membrane (*211*). It is difficult to regulate their concentrations on the plasma membrane. In our assay system, we maintained similar densities of CLASP molecules on membrane and compared their clathrin assembly potential.

All CLASPs have different types and numbers of clathrin binding motifs at their C-terminal tail. At least two clathrin binding sites required for efficient clathrin assembly on SMrT tube by

epsin (*96*). In our assay condition, Epsin, AP180, Dab2, ARH are sufficient to assemble clathrin whereas GFP-AP2 β subunit, β-arrestin and NUMB unable to assemble clathrin on SMrT templates. AP180, Dab2, ARH have two or more than two clathrin binding sites which may be required to cross link clathrin molecules on the membrane. Despite having multiple clathrin binding sites on His-GFP AP2 β subunit, it is unable to assemble clathrin on membrane surface. Our result is contradictory to the previously reported results where AP2 could assembled clathrin on GUV surface or in solution (*80, 181*). This may be due to complete washing of unbound AP2 β subunit from solution in our assay condition and is representing the incapability of clathrin assembly by membrane recruited AP2 β.

The patterns of clathrin assembly and clustering of CLASPs are different across Epsin, AP180, Dab2, ARH. AP180 and Dab2 have tight clathrin assembly and shows high intensity of clathrin in clathrin foci compared to clathrin assembly mediated by epsin and ARH. AP180 and Dab2 are also get clustered on the membrane compared to epsin and ARH. There may be multiple factors which decide these patterns of clathrin assembly. These could be number and types of clathrin binding motifs. Distance between clathrin binding motifs and structural flexibility of CLASP C-terminal could have role in the pattern of clathrin assembly reaction (*91*). These factors need to be address.

Our assay allowed us to compare kinetics of CLASP-mediated clathrin assembly. Onset time and time constant (τ) for clathrin assembly are two important kinetic parameters which can be compared with the initiation time and lifetime of clathrin-coated pit formation process respectively in cells. These parameters are affected by the affinity and specificity of clathrin binding motif with the N-terminal domain (NTD) of clathrin. There could be other factors which affect these parameters as mentioned above. Clathrin NTD has weak binding affinity for AP180, β-arrestin clathrin binding motifs (*1*)but has higher affinity for W box (*2*). Dab 2 is showing early initiation of clathrin assembly compared to other CLASPs. This may be because of two different CBMs (W box and clathrin binding motif 1) increase avidity and on rate of clathrin recruitment.

Clathrin terminal domain has redundancy for CLASPs motifs. There are CLASPs molecules which have single clathrin binding motif but are unable to assemble clathrin like β arrestin, AP2 β subunit. Dab2 induce clustering of these CLASPs get clustered in clathrin

assembly. This indicates the role of Dab2 and clathrin in CLASP clustering/sorting during clathrin-coat assembly reaction.

AP2 is an essential CLASP molecule in CME and its depletion stops CME in cells (*68*, *69*), but it is not showing clathrin assembly in our assay system. Dab2, AP180, epsin, ARH have different types and numbers of AP2 binding motifs and are sufficient to assemble clathrin on the membrane. These motifs can interact with AP2 and may get recruited at the site of CCP. In AP2 KD cells, CCPs could not get matured. This may be due to incapability of these CLASPs to recruit at the site of CCP. Presence of multiple binding motifs increases avidity of these CLASPs for AP2 and clathrin which subsequently affect the timing of CLASPs recruiting at the CCP. So the major role of AP2 is to regulate CCP formation by controlling recruitment of other CLASPs at the CCP sites which result in accumulation of clathrin assembly CLASPs ("CLASP A"). This leads to clathrin assembly on membrane by clathrin assembling CLASP molecules with clustering of those CLASPs member which are not capable to assemble clathrin. This shows the loading of cargo during the formation of clathrin-coated pit and direct role of clathrin in CLASP clustering/sorting.

AP180 and Dab2 have differences in their clathrin assembly onsets (initiation times) but these proteins get co-cluster during clathrin assembly on SMrT templates. This may be due to simultaneous binding of AP180 and Dab2 to clathrin and getting cluster in the same clathrin foci. This mechanism may indicate the role of clathrin in the recruitment of maximum numbers and types of CLASPs in the CCP.

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