Biochemical and functional characterization of connecdenn and its DENN domain in intracellular trafficking

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Abstract

Endocytosis and vesicle trafficking are essential for cellular division, differentiation and survival. Following endocytosis, cargo is delivered to the sorting endosome, where it is selected for degradation or recycled back to the cell surface. The various trafficking steps within the endosomal system are regulated by Rabs, small GTPases, which toggle between an inactive GDP-loaded conformation and an active GTP-loaded conformation. Clathrin-mediated endocytosis (CME) allows cargo internalization and coupling to the endosomal system via formation of clathrin-coated vesicles (CCVs). Connecdenn was identified as a component of clathrin-coated vesicles (CCVs) isolated from developing rat brain. The C-terminus of connecdenn is predicted to be unstructured and encodes several binding motifs that bind to the CME proteins intersectin1, endophilinA1 and AP-2. Connecdenn is enriched in brain, localizes to synapses, and its knockdown in hippocampal cultures inhibits synaptic vesicle recycling. Connecdenn enriches strongly on CCVs and is stably associated with the CCV membrane after removal of its clathrin coat. The stable membrane association of connecdenn depends on the N-terminal DENN domain, an evolutionary conserved but uncharacterized tri-partite domain. The DENN domain also functions as a guanine nucleotide exchange factor (GEF) for Rab35, which regulates cargo recycling. In fact, in non-neuronal cells, connecdenn and Rab35 do not regulate CME but control the recycling of cargo from sorting endosomes. Curiously, connecdenn/Rab35 specifically control the recycling of MHCI, which enters the cell independently of CME but do not influence the trafficking of the transferrin receptor, a prototypical CME cargo. Overall, my thesis work on connecdenn has allowed us to appreciate three important conclusions. First, connecdenn is a CCV protein that functions at the neuronal synapse to regenerate SVs. Second, connecdenn can function downstream of CME where it activates Rab35 to control cargo-specific recycling routes. Finally, and importantly, the DENN domain of connecdenn is a GEF for Rab35. This leads the way to study DENN domains of other proteins in respect to Rab GTPase function to better understand trafficking pathways and mechanisms.

Résumé

Le trafic intracellulaire est un processus essentiel pour la division, différenciation et survie cellulaire. Suite à l'endocytose, le cargo est acheminé vers l'endosome de triage qui le dirigera vers la voie de dégradation ou le recyclera à la surface de la cellule. Le trafic endosomal est régi par les petites GTPases Rab, basculant entre la conformation inactive GDP-chargé et active GTP-chargé. Dans l'endocytose tributaire par la clathrine (ETC), la formation de vésicules tapissées de clathrine (VTC) permet l'internalisation du cargo et son couplage au système endosomal. La protéine Connecdenn a été identifiée comme composante des VTCs. L'extrémité C-terminale de Connecdenn est prédite etre non structurée et code plusieurs motifs d'interaction avec les protéines impliquées dans l'ETC comme intersectin1, endophilinA et AP-2. Connecdenn est enrichie sur les VTCs. Lorsque le manteau de clathrine est dissocié, l'association de Connecdenn avec la membrane des VTC demeure stable. Cette association et son recrutement dans les VTCs s'effectuent grâce à son extrémité C-terminale et s'avèrent nécessaires pour le recyclage des vésicules synaptiques. Par contre, l'association de Connecdenn avec la membrane des VTCs dépend de son domaine DENN N-terminal, dont la fonction n'a pas encore été caractérisée. Ce domaine fonctionne également comme un facteur d'échange guanine (FEG). En fait, dans les cellules non-neuronales, Connecdenn et Rab35 ne régularisent pas l'ETC, mais plutôt le recyclage du cargo à partir des endosomes de triage. De plus, l'épuisement de Connecdenn ou Rab35 perturbe spécifiquement le recyclage de MHCI, dont l'internalisation n'est pas régie par l'ETC. Toutefois, l'absence de Connecdenn ou de Rab35 n'a aucune influence sur le trafic du récepteur de la transferrine, cargo typique de l'ETC. Mon travail sur Connecdenn se résume a trois conclusions generales; Connecdenn est une protéine associée à l'ETC fonctionnant dans le recyclage des vésicules synaptiques, Connecdenn peut fonctionner en aval de l'ETC où il activera Rab35 permettant le recyclage du cargo venant de différentes voies d'endocytose à l'endosome de triage et finalement, le domaine DENN de Connecdenn est une FEG pour Rab35. Cette découverte débute l'étude des domaines DENN et leur implication dans la régulation des Rab, permettant une meilleure compréhension des mécanismes gouvernant le transport intracellulaire.

List of abbreviations

a.a.	amino acid
AP	Adaptor Protein complex
ARH	Autosomal Recessive Hypercholesterolemia
BAR	Bin1/amphiphysin/Rvs167
CCV	Clathrin-Coated Vesicle
CCP	Clathrin-Coated Pit
Cdc42	Cell-Division Cycle 42
CHC	Clathrin Heavy Chain
CLC	Clathrin Light Chain
CME	Clathrin-Mediated Endocytosis
Dab2	Disabled2
DENN	Differentially Expressed in Neoplastic versus Normal cells
DH	Dbl Homology
EEA1	Early Endosome Antigen 1
EGFR	Epithelium Growth Factor Receptor
EHD	ENTH homology domain
EM	Electron Microscopy
Eps15	Epidermal growth factor pathway substrate 15
Epsin	EPS15 interacting protein
GAP	GTPase Activating Protein
GDP	Guanosine DiPhosphate
GEF	Guanine nucleotide Exchange Factor
GTP	Guanosine Tri-phosphate
GTPase	Guanosine Tri-phosphatase
KCl	Potassium Chloride
KD	Knockdown
kDa	kiloDalton
HOPS	Homotypic fusion and vacuole protein sorting complex

Hz	Hertz
M6PR	Mannose-6-phosphate receptor
MADD	MAP kinase-Activating Death Domain protein
MHCI	Major Histocompatibility Class I
NECAP	adaptiN-Ear-binding Coat-Associated Protein)
NPF	Asparagine-Proline-Phenylalanine
N-WASP	Neuronal-Wiskott Aldrich Syndrome Protein
PH	Pleckstrin Homology
PXXP	Proline-X-X-Proline, X is any amino acid
Rab	Ras-associated in Brain
Rac	Ras-related C3 botulinum toxin substrate
RME	Receptor-mediated endocytosis
RTK	Receptor Tyrosine Kinase
TfnR	Transferrin Receptor
TGN	Trans-Golgi Network
TRAPP	TRAnsport Protein Particle protein complex
TrkA	Tyrosine kinase, receptor, type A, also known as Neurotrophic(N)TRK
SH3	Src Homology 3
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptors
SV	Synaptic Vesicle
VPS	Vacuolar Protein Sorting

List of figures and tables

Contribution of authors

Chapter 2 - Connecdenn, a novel DENN domain-containing protein of neuronal clathrin-coated vesicles functioning in synaptic vesicle endocytosis

The rational for this project was devised by Dr Peter S. McPherson and myself. Dr Valerie Legendre-Guillemin generated the shRNA viral transfert vector and did the preliminary tests to evaluate the knockdown efficiency against connecdenn. The virus was produced by Dr Beverly L. Davidson. Excellent technical assistance was provided by Jacynthe Philie.

Contribution of figures:	Patrick Allaire	figures 1, 2, 4, 5a, 5b, 6, 7 and 8b
	Dr Brigitte Ritter	figures 2b, 3a/b
	Dr Sebastien Thomas	figure 8a/c/d
	Jonathon Burman	figure 5c
	Drs Alexei Yu Denisov	figure 3c/d

Chapter 3 - The connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes

The rational for this project was devised by Dr Brigitte Ritter and myself with the assistance of Dr Peter S. McPherson. miRNA lentiviruses to knockdown connecdenn and Rab35 was generated and tested by Dr Brigitte Ritter. Excellent technical assistance was provided by Jacynthe Philie.

Contribution to figures:	Patrick Allaire	figures 1, 2, 3, 4, and suppl.1
	Dr Claudia Dall' Armi	figure 1E
	Andrea L. Marat	figure 3E
	Dr Brigitte Ritter	figures 4, 5, 6, 7, suppl. 2 to 6

Chapter 1 - Introduction

Rationale and objectives of the research

The plasma membrane of the cell is a highly dynamic interface that allows cells to sense their environment via plasma membrane receptors. These receptors transmit extra-cellular stimuli into the interior of the cell where they are integrated, allowing the cell to adapt to changes in the environment. This adaptation crucially depends on the modulation of the plasma membrane composition, which is achieved through endocytosis (internalization) and secretion of the plasma membrane components. Endocytosed molecules traffic to various intracellular compartments where their fate is decided between recycling back to the plasma membrane or degradation. The proper function of this trafficking system is crucial for cellular homeostasis, division, differentiation, and function. Consequently, understanding the mechanisms of the various steps that involve intra-cellular trafficking such as the formation of cargo carriers during endocytosis and their delivery to specific compartments is vital. The objective of my research was to identify and characterize novel proteins functioning in one form of endocytosis named clathrin-mediated endocytosis.

Review: From clathrin-mediated endocytosis to trafficking of recycling membranes and cargo via the endosomal system

A. Endocytosis

Many forms of endocytic routes exist that have evolved for uptake of specific cargo. Endocytosis can be broadly subdivided into two groups based on uptake volume, with large volumes entering through phagocytosis and macropinocytosis, and small volumes by pinocytosis. Phagocytosis is a specialized form of endocytosis used by macrophages to remove various macromolecules, viruses, cells or cellular debris whereas macropinocytosis is restricted to fluids. Phagocytosis sends its resulting vesicles directly to lysosomes, organelles that are specialized in degradation, while macropinocytosis sends its vesicles to the cargo-sorting organelles in the endosomal system. In pinocytosis, resulting cargo carriers are much smaller and generally thought to converge with the sorting endosome, a cargo-sorting organelle. This pathway is used for the uptake of nutrients such as transferrin and for the control of the activity of plasma membrane receptors and their expression levels. It is also required for cellular processes such as cell division, cellular migration, and neurite outgrowth. Except for clathrin-mediated endocytosis (CME), the mechanisms for the various forms of pinocytosis are not well understood and only few key regulatory proteins and cargo have been identified. In addition, certain types of cargo can often use different endocytic routes depending on the cellular and stimulatory context. This is especially observed for receptor tyrosine kinases (RTKs) such as the epithelium growth factor receptor (EGFR). Other cargo such as GPI-linked proteins that enter through the caveolae pathway or the transferrin receptor that enters through CME are pathway-specific cargo and are thus good markers to study these endocytic pathways. Table 1.1 summarizes the key points of the known endocytic pathways, associated cargo and molecular requirements.

	GTPase dependencies				
Route	**Rab5**	**Dynamin**	**Other GTPases**	**Associated Cargo**	**Morphology**
Clathrin-mediated	yes	yes	Arf6	TfnR, RTKs, GPCR, Anthrax toxin	Vesicular
Caveolin1-mediated	yes	yes	Cdc42	GPI-linked proteins, CTxB, SV40	Vesicular/Tubular
Flotilin-dependent	unknown	yes	unknown	CD59, proteoglycan, CTxB	Vesicular
Arf6-dependent	yes	no	Arf6	MHCI, CD1, CD59, carboxypeptidaseE	Vesicular/Tubular
GLIC/GEEC	unknown	Unknown	Cdc42/Arf1	GPI-linked proteins, CTxB	Tubular/Ring-like

Table 1.1 Overview of endocytic routes and associated cargo. Endocytic routes that do not use protein coats are in *italics*. GTPases are proteins that have functions associated with guanine triphosphate (GTP) nucleotide binding and hydrolysis. Dynamin is a large GTPase that pinches vesicles off the plasma membrane upon GTP hydrolysis. Arf1, Arf6, Cdc42 and Rab5 are molecular switches of the small GTPase superfamily (discussed below) that allow formation of a specific pathway upon activation by binding GTP. TfnR: transferrin receptor, RTK: receptor tyrosine kinase, GPCR: G-protein coupled receptor, GPI-linked: Glycosylphosphatidylinositol-linked, CD1 and CD59: cluster of differentiation 1 and 59, CTxB: cytotoxin B, SV40: Simian vacuolating virus 40.

CME is the best understood form of endocytosis. During CME, various transition structures such as clathrin-coated pits (CCPs) and clathrin-coated vesicles (CCVs) are readily recognizable with their distinct poly-pentagonal/hexagonal lattices or soccer ball-like appearance (figure 1.1a/b). This appearance is the result of the polymerization of clathrin triskelia, trimers formed by three clathrin heavy chains in association with clathrin light chains, into an electron dense scaffold (figure 1.1a) that holds the invaginating membrane in place, preventing it from collapsing back into the plasma membrane (figure 1.1c) [1-4].

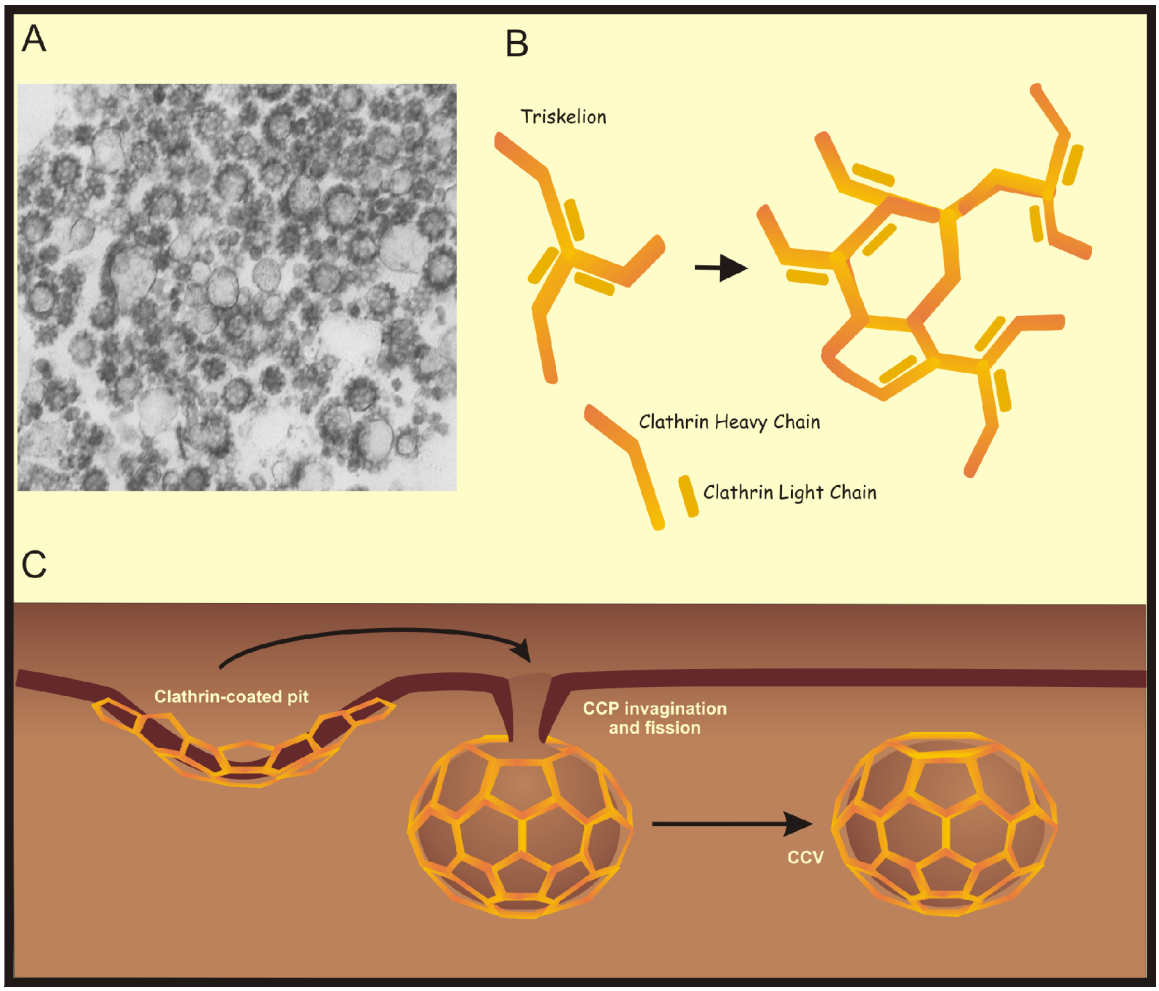

Figure 1.1 Clathrin-coated structures. A. Electron-microscopy micrograph of CCVs purified from brain, magnified 30,000 times. **B.** Cartoon example of a clathrin triskelion structure that polymerizes into pentagons and hexagons. The short bar is the clathrin light chain and the kinked bar attached at the center to two other kinked bars is the clathrin heavy chain. **C.** Cartoon representation of the various clathrin structures found in the cell. Clathrin triskelia are recruited to plasma membrane micro-domains for the formation of a clathrin-coated pit. With the help of a variety of other endocytic proteins, the pit is invaginated into a vesicle and cleaved off as a clathrin-coated vesicle.

Within the last years, several proteomics studies on isolated CCVs enabled us to appreciate the protein complexity of this organelle as well as observing unexpected findings [5, 6]. The proteins involved in CME can be sub-grouped based on their

functional roles and are recruited or activated during specific stages to allow progression from cargo recruitment and formation of CCPs to CCV budding. Apart from clathrin, which provides the structural aspect of CME, other CME proteins are grouped as adaptors or accessory proteins [4, 5]. All adaptors share the ability to bind the plasma membrane in a PI(4,5)P_2-dependent way, to recruit cargo into CCPs, to recruit clathrin and enhance its polymerization at the growing CCP, and finally, to recruit a vast array of accessory proteins. Because of their central role, adaptor proteins along with clathrin, which also binds many accessory proteins, are considered organisatory hubs and interfering with their function dramatically inhibits CME [7, 8]. Table 1.2 lists adaptor proteins involved in CME along with some of their specific function.

Adaptor	Domain	Motifs recognized	Cargo example	Reference
AP-2	μ2	YXXΦ and NPXY	TfnR, EGFR	[9, 10]
AP-2	α2/σ2	(DE)XXXL(LI)	MHCII	[11]
AP-2	β2-ear	DE_nX_{1-2}FXX(FL)XXXR	β-arrestin, ARH, Epsin	[12]
β-Arrestins	N-domain	phoshorylation	GPCRs	[13, 14]
Dab2/ARH	PTB domain	FXNPXY	LDLR	[15],[16]
Numb	PTB domain	FXNPXY	LDLR, EGFR, Notch	[17, 18]
Epsin/Eps15	UIM	ubiquitined cargo	EGFR	[19]
HIP1/HIP1R	unknown	unknown	AMPAR (GlutR)	[20]

Table 1.2. CME adaptors and cargo recognition. Domain (column 2) refers to the part of the adaptor (column 1) that recognizes a motif (column 3) in the cargo (column 4) to be recruited into clathrin-coated pits. Motifs can be a specific amino acid sequence, or via a phosphorylated or ubiquitinated amino acid, usually within a specific sequence. AP-2: adaptor protein 2, Dab2: disabled 2, ARH: (autosomal recessive hypercholesterolemia, HIP1/HIP1R, PTB: phosphotyrosine binding, TfnR: transferrin receptor, MHCII: major histocompatibility complex II, VMAT: vesicular monoamine transporter, GCPR: G-protein-coupled receptor, LDLR: low density lipoprotein receptor, EGFR: epithelium growth factor receptor, GlutR: glutamine receptor. UIM: ubiquitin-interacting motif. X indicates any amino acid,

Adaptor protein 2 (AP-2) is the most important adaptor in CME and also required for the efficient function of other adaptors as in fact, these other adaptors bind to AP-2 [12]. AP-2 interacts with at least 22 other accessory proteins [8], which is more than double than any of the other proteins functioning in CME. Thus, AP-2 knockdown severely inhibits endocytosis of CME-specific cargo such as the transferrin receptor and also affects endocytosis of cargo utilizing other adaptors such as ARH, Dab2, β-arrestins, and HIP1 [21]. AP-2 is a hetero-tetrameric protein complex and belongs into the AP family comprising AP-1 to 4. AP-2 is composed of two large, similarly structured subunits α- and β2-adaptin, and two small subunits σ2- and μ2- adaptin (figure 1.2). In general, σ2-adaptin provides stability of the hetero-tetramer, while the μ2 subunit binds to protein cargo and $PI(4,5)P_2$. α- and β2-adaptin are organized into two domains, the trunk and ear, connected by a flexible linker. The ears are further divided into the platform and sandwich sub-domains, each serving as a protein interaction interface recognizing specific peptide motifs. Both, α- and β2-ear recruit various accessory proteins required for CME. In addition, the α-trunk recruits AP-2 to the plasma membrane by binding to $PI4,5P_2$. The β2-ear binds CHC, ARH, Dab2, and β-arrestin. The most extensive group of proteins involved in endocytosis are the accessory proteins. As mentioned, these proteins are recruited to CCPs via peptide motif interactions with the α- and β2-ear of AP-2, with other adaptors, and with the clathrin heavy chain. Further, some accessory proteins are indirectly recruited via binding to other accessory proteins, often through the interaction of PXXP motifs with SH3 domain proteins (reviewed in [5]).

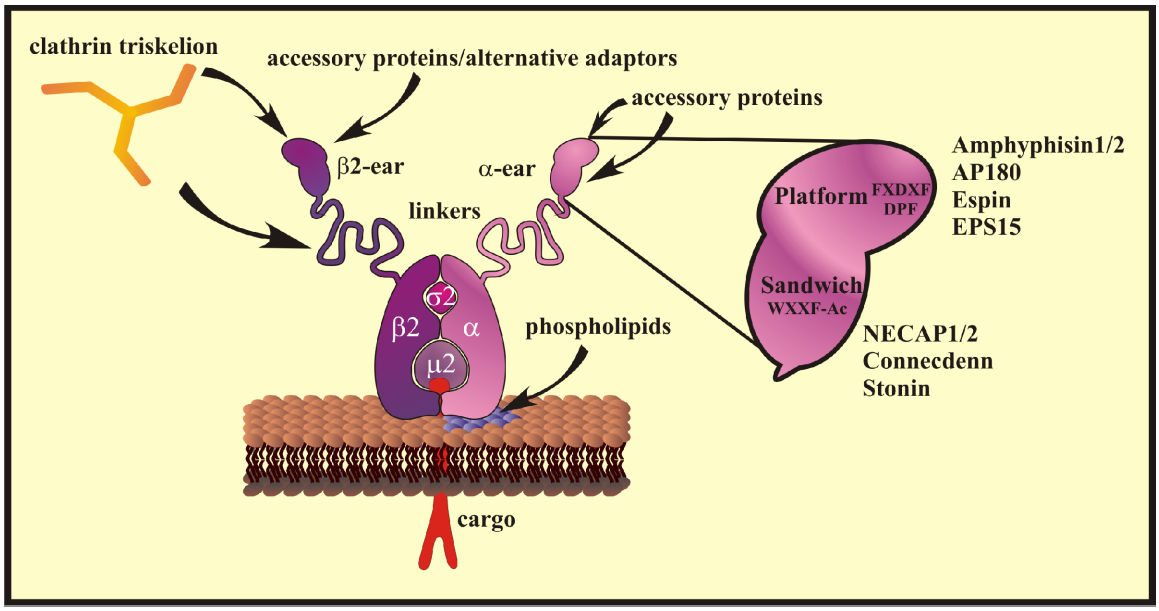

Figure 1.2 AP-2 structure and function. AP-2 is a hetero-tetramer like the other family members AP-1, AP-3, and AP-4, which all share a similar structural organization and function. Arrows indicate binding regions for indicated protein(s) or phospholipids. Note that the μ2 subunit binds both cargo and PI(4,5)P2, similar to the α-trunk (not indicated). The σ2 subunit serves mainly to stabilize the AP-2 complex. The enlarged α-ear domain indicates the two globular sub-domains, which serve as binding sites for three distinct types of amino acid interaction motifs. The platform sub-domain binds FXDXF (where X is any amino acid) or DP(F/W) motifs, whereas the sandwich sub-domain binds WXXF-Ac (where X is any amino acid and Ac being an acidic amino acid or the C-terminal carboxy group). Examples of accessory proteins binding the platform or sandwich sub-domains are indicated at the corresponding region. Note that the β2-ear also binds accessory proteins as well as alternative adaptors.

CME events can be roughly described with 5 steps from CCP formation to CCV un-coating. Each step requires the function of a specific set of proteins for the process to continue (figure 1.3).

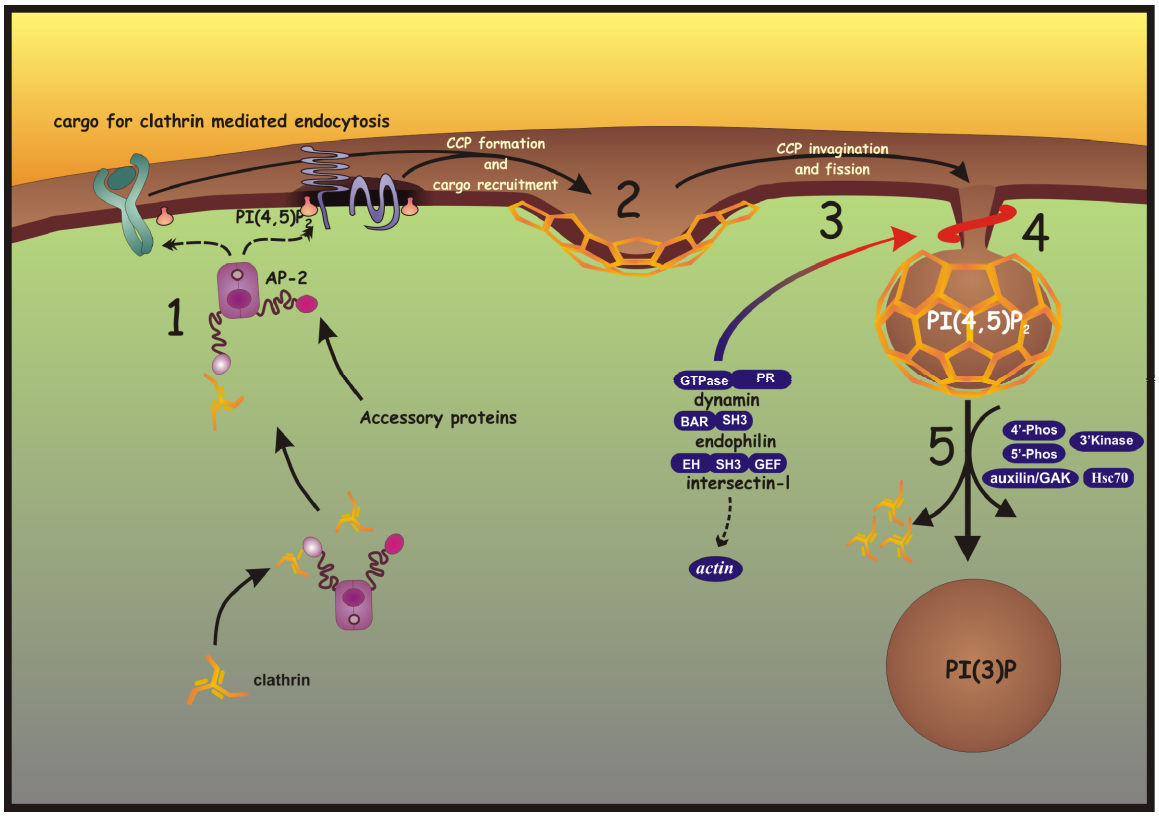

Figure 1.3 Sequence of events during clathrin-mediated endocytosis. See text on page 18-19 for details. 1. Formation of CCP. 2. Recruitment of cargo to pre-existing CCPs. 3. Invagination of CCP. 4. Recruitment of dynamin and vesicle release. 5. Uncoating of CCVs.

1. Formation of CCPs: regions of high $PI(4,5)P_2$ in the plasma membrane recruit AP-2 [22, 23]. This in turn leads to the recruitment of clathrin triskelia and accessory proteins such as epsin, which contains a BAR domain that deforms the plasma membrane and aids in membrane invagination [24].

2. Recruitment of cargo to pre-existing CCPs: Cargo ready to be endocytosed is recognized by AP-2 or other adaptors and recruited into pre-existing CCPs. This leads to a concentration of adaptors and accessory proteins within the pit, increasing the efficiency of pit formation and likelihood of successful endocytosis [25, 26]. One positive feedback loop in this process involves AP-2 α-ear-mediated recruitment of the accessory protein kinase AAK1, which in turn phosphorylates μ2-adaptin and thereby

enhances the affinity of AP-2 for $PI(4,5)P_2$, resulting in a stabilization of AP-2 in the CCP [27].

3. Invagination of CCPs: As cargo is further accumulated, various accessory proteins such as the BAR domain containing protein amphiphysin are recruited. With its BAR domain, amphiphysin has the ability to negatively bend membranes through electrostatic membrane interactions [28]. This, with the concerted recruitment of proteins functioning on actin such as intersectin1-l and HIP1, which locally remodel the actin cortex to release plasma membrane tension and to create force to pull the vesicle inward, allows for membrane invagination [29, 30]. These steps are coordinated with the polymerization of clathrin triskelia on the invaginating membrane to prevent it from collapsing back into the membrane [26].

4. Recruitment of dynamin and vesicle release. When the pit is deeply invaginated, the edges of the pit will come in close proximity, forming a neck-like structure between the deeply invaginated pit and the plasma membrane. This creates local accumulation of amphiphysin. At its C-terminus, amphihysin bears a SH3 domain that enables it to recruit dynamin and to stimulate its activity [31]. Dynamins are a family of large GTPases with membrane-tubulating and fission properties. As dynamin accumulates around the neck, it polymerizes into ring-like structures around it. Dynamin polymerization activates its intrinsic GAP function and conversion of GTP into GDP causes a change in conformation that allows the dynamin ring to stretch the neck and cleave off the vesicle [32-34].

5. Uncoating of CCVs. During CME progression, AP-2 recruits hRME-6, an activator of the small GTPase Rab5 [35]. GTP-bound Rab5 generates PI(3)P by recruiting PI(4)P and PI5P phosphatases and PI(3)P kinases to the vesicle that together convert $PI(4,5)P_2$ to PI3P [36, 37]. The decrease in $PI(4,5)P_2$ lowers the affinity of AP-2 for the membrane, thereby destabilizing the clathrin coat. Concomitantly, increasing levels of PI(3)P recruit auxilin via its PI(3)P-binding domain PTEN [38, 39]. Auxilin in turn recruits, through its

DnaJ domain, Hsc70, a chaperon with ATPase activity that causes efficient clathrin coat removal [40, 41]. Since there is no physical barrier between the CCP membrane and the plasma membrane, PI(3)P can rapidly diffuse out of the forming CCP such that no appreciable amounts of PI(3)P accumulates before vesicle fission. During CCP maturation, $PI(4,5)P_2$ thus still remains abundant, preventing premature coat-removal. Once a physical barrier is established through the dynamin ring formation or CCV release, PI(3)P can accumulate in functional amounts to trigger coat destabilization and removal [38].

B. Formation of the endosomal system

The endosomal system encompasses various inter-related organelles of specific function, which serve as signaling platforms and in the sorting of cargo (also referred to the early endosome) and membrane components. The sorting leads to two possible routes: through the recycling pathways back to the plasma membrane or towards the lysosome in the degradation pathway [42]. Both pathways require specific protein components for their function, which are delivered from the trans-Golgi network (TGN), the source of newly synthesized membranes and trans-membrane proteins [43]. Consequently, the formation of the endosomal system is the results of membrane flow incoming from the plasma membrane and outgoing from the Golgi.

The TGN is the exit site for the secretory pathway and specific cargo targeted to the endosome network. The constitutive pathway the secretion pathway originates from the endoplasmic reticulum (ER), where all new lipids and transmembrane proteins are incorporated into the membrane. From the ER, cargo is trafficked to the cis-Golgi network through Rab1-controlled vesicle delivery [44]. As the cargo traffics through the various Golgi sub-compartments (cis-, median-, trans-Golgi and TGN) they may undergo specific post-translational modifications for sorting in the TGN for constitutive or regulated exit [45].

Following endocytosis, the resulting endocytic vesicles become PI(3)P- and Rab5-positive, a pre-requisite for integration into the endosomal system that allows endocytic vesicles to fuse with each other or with sorting endosomes [46-48]. Within the early endosome, cargos destined for recycling are sequestered and leave toward the Rab11-positive recycling endosome [49]. From the recycling endosome, cargo is sent back to the plasma membrane in a Rab11/Rab22-dependent manner and the transferrin receptor is the best described cargo for this pathway [50-54]. Alternatively, recycling cargo may be sequestered into tubular structures emanating from the sorting endosome, where they may be sent to other poorly defined recycling compartments such as the Rab22 endosomes. Alternatively, cargo in sorting endosomes may directly be sent back to the plasma membrane in a still debated mechanism, possibly involving Rab4 or Rab35 and EHD1. The EHD (ENTH homology domain) protein family consists of four members that have ATPase activity and are proposed to function similarly to dynamins [55]. As such, they have been incorporated into the dynamin super-family [55].

Cargo targeted for degradation remains in early endosomes, which mature to late endosomes and eventually lysosomes, a digestion organelle where vesicular membrane, trans-membrane proteins and their cargo, and the soluble vesicle content are degraded. The process requires the sequential function of Rab5, Rab7, and Rab9 as well as phospholipid conversion from PI(3)P to $PI(3,5)P_2$. This degradation pathway regulates trans-membrane protein turnover and receptor RTK activity and signaling. As such, most RTKs are targeted to the degradation pathway after their activation, which is well described for EGFR [56]. Under specific conditions, some RTKs such as EGFR and TrkA are used to create signaling endosomes, which carry activated kinases back to the nucleus in order to modulate gene expression [57].

The transport of the various cargos to specific compartments requires that they be sorted properly. Sorting of cargo occurs via peptide sorting motifs that are encoded in the cytosolic portion of transmembrane proteins. As for $PI(4,5)P_2$ in CME, the exit from the Golgi apparatus requires PI(4)P to recruit the budding machinery for exit to occur. In general, cargo flows through the constitutive default pathway unless sorted out into the selective pathway to endosomes. Clathrin, AP-1, and AP-3 are the central components of

the coat complexes of the selective pathway. The sorting requires tyrosine- and di-leucine-based peptide motifs that direct cargo into CCPs in a AP-1/Arf1-dependent manner [58]. Vesicles derived from these CCVs are targeted directly to the endosomal system where cargo is then re-sorted and trafficked towards the lysosome. Soluble proteins required for lysosomal function bind to specific receptors such as the M6PR, which enters CCVs [59, 60]. When M6PR and its cargo reach the acidic late endosomes, the cargo is released into the vesicle and subsequently transported to the lysosomes. At late endosomes, M6PR is sorted out of the degradation pathway and recycled back to the TGN [61]. The endosomal system thus relies on the endocytic and exocytic trafficking systems for the establishment of the various functional compartments (figure 1.4)

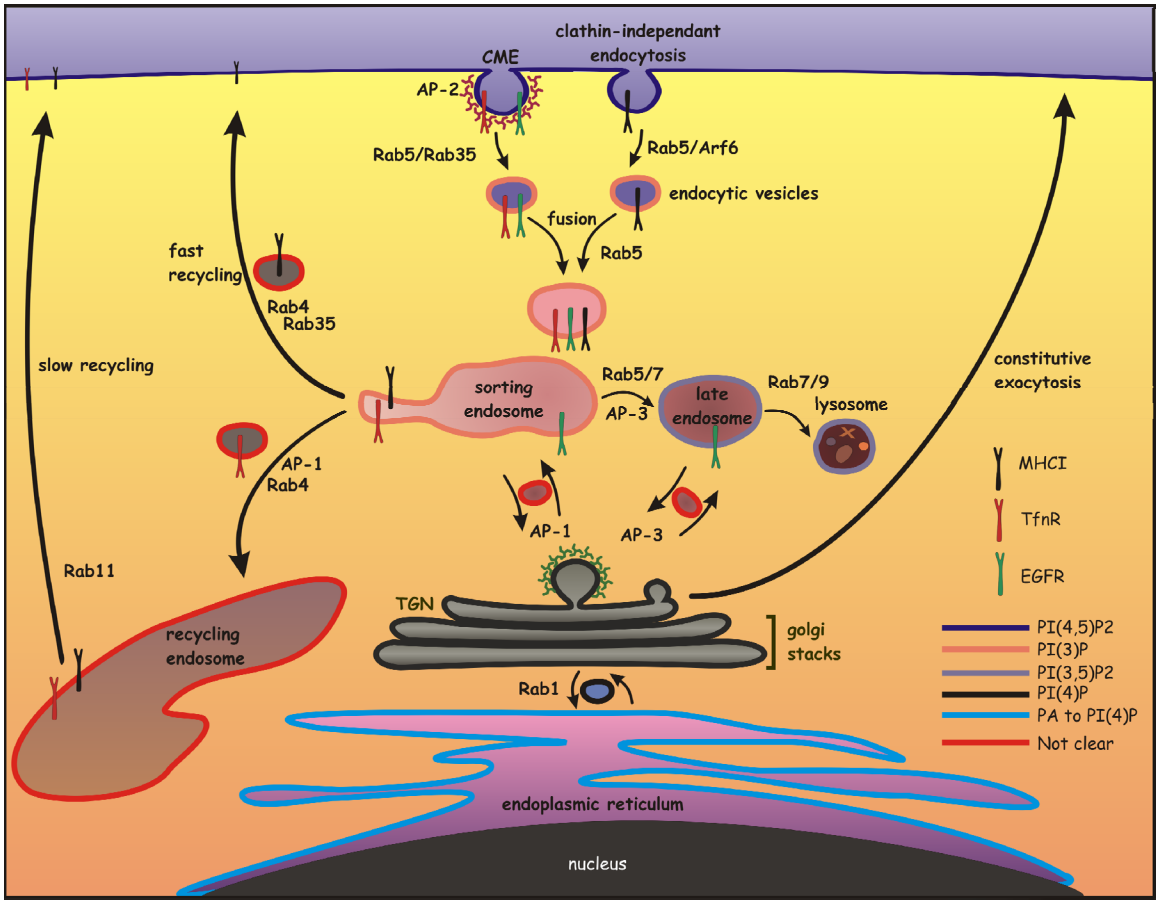

Figure 1.4 The endosomal system. The system is formed by the convergence of membrane from the plasma membrane and the TGN. It serves to sort and regulate the flow of membrane and its components to their final destination for function or degradation. Illustrated are the common, best studied trafficking pathways and their regulators. The various compartments have a defined, specific phospholipid composition, which supports the recruitment of proteins required for their function.

C. Control of endocytic vesicle traffic via Rab proteins

The maintenance and functional integrity of the various compartments illustrated in figure 1.4 is controlled in part by a family of small molecular switches called Rab GTPases. They tightly regulate the various transport steps of cargo vesicles between a donor membrane such as the plasma membrane and an acceptor membrane such as the sorting endosome. The Rab family is part of the Ras super-family of small GTPases, which is divided into 5 families, Ras, Rac, Rab, Arf, and Sar1/Ran. In humans, at least 63 Rab genes have been identified, many of which are conserved from yeast to human [62]. Rab GTPases are present throughout eukaryotes including 11 members in *S. cerevisiae*, 29 in *C. elegans* and 26 members in *D. melanogaster* [62-64]. In addition, mammalian Rab genes generally consist of several exons, and alternative splicing occurs.

Biology of Rab of activation and recycling

Like other small GTPases, Rabs cycle between an inactive GDP-bound form and active GTP-bound form (figure1.5). When GDP-bound, Rabs are generally soluble in the cytoplasm. However, in their GTP-bound form, Rabs localize to the cytosolic face of membranes, where they regulate various membrane trafficking steps by recruiting specific sets of effector proteins. The membrane localization is in part the result of the insertion of their C-terminal prenylated tail. At their C-terminus, Rabs contain a cysteine-based motif for post-translational modification (CXXX, CC, CXC, CCXX or CCXXX), where X is any amino acid) where one or two hydrophobic geranylgeranyl groups are added. This modification requires two sequential steps, first the recognition of the newly synthesized Rab by the Rab escort protein (REP), which then presents the GTPase to the Rab geranylgeranyl transferase (RGGTase) [65]. REP uses a hydrophobic pocket to hide the Rab-bound geranylgeranyl group, thus functioning as a chaperone to keep the Rab soluble in the cytosol. Finally, the REP-Rab complex is delivered to the appropriate membrane via a mechanism still not understood. However, it has been suggested that the targeting relies on membrane receptors or complexes that not only recognize the REP-Rab dimer but also dislocate the lipid modification from the lipid

binding pocket of the REP such that the Rab can be inserted into the membrane. To date no such receptor has been identified biochemically for Rab-REP but GDP/GTP exchange factors (GEF) have been suggested to be part of the recruitment process as delivery is accompanied by the exchange of GDP with GTP and the release of REP. Rab inactivation occurs upon hydrolysis of the bound GTP to GDP. Because the intrinsic enzymatic rate of GTP hydrolysis is very low, Rabs, like other small GTPases typically require a GTPase-activating protein (GAP), which increases the rate of hydrolysis. The membrane-anchored, GDP-bound Rab is then recognized by the Rab GDP-dissociation inhibitor (GDI), which extracts the geranylgeranyl tail from the membrane and brings the Rab back in solution. The GDI thus serves as a recycling factor for Rabs [66].

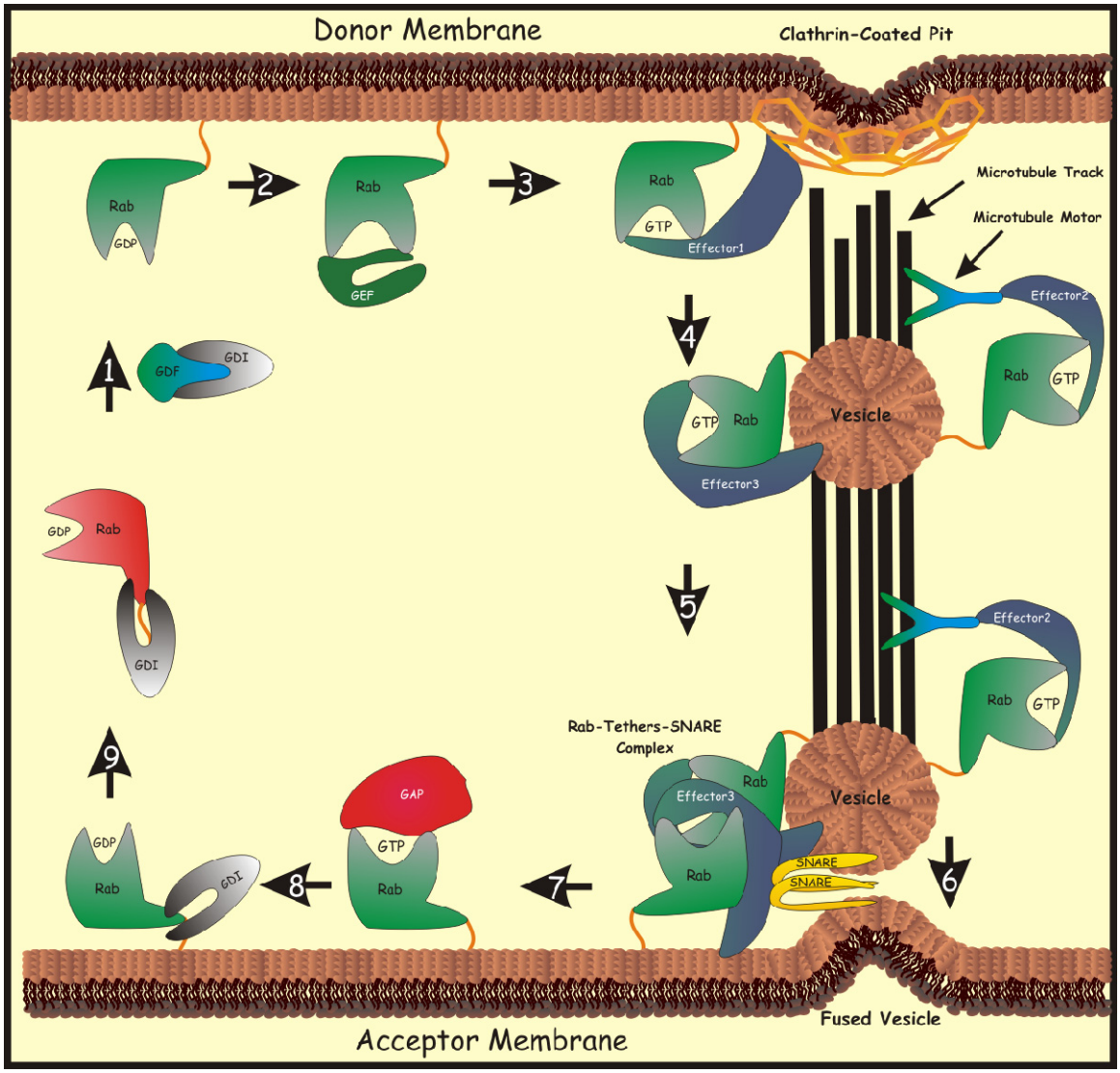

Figure 1.5 Rab recycling and function. The arrows indicate the various Rab states or effector functions. **1)** The GDF (GDI dissociating factor) removes the GDI (GTPase dissociating inhibitor) from the GDP-loaded Rab. This allows the prenylated tail of the Rab to insert into the membrane. **2)** A GEF then binds the GDP-loaded Rab, destabilizing the affinity for GDP in favor of exchange for the more abundant GTP. **3)** GTP-Rab allows for effector recruitment. Effector 1 prepares the budding vesicle for subsequent events (e.g. phospholipid conversion to recruit other factors once the protein coat is removed. **4)** Effector 2 links the vesicle to microtubule motors to allow vesicle transport while tethering factors (effector 3) are recruited onto the moving vesicle **5)** The vesicle tethers to its target site and engages the SNARE complex. **6)** Membrane fusion occurs. **7)** A GAP eventually recognizes the active Rab and provokes GTP hydrolysis to GDP. **8)** The inactive GDP-Rab is recognized by the GDI, and **9)** extracted from the membrane into the cytosol.

Structure-function analysis of Rab GTPases

The Rab family, like other small GTPases, shares a common structural core called the G-domain, which shows significant sequence similarity. The G-domain consists of five parallel and one anti-parallel β-sheet surrounded by five α-helices (figure 1.6). The components responsible for guanine nucleotide- and Mg^{2+}-binding, as well as GTP hydrolysis, are located in the five loops that connect the α-helices and β-strands. The amino acid residues that come together in space to form this active site are closely associated with either the phosphate groups of the bound nucleotide and the Mg^{2+} ion or the guanine base and are highly conserved within the entire Ras superfamily. These conserved residues can be used to recognize any small GTPase. Crystallographic analysis of the structure of small GTPases in the GDP- and GTP-bound state shows that the proteins adopt two different conformations, with the major differences occurring in regions named switch I (amino acids in loop 2) and switch II (amino acids in loop 4, α-helix 2, and loop 5). Both switch regions are crucial for binding to GEFs, effectors, and GAPs [66, 67].

Sequence alignment studies identified five amino acid stretches that are preserved in the Rab family and that distinguish them from the other four families of small GTPases. These stretched are named Rab family (RabF) 1 to 5 and are used to identify novel Rab genes/proteins (figure 1.6). The five RabF motifs are grouped in and around switch regions I and II. In addition to these, four other amino-acid stretches have been used to further subdivide the Rab family into ten sub-families, namely Rab sub-family (RabSF) 1 to 4. The RabSF regions are situated on two different surfaces of the GTPases and are proposed to allow for specific effector binding shared within the sub-family [63, 68]. Therefore, effectors and specific regulators bind both to the RabF motifs in the switch I and II regions to discriminate between active and inactive conformations, while using the RabSF regions to create/enhance specificity. Consequently, sub-family members often show redundancy in function. The Rab C-terminus however, which is implicated in sub-cellular targeting, shows the greatest sequence variation.

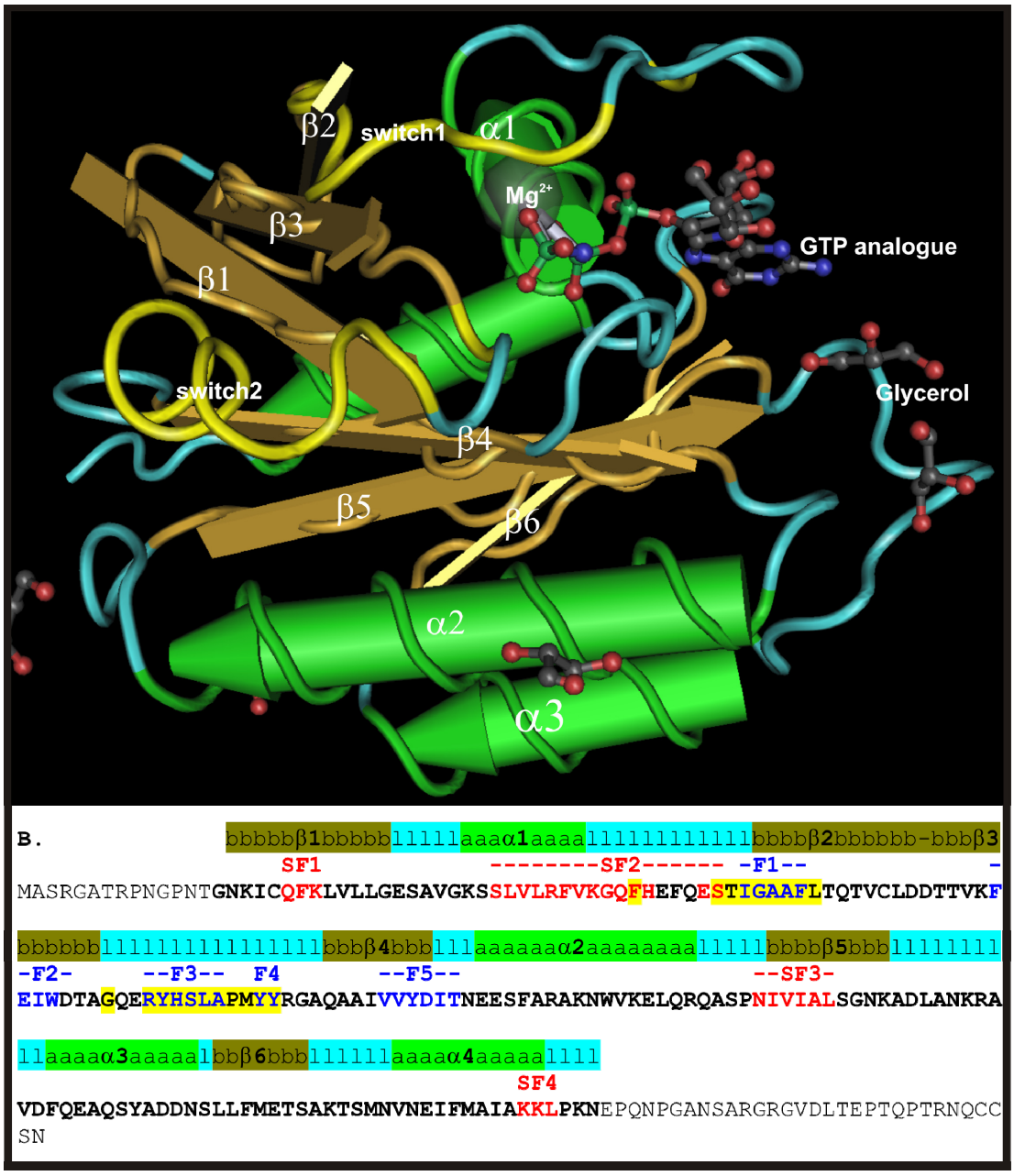

Figure 1.6 Structure analysis of human Rab5A. **A.** Crystal structure of human Rab5A bound to the GTP analogue GppNHp (GTPase domain amino-acid 16 to 185, bold in B). The Mg^{2+} ion (light grey sphere) highlights the GTP-binding pocket. α-helixes are in green and β-strands are in brown. The two switch regions, situated on both sides of the nucleotide-binding pocket, are in yellow. **B.** Amino acid sequence of human Rab5A. The colored bar above the sequence represents the coding regions for β-strands (brown), α-helixes (green) and loops (blue). RabF (blue) and SF (red) signature motifs placed between code bar and sequence. On the sequence, switch regions are in yellow. GEFs, GAPs and effectors make contact with the switch regions depending on their conformation.

Rab effectors - mediators of function

Rab function is mediated by proteins or protein complexes bound to the GTP loaded conformation. These proteins or protein complexes, named Rab effectors, execute a variety of functions that together define the cellular role of individual Rab GTPases.

1. Effectors can bind motor proteins or motor adapters for intracellular transport

Following budding, the resulting vesicle must be transported to their target site in order to deliver their cargo. The cell contains a network of actin and microtubule tracks on which organelles can be actively transported when tethered to either actin motors (myosins) or microtubule motors (kinesins or dyneins). A number of studies have implicated Rabs and their effectors in the regulation of this transport step. For example, Rab5 regulates the transport of early endosomes toward the peri-nuclear area via recruitment of its effector hVPS34, a phosphatidylinositol-3-OH kinase [69, 70]. hVPS34 binds to the kinesin KIF16B and allows for the transport of early endosomes to the plus end of microtubules [70]. Disruption of the interaction of hVPS34 with Rab5 leads to peri-nuclear accumulation of early endosomes, delay in receptor recycling to the plasma membrane and acceleration of degradation [69, 70]. Additionally, active Rab5 has also been linked to vesicle transport through its effector Htt-associated protein 40 (HAP40) [71, 72]. Another interesting example is provided by MADD/DENN, which functions both as a GEF and an effector for Rab3 in regulating axonal transport of vesicles. MADD/DENN enables transport of Rab3 vesicles by bridging them to the motor proteins KIF1Bbeta and KIF1A [73, 74].

2. Effectors in vesicle tethering

Once the vesicle has reached its destination, it is tethered to its target membrane by tethering factors. Tethering factors can be divided into two groups: long coiled-coil proteins and large multi-subunit complexes. The former includes p115 (Uso1p in yeast), early endosome antigen 1 (EEA1), and the Golgins. The latter group includes the exocyst (Sec6/8 complex), TRAPP-I and TRAPP-II, the conserved oligomeric Golgi complex (Sec34/35p complex), and the homotypic fusion and vacuole protein sorting

(HOPS)/vacuole protein sorting (VPS) complex [75]. EEA1, a Rab5 effector, has been well studied in the formation of sorting endosomes from endocytic vesicles. As mentioned above, Rab5 is recruited to forming endocytic vesicles and supports the conversion of $PI4,5P_2$ into PI3P [36]. PI3P in turn recruits EEA1 through its FYVE domain and EEA1 is further stabilized on the vesicle through simultaneous Rab5 interaction [48, 76]. As vesicles converge together or with EEA1-enriched sorting endosomes, the coil-coil domains of EEA1 molecules on the two different compartments homodimerize, and with the help of SNAREs (see below), lead to homotypic fusion. In contrast, heterotypic tethering occurs when the target membrane harbors tethering factors or receptors that are competent for binding with the incoming vesicle such as in synaptic vesicle tethering to the plasma membrane [77]. The tethering process allows for spatial and functional specificity necessary for compartment function.

3. Effectors in membrane fusion

Membrane fusion is performed by members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) family. The members are classified as Qa-Qb-Qc and R-SNAREs based on a conserved glutamine (Q) or arginine (R) residue in the SNARE motif, an unstructured sequence that becomes structured once the right SNAREs are paired [78]. The Qa-Qb-Qc and R-SNAREs can be found on either the vesicles or the target membrane and for fusion to occur, one of each Qa-Qb-Qc and R-SNARE is required. Rabs do not bind directly to SNAREs but indirectly via an effector. For example, the Rab5 effector EEA1 binds to the SNARE syntaxin-13, which is another factor required for homotypic fusion [79].

Fusion and tethering are independent events that can be distinguished both *in vivo* and *in vitro*. *In vitro*, while SNAREs will fuse liposomes, tethering factors will only lead to a clustering [75]. *In vivo*, however, tethering is required for SNARE-dependent membrane fusion [80]. Consequently, SNAREs are not believed to play a significant role in the specificity of tethering.

4. Effectors and cargo sorting

The existence of multiple exit sites within an organelle, like the sorting endosome, for delivery of cargo to specific sub-cellular destinations requires cargo sorting. Generally, Rabs are not considered to be involved in this process but some examples exist. Rab7 and Rab9, better understood in the generation of lysosomes, are also required for recycling of hydrolase-free M6PR from late endosomes back to the TGN. Rab7 recruits the VPS sub-complex, which binds M6PR. The VPS sub-complex, is composed of Vps26/29/35 and is part of the larger retromer complex, which recycles cargo back to the TGN [81]. Rab9 also functions to recycle M6PR via its effector TIP47 (tail-interacting protein of 47kDa). TIP47 binds the cytoplasmic tail of M6PR and this provides a second exit route for M6PR to recycle back to the TGN [82, 83].

Thus, the large number of functions that a Rab can mediate highlights their importance in intra-cellular trafficking. And, indeed, de-regulation of Rab function is associated with various types of diseases [84-88].

D. Control of the synaptic vesicle cycle by Rab proteins

The neuronal synapse is a specialized communication apparatus established between pre-synaptic axon terminals and the post-synaptic dendrites that relays action potentials. In the chemical synapse, the action potential causes fusion of synaptic vesicles (SVs) to the axon terminal membrane to release their neurotransmitter content. The neurotransmitter diffuse toward the dendrites where they bind receptors that will regenerate the action potential. It is thus crucial for neuronal communication to maintain the pool of synaptic vesicles to properly relay signals. This is done by the recycling of synaptic vesicle membrane from the plasma membrane either by clathrin-mediated or bulk clathrin-independent endocytosis [89]. The fusion and retrieval of synaptic vesicle membrane is tightly coupled such that fusion of SVs is followed immediately by their endocytosis. This forms the basis of the synaptic vesicle cycle. SVs are tethered to and fuse with the plasma membrane in what is called the active zone, whereas their endocytosis occurs in the peri-active zone, an area in which numerous endocytic proteins are enriched [90]. Specific cytoskeletal proteins define the two areas and prevent diffusion of the compartments. This compartmentation allows for rapid and efficient recycling by functionally separating and concentrating regulatory proteins for fusion and endocytosis. Finally, endocytosis in the peri-active zone is associated with extensive actin-remodeling, which is thought to provide an additional network of actin tracks to allow for more efficient removal of SVs from the peri-active zone, enhancing endocytosis and overall speed of the cycle (figure 1.7) [91-93].

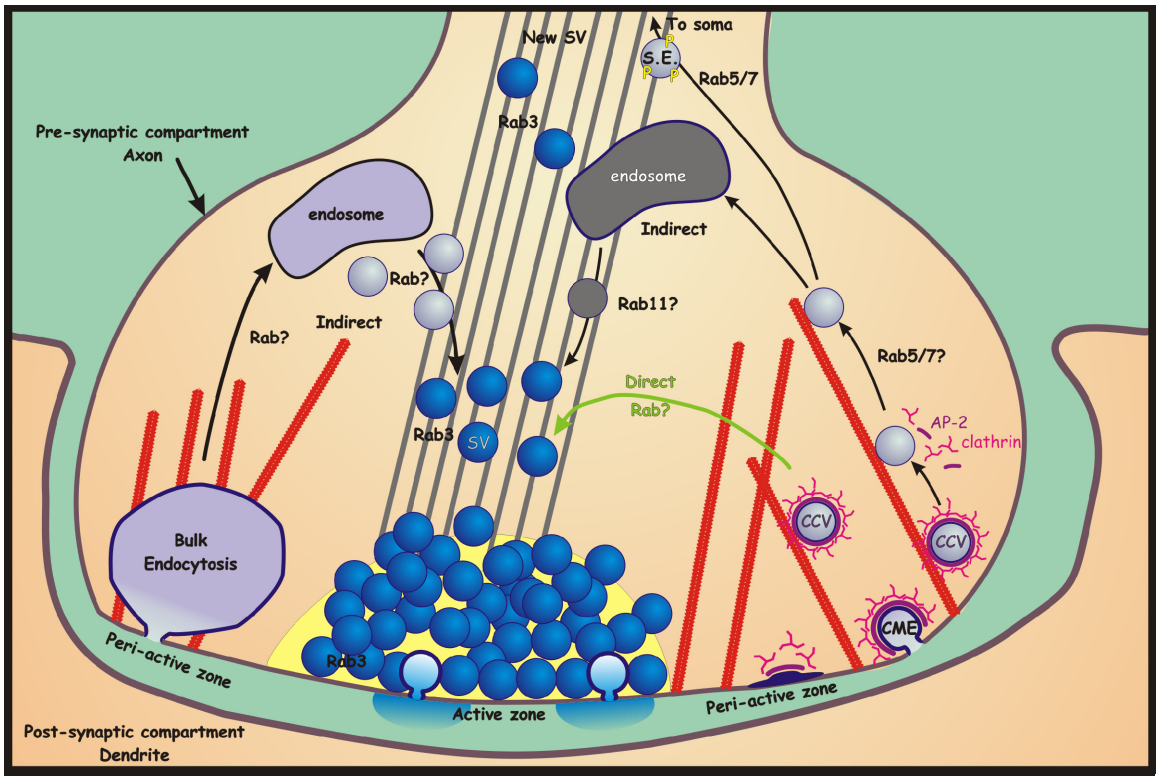

Figure 1.7 Synaptic vesicle regeneration. After SV fusion, SVs can be endocytosed via clathin-dependent (right) or clathrin-independent mechanisms (left). The trafficking of the endocytic vesicle can then take different route to regenerate the SV population. In activity-dependent SV recycling, the endocytic vesicle is directly transported back to the active zone, while being refilled with neurotransmitter to become a new SV. This Rab3-dependent regeneration process is the typical SV cycle. Alternatively, endocytic vesicles may merge with an endosomal compartment via Rab5 function. From there, new vesicles can bud off, be refilled with neurotransmitters and transported to the active zone as new SVs. This indirect route for SV regeneration is not coupled to a burst of exocytosis following nerve depolarization and thus functions either in parallel to or in the absence of the direct SV cycle. Red bars are actin tracks and grey bars are microtubule tracks

Until recently, no endosomal intermediates have been detected in electron microscopy micrographs of the central nervous system (CNS) synapse, and it was therefore believed that SVs recycle directly from the membrane [94-99]. Synaptic terminals in the CNS contain between 100 to 200 SVs per active zone, yet, neuronal activity in these circuits can generate up to 100 action potentials per second (100 Hertz), which would rapidly

delete the SV population unless a rapid mechanism of recycling exists. CME, which is the best studied pathway to directly recycle SVs functions on a timescale of approximately only 15 seconds [97]. This is insufficient to maintain the necessary SV population during high stimulation. Thus, a faster method, called kiss-and-run was proposed, in which SVs fuse but do not completely collapse into the membrane. This scenario would allow for a faster recycling requiring no or a less complex membrane recovering machine. However, this mode of SV recycling only been suggested through indirect observation and its existence remains heavily debated [100]. Lately, several reports using hippocampal neurons knocked down for clathrin, AP2 or from dynamin knockout animals provided direct morphological evidence for an indirect recycling of SVs from endosomal intermediates during and after high frequency stimulation [94, 95, 97-99]. The endosomal intermediates are the result of bulk membrane endocytosis that is triggered during high frequency stimulation. The molecular determinants involved in this pathway are mostly unknown but may involve AP-1, AP-3, clathrin, dynamin and certain Rabs associated with recycling such as Rab11.

In the SV cycle, CME and exocytosis are far better understood than the mechanisms underlying the conversion of endocytic vesicles or endosomal intermediates into SVs, in which Rab proteins likely play a crucial role. The best studied step is the exocytosis of SVs, which is thought to be mainly regulated by the Rab3 sub-family (Rab3A, Rab3B, Rab3C, and Rab3D) and secondarily, the Rab11 sub-family (Rab11A, Rab11B, and Rab25). The reported functions of Rab3 proteins in exocytosis encompass transport, tethering, and fusion of SVs at the active zone [73, 101, 102]. Knockout studies of Rab3 sub-family members have shown remarkable redundancy in their function [103]. Electrophysiological studies in Rab3A$^{-/+}$, B$^{-/-}$, C$^{-/-}$ and D$^{-/-}$animals demonstrated that Rab3 functions in stimulated SV exocytosis but not in spontaneous release. This demonstrated that the Rab3 sub-family is not essential for synaptic membrane trafficking but functions to modulate the basic release machinery, implying the participation of other Rabs in exocytosis.

The function of Rab3 in exocytosis has been mostly attributed to its effector RIM1α/2α, which helps in organizing multi-protein complexes that in turn engage the SNAREs syntaxin and SNAP-25, thereby directly mediating SV fusion at the active zone [104-106]. Recently, however, a series of experiments have also shown a role for Rab3 in trafficking of SVs on microtubules. The multidomain protein DENN (Differently Expressed between Neoplastic and Normal cells) functions as both a GEF and effector for Rab3 [73, 107, 108]. The protein contains an N-terminal DENN domain and in its C-terminal region a death domain. The specific requirement for the GEF activity has not yet been determined. However, the effector role was recently localized within the N-terminal DENN domain, which is required to bind Rab3. Via its death domain, DENN also binds KIF16, thus bridging Rab3 vesicles to microtubule assisted movement [73]. A similar phenotype was observed in *C. elegans* for mutations in Aex-3, the worm homolog of MADD/DENN [74]. Consequently, Rab3, apart from its role in calcium-triggered SV release, is also involved in SV transport toward the active zone.

Besides the Rab3 sub-family, only members of the Rab11 sub-family have been shown to directly impair stimulus-induced exocytosis of SVs [109]. However, these studies have been done in PC12 cells, and contrary to CNS neurons, SV recycling in PC12 has a well defined endosomal intermediate [110-112]. This endosomal intermediate could serve as a specialized recycling compartment, where Rab11 would function similarly to its established role on the recycling endosome in non-neuronal cells. Interestingly, Rab11 is enriched on purified SVs similar to Rab3 [113], suggesting that endosomal intermediates do form during the SV cycle in CNS neurons. The current data calls for a closer investigation of the function of Rab11 in the SV cycle in the CNS synapse.

In addition to Rab3 and Rab11, Rab5 and Rab7 are also enriched on SVs [113]. The function Rab5 on endocytic vesicles suggests that it could have a role in the SV cycle. Over-expression and Rab5 effector knockdown studies in hippocampal neurons, *D. melanogaster* photoreceptor cells, and *C. elegans* CNS neurons demonstrated that Rab5 had some effect on recycling, SV size and maintenance of the SV pool [114, 115]. However, the detail of its function in SV recycling, is vague and because over-expression did not affect activity-induced recycling it has been suggested that Rab5 may function to

support and/or replenish the SV pool during inactivity. Finally, the function of Rab7 in the SV cycle has not been studied but together with Rab5, it is involved in the generation of signaling endosomes. The signaling endosome is an endosome generated at the tip of the axon that carries activated plasma member RTKs such as TrkA back to the cell body where kinases are activated to transmit signals for neuronal differentiation and survival [116-118]. It is thus possible that a specific portion of SVs may be redirected via Rab5/Rab7 mechanisms back to the cell center for neuronal survival instead of use in synaptic transmission. Alternatively Rab5, 7, and 11 may function to recycle SVs from endosomal intermediates formed after bulk endocytosis and thus might be a part of the SV cycle.

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Research Rational

CME is used for house keeping function and also in specialized forms in various tissues and during development. Consequently, determining the basic and variable components of CCVs will help us to better understand how CME serves these functions. CCVs are intermediary organelles formed during CME and can be efficiently purified. Recent developments in proteomics allows for analysis of protein components of sub-cellular protein complexes and organelles such as CCVs. In fact, the recent proteomic analysis of rat brain and liver CCVs led to the realization of unexpected functions of the clathrin light chains and identification and characterization of several uncharacterized proteins such as enthoprotin, RME-8, and NECAPs. To better understand the composition and function of CCVs in brain during developing, I developed a protocol to purify CCVs from post-natal day 5 rat brains in order to identify novel proteins not present in CCVs isolated from adult brain. This led to the identification and characterization of connecdenn, and more importantly, to the discovery of a new domain with guanine nucleotide exchange activity toward the small GTPase Rab35.

Chapter 2. Connecdenn, a novel DENN domain-containing protein of neuronal clathrin-coated vesicles functioning in synaptic vesicle endocytosis.

Abstract

Clathrin-coated vesicles (CCVs) are responsible for the endocytosis of multiple cargo, including synaptic vesicle membranes. We now describe a new CCV protein, termed connecdenn, that contains an N-terminal DENN (differentially expressed in neoplastic versus normal cells) domain, a poorly characterized protein module found in multiple proteins of unrelated function and a C-terminal peptide motif domain harboring three distinct motifs for binding the α-ear of the clathrin adaptor protein 2 (AP-2). Connecdenn coimmunoprecipitates and partially colocalizes with AP-2, and nuclear magnetic resonance and peptide competition studies reveal that all three α-ear-binding motifs contribute to AP-2 interactions. In addition, connecdenn contains multiple Src homology 3 (SH3) domain-binding motifs and coimmunoprecipitates with the synaptic SH3 domain proteins intersectin and endophilin A1. Interestingly, connecdenn is enriched on neuronal CCVs and is present in the presynaptic compartment of neurons. Moreover, connecdenn has a uniquely stable association with CCV membranes because it resists extraction with Tris and high-salt buffers, unlike most other CCV proteins, but it is not detected on purified synaptic vesicles. Together, these observations suggest that connecdenn functions on the endocytic limb of the synaptic vesicle cycle. Accordingly, disruption of connecdenn interactions with its binding partners through overexpression of the C-terminal peptide motif domain or knock down of connecdenn through lentiviral delivery of small hairpin RNA both lead to defects in synaptic vesicle endocytosis in cultured hippocampal neurons. Thus, we identified connecdenn as a component of the endocytic machinery functioning in synaptic vesicle endocytosis, providing the first evidence of a role for a DENN domain-containing protein in endocytosis.

Material and Methods

Antibodies and peptides. Monoclonal antibodies against the following proteins were from the indicated commercial sources: α-adaptin [clone 8 (BD Transduction Laboratories, Lexington, KY) and clone AP.6 (Upstate, Charlottesville, VA)], dynamin, and Na^+/K^+ ATPase α-1 (Upstate), clathrin-heavy chain (CHC) (BD Transduction Laboratories), Flag (M2; Sigma, St. Louis, MO), postsynaptic density-95 (PSD-95) (mAb-045; Affinity BioReagents, Golden, CO), actin (Chemicon, Temecula, CA), and synaptophysin (Sigma). Polyclonal antibodies recognizing endophilin A1 [119], intersectin-s and -l (2173) [120], and NECAP 1 [121] were previously described. A polyclonal antibody against synaptotagmin was described previously [122] and was a gift from Dr. Pietro De Camilli (Yale University, New Haven, CT). Polyclonal sera recognizing connecdenn were raised in rabbits (3775 and 3776) against a synthetic peptide containing amino acids 1002–1016 of the mouse connecdenn protein with an added N-terminal cysteine (CVEQLRRQWETFE) coupled to keyhole limpet hemocyanin (KLH). Polyclonal sera recognizing clathrin-light chain a and b (CLCa/b) were raised in rabbits (4045 and 4046) against a synthetic peptide encoding amino acids from the conserved domain with an added N-terminal cysteine (CEEDPAAAFLAQQESEIAGIEND) coupled to KLH. Peptides used for competition assays were as follows: DPW peptide from epsin 1, CSDPWGSDPWG; FXDXF peptide from amphiphysin 1, CSFFEDNFVPE; and WXXF-acidic peptide from NECAP 1, CQAPQPSNWVQF. All synthetic peptides were purchased from Howard Hughes Medical Institute/Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT).

Expression constructs. Bacterial expression constructs for the adaptin ear domains of adaptor protein 1 (AP-1) and AP-2 were described previously [121]. Glutathione *S*-transferase (GST) fusion proteins of the -ear point mutations R707A, F740A, and Q782A were described previously [121], and point mutation R707A was generated using the megaprimer approach [123] with the oligonucleotide GCGCGAATTCGGCTCTGAAGACAACTTTGCCGCGTTTGTTTGTAAAAATAATG

GTG. GST fusin proteins of the various Src homology 3 (SH3) domains of intersectin ([124] and the SH3 domain of amphiphysin I [125] and II [126], endophilin A1 [119], and PKC and CK2 substrate in neurons 1 (PACSIN 1) [127] were described previously. The PACSIN 2 construct was a generous gift from Dr. Markus Plomann (University of Cologne, Cologne, Germany). Flag-tagged full-length connecdenn (residues 1–1016) was generated by PCR from the full-length mouse expressed sequence tag clone (gi31542026) using forward primer CGCGAATTCATGGGCTCCAGGATCAAG and reverse primer CGCGTCGACTCACTCAAAGGTCTCCCAC with subcloning into pCMV-Tag-2B. The C-terminal peptide motif domain (residues 372–1016) was made the same way using the forward primer CGCGAATTCCTAGACCTTCTCAATTCCG and the reverse primer from above with cloning into peGFP-C2. All constructs were verified by sequence analysis.

Binding studies. Transfected HEK-293 cells were scraped in HEPES buffer (10 mM HEPES-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulphonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin), incubated with 1% Triton X-100 and 150 mM NaCl for 30 min at 4°C, and then centrifuged for 30 min at 205,000 x *g*. Aliquots of the supernatant were incubated for 1 h at 4°C with GST fusion proteins precoupled to glutathione-Sepharose before washing in HEPES buffer with 1% Triton X-100 and 150 mM NaCl. Adult rat brain was homogenized in HEPES buffer and centrifuged at 800 x *g* for 5 min. The supernatant was incubated with 1% Triton X-100 and 33 mM NaCl for immunoprecipitation assays or 150 mM NaCl for GST pull-down assays for 30 min at 4°C and then centrifuged for 30 min at 205,000 x *g*. Aliquots of the supernatant (2 mg) were incubated for 1 h at 4°C with GST fusion proteins precoupled to glutathione-Sepharose before washing in HEPES buffer with 1% Triton X-100 and 150 mM NaCl. For competition experiments, extracts were incubated for 1 h at 4°C with 400 pmol of GST–-ear in the presence of a 300- to 1000-fold molar excess of peptide as indicated. For coimmunoprecipitation studies, 2 mg aliquots of soluble brain extract were incubated with 5 μl of AP.6 antibody or 10 μl of 3775 or 2173 sera as well as protein G- or protein A-Sepharose beads, as appropriate. The samples were incubated for 2 h at 4°C before washing in HEPES buffer with 1% Triton X-100 and 33 mM NaCl.

Subcellular fractionation and extraction studies. SVs were purified as described previously [128]. CCVs were purified from rat brain in buffer A (100 mM MES, pH 6.5, containing 1 mM EGTA and 5 mM $MgCl_2$) as described previously [129]. For the extraction of coat proteins, 50–100 μg aliquots of CCVs were centrifuged at 200,000 x *g* for 15 min. The pellets were resuspended in 100 μl of buffer A, Tris buffer (1:1 mixture of buffer A and 1 M Tris, pH 9.5), buffer A with various concentrations of NaCl, or pH 11 buffer (25 mM Na_2CO_3, pH 11) and incubated for 30 min on ice. The samples were centrifuged at 200,000 x *g*, and the pellets were resuspended in buffer A and analyzed in parallel with the supernatant fraction. In other cases, CCVs in buffer A and CCVs extracted with Tris buffer were loaded on the top of linear 20–50% sucrose gradients prepared in buffer A and Tris buffer, respectively, and were centrifuged in a Sorvall (Newtown, CT) AH629 rotor at 145,000 x *g* for 1.5 h. The gradients were fractionated from the bottom, and gradient fractions were analyzed by Western blot.

Nuclear magnetic resonance spectroscopy and α-ear/peptide complex modeling. The preparation of α-ear for nuclear magnetic resonance (NMR) was described previously [130-132]. NMR spectra were acquired at 30°C on 800 MHz Varian (Palo Alto, CA) Unity Inova spectrometer. Detailed analysis of peptide binding to the α-ear was performed by comparison of chemical shifts for backbone amide signals in ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra. HSQC spectra were recorded at 1:25, 1:5, 1:2, 1:1, and 2:1 peptide/protein ratios that were confirmed by UV concentration of both components and intensity of H1 (W) signals in one-dimensional NMR. The HADDOCK approach [133] was used to model the binding of the WETFE residues from the connecdenn peptide to the α-ear using the general protocol described previously [132]. Additional hydrogen bond restraint between the side-chain oxygen of E^{+1} in the peptide and $NH3^{+}$ group of α-ear K727 was added based on X-ray structure of similar WVTFE/α-ear complex [134] and our α-ear mutagenesis data. The figure was generated using the MOLMOL program [135].

Localization studies. Immunofluorescence analysis of rat hippocampal neurons at 21 d *in vitro* (DIV) was performed as described previously [136]. COS-7 cells were plated on

poly-L-lysine-coated coverslips and transfected with Flag-tagged connecdenn using GeneJuice (Novagen, Madison, WI). After overnight expression, cells were fixed with 3% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, and then incubated with primary antibodies for 1 h at room temperature. Cells were subsequently washed and incubated with appropriate secondary antibodies before visualization. Quantification of colocalization was performed with the RG2B colocalization plug-in of NIH ImageJ software, a public domain Java image processing and analysis program inspired by NIH Image for Macintosh (http://rsb.info.nih.gov/nih-image/about.html).

FM4-64 uptake studies. For overexpression studies, hippocampal neurons were transfected after 7–8 DIV with green fluorescent protein (GFP) alone or GFP tagged to the peptide motif domain of connecdenn (residues 372–1016). Endocytosis of SVs was measured at 21–28 DIV using the fluorescent lipid membrane marker FM4-64 *N*-[3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)hexatrienyl)pyridinium dibromide] (10 μM). SV labeling was performed by bath application of 40 mM KCl plus FM4-64 in HBSS for 90 s followed by 2 min in the presence of HBSS plus FM4-64. Background fluorescence was quenched with a 2 ml/min perfusion of HBSS plus ADVASEP-7 (1 mg/ml) for 10 min. To quantify SV endocytosis, we analyzed FM4-64 fluorescence images with NIH ImageJ software. The RG2B colocalization plug-in was used to select GFP-labeled varicosities that colocalized with FM4-64 staining. The colocalized regions were outlined, and the resulting regions of interest (ROIs) were saved and applied on original raw images for measurement of total FM4-64 fluorescence per ROI. The colocalized ROIs were then subtracted, and the remaining FM4-64 punctae, corresponding to active synapses from nontransfected neurons, were measured identically.

For loss-of-function studies, three small interfering RNAs (siRNAs) matching selected regions of mouse connecdenn, with dT overhangs already annealed, were synthesized by Qiagen (Hilden, Germany). siRNA#1 was not effective on transfected mouse connecdenn and was not used further. siRNA#2 (GGCCCAGGCTGCTTTCTTT) and siRNA#3 (GAGCTGCTTCTGTATCTTA) were effective in knocking down transfected mouse connecdenn, with siRNA#3 the most effective in knocking down endogenous connecdenn

in COS-7 cells (data not shown). This sequence was thus selected for generation of short hairpin RNA (shRNA) packaged in a lentivirus delivery system, which was prepared as described previously [137]. Briefly, PCR was used to amplify a mouse U6 promoter followed by a stem–loop–stem structure encoding the sequence of siRNA#3. The forward primer matching the mouse U6 promoter had the sequence GCGCAATTGCGGGAAAACTGAATAAGAG. The reverse primer, matching the 3' end of the U6 promoter followed by the siRNA antisense strand, a loop, the siRNA sense strand, and an RNA Pol III termination sequence, had the sequence GCGCAATTGAAAAAAAGAGCTGCTTCTGTATCTTAT-GACAGGAAGTAAGATACAGAAGCAGCTCTTCAAAACAAGGCTTTTCTCCAAG G. The resulting PCR fragment was cloned into a shuttle vector that was used to generate feline immunodeficiency virus (FIV) expressing the connecdenn shRNA and GFP as described previously [137]. Control virus expressing GFP alone was described previously [137]. Hippocampal neurons were transduced with a multiplicity of infection (MOI) of 3 (GFP/connecdenn shRNA) or 10 (GFP alone) at 14 DIV, and cells were lysed at 21 DIV in SDS gel sample buffer to generate extracts for Western blot analysis. Alternatively, hippocampal neurons were transduced with one-fifth the MOI used for Western blot and were processed for FM4-64 uptake as described for transfection experiments.

Results

Identification of connecdenn

Connecdenn was identified through the presence of the sequences DPF, FSDVF, and WETFE (Fig. 2.1, dark gray ovals) that match consensus α-ear-binding motifs. Connecdenn also contains seven proline-rich stretches with potential SH3 domain-binding motifs (Fig. 2.1, light gray rectangles). At the N terminus, connecdenn contains a DENN domain composed of tandem upstream (u) DENN, DENN, and downstream (d) DENN modules (Fig. 2.1). Connecdenn is detected in multiple species with the mouse protein 27 and 21% identical throughout its length to fly and worm orthologs, respectively. Homology is found in small blocks throughout the proteins with the best conservation in the DENN domain (39 and 33% identity of the DENN domain of mouse with fly and worm orthologs, respectively). The annotated mouse connecdenn has been entered into GenBank under the accession number DQ448594.

Connecdenn is an AP-2-binding partner

Immunoprecipitation of AP-2 from brain extracts leads to a robust coimmunoprecipitation of connecdenn (Fig. 2.2*A*). In pull-down assays, connecdenn from brain extracts binds to GST–α-ear but not to GST fusion proteins encoding the ß2-ear or the ear of the α-adaptin subunit of AP-1 (Fig. 2.2*B*). Flag-tagged connecdenn also binds to GST–α-ear (Fig. 2.2*C*). At low levels of expression in COS-7 cells, Flag-tagged connecdenn displays a punctate pattern that partially colocalizes with endogenous AP-2, a marker of plasma membrane CCPs (Fig. 2.2*D*). Quantification using the RG2B colocalization plug-in of NIH ImageJ software reveals that 44.7% of all connecdenn puncta are colocalized with AP-2 (20 cells from two separate experiments). Given the density of the immunopositive punctate, we rotated the relative orientation of the images by 2°, after which 16.4% of the punctae overlapped with AP-2. Thus, a significant fraction of Flag-tagged connecdenn is localized to CCPs.

We next sought to identify the determinants within connecdenn responsible for α-ear interaction. The α-ear is a bi-lobed structure with FXDXF and DPF/W motifs binding to a site in the platform subdomain and WXXF-acidic motifs binding to a site in the sandwich subdomain [131, 132]. Mutation to alanine of sandwich subdomain residues involved in interactions with WXXF-acidic motifs, including K727, F740, and Q782 [132], interferes with α-ear binding to connecdenn and NECAP 1, whereas mutation of R707, shown not to be involved in binding [138], has no influence (Fig. 2.3*A*). These studies demonstrate an important contribution of the connecdenn WXXF-acidic motif to -ear interaction. However, the residual binding of connecdenn to F740A and Q782A, in the face of no binding of NECAP 1 (Fig. 2.3*A*), which contains WXXF-acidic only [121], suggests that connecdenn can use other α-ear-binding motifs. To address this issue, we performed peptide competition experiments. Addition of DPW, FXDXF, or WXXF-acidic peptides alone, at 300:1 or 1000:1 molar ratio to GST–α-ear fusion protein in pull-down assays, had little or no influence on the binding of the α-ear to connecdenn (Fig. 2.3*B*). Moreover, simultaneous incubation of DPW and FXDXF peptides also has little effect on connecdenn binding (Fig. 2.3*B*). In contrast, DPW or FXDXF peptides do strongly decrease binding when combined with the WXXF-acidic peptide, and a complete block in binding is observed when all three peptides are added simultaneously (900:1 total molar ratio to the fusion protein, whereas individual peptides were added as high as 1000:1 without effect) (Fig. 2.3*B*). Thus, WETFE is the predominant sequence mediating connecdenn binding to AP-2, but the DPF and FSDVF sequences also contribute.

We next examined the interaction of a WETFE peptide from connecdenn with the α-ear by NMR. Interestingly, the chemical shift changes that occur on the α-ear in the presence of the connecdenn peptide, which demonstrate a direct interaction, resemble those seen with a WVQF-COO^{-} peptide from NECAP 1 (Fig. 2.3*C*). Thus, the model of the connecdenn peptide docked to the α-ear (Fig. 2.3*D*) is similar to that seen previously for NECAP [132].

Connecdenn interacts with endocytic SH3 domain proteins

Connecdenn contains a series of PXXP core sequences, suggesting that it is a binding partner for SH3 domains. We thus performed pull-down assays using GST–SH3 domains from multiple endocytic proteins. Flag-tagged connecdenn binds the SH3 domain of endophilin A1 and the SH3A and SH3C domains of the endocytic adaptor protein intersectin (Fig. 2.4*A*). The specificity of connecdenn interactions with SH3 domains is indicated by the lack of binding to the SH3 domains of amphiphysin I and II, PACSIN 1 and 2, and the SH3B, SH3D, and SH3E domains of intersectin (Fig. 2.4*A*). Dynamin demonstrates the expected pattern of binding in these experiments (Fig. 2.4*A*). Intersectin has two splice variants, intersectin-short (s) and intersectin-long (l), that share the five SH3 domains, with intersectin-l containing additional C-terminal sequence [120, 139]. Endophilin A1 comprises an N-terminal BAR (Bin1/amphiphysin/Rvs167) domain coupled to the C-terminal SH3 domain [140-142]. Like connecdenn (Fig. 2.5), intersectin-l and endophilin A1 are enriched in brain and are present in the presynaptic compartment [119, 120, 141, 143, 144]. Interestingly, connecdenn coimmunoprecipitates with intersectin-s/intersectin-l and endophilin A1 from brain extracts (Fig. 2.4*B*,*C*). The relatively low amount of endophilin recovered in the immunoprecipitated sample relative to the starting material likely results from the fact that endophilin A1 is much more abundant than connecdenn, and, as such, only a fraction of endophilin A1 in the sample can be coimmunoprecipitated. Thus, connecdenn is a component of an endocytic complex involving AP-2 and synaptic SH3 domain-bearing proteins.

Connecdenn is a brain-enriched protein at the synapse

When equal protein amounts of tissue extracts are analyzed, connecdenn is detected at higher levels in brain and testis than in other tissues (Fig. 2.5*A*). Within brain, connecdenn is detected in all brain regions examined with variable expression levels (Fig. 2.5*B*). To examine the localization of connecdenn, we performed confocal

immunofluorescence microscopy of mature hippocampal neurons at 21 DIV. Connecdenn is detected in neuronal cell bodies and can also be seen to extend into dendrites (Fig. 2.5*C*). In addition, brighter connecdenn punctae are detected that are situated along dendrites and that colocalize in part with synaptophysin, a presynaptic marker (Fig. 2.5*C*). Connecdenn punctae also partially colocalize with PSD-95, a marker of the postsynapse, but more often are found adjacent to PSD-95-positive structures (Fig. 2.5*C*). Quantification reveals that 45.0% of all synaptophysin-positive puncta contain connecdenn (23 cells from five experiments), whereas only 19.4% of PSD-95-positive puncta are colocalized with connecdenn (16 cells from three experiments). These data demonstrate that a pool of connecdenn is present at the synapse, with a proportion of the synaptic pool in the presynaptic compartment.

Connecdenn has a stable association with CCV membranes

The presence of connecdenn in the presynaptic compartment and its binding to the α-ear of AP-2 prompted us to test whether the protein was present on CCVs. Interestingly, connecdenn is enriched on CCVs to the same extent as CHC and AP-2 (Fig. 2.6*A*). Moreover, it shows the same distribution pattern as these two key CCV components throughout the various fractions of the subcellular fractionation procedure (Fig. 2.6*A*). In contrast, the abundant SV membrane proteins synaptotagmin and synaptophysin are enriched on CCVs but only by approximately twofold to threefold (Fig. 2.6*B*), similar to what has been reported previously [145, 146].

The majority of peripheral membrane proteins of CCVs are stripped from the vesicles under conditions that remove the clathrin coat. Interestingly, connecdenn resists extraction with 0.5 M Tris, which removes clathrin coats (Fig. 2.7*A*) [147]. Connecdenn is stripped from CCVs by pH 11.0, confirming that it is not an integral membrane protein like synaptophysin (Fig. 2.7*A*). The association of connecdenn with CCVs stripped of the clathrin coat is stable through a range of NaCl concentrations up to 1 M, indicating that the interaction is likely hydrophobic in nature (Fig. 2.7*B*). It is conceivable that, under the stripping conditions used, connecdenn is removed from the membrane but pellets as a

result of aggregation. We thus ran stripped CCVs on sucrose gradients to separate the coats from the vesicles. When CCVs are run in control conditions (buffer A), CHC, synaptophysin, and connecdenn comigrate and move deeply into the gradient (Fig. 2.7*C*). In stripped CCVs, there is significantly less CHC, and the pool that remains migrates at the top of the gradient, likely attributable to the fact that it comes off the vesicles during the centrifugation step. In contrast, connecdenn and synaptophysin comigrate into deeper gradient fractions, although they do not migrate as fast as intact CCV, likely attributable to the loss of density after removal of the clathrin coat (Fig. 2.7*C*). These data support the conclusion that connecdenn remains associated with the vesicles after stripping of the clathrin coat. Interestingly, connecdenn is absent from highly purified SVs (Fig. 2.7*D*). Together, these data demonstrate that connecdenn is a CCV protein, suggesting that it is involved in endocytic traffic in the synapse.

Disruption of connecdenn function inhibits SV endocytosis

To address whether the interaction of connecdenn with its endocytic protein-binding partners is necessary for CME of SVs, we assessed SV uptake using the styryl dye FM4-64 in cultured hippocampal neurons. Neurons were transfected with GFP or GFP–connecdenn peptide motif domain, and FM4-64 uptake was performed and quantified as described in detail in Materials and Methods. FM4-64 uptake in GFP–connecdenn peptide motif domain expressing boutons was 45.9 ± 6.3% of that seen in nontransfected boutons from the same fields (10 fields from three independent cultures), whereas for GFP alone, uptake was 88.2 ± 13.3% of that seen in nontransfected boutons (12 fields from three independent cultures). The effect of the GFP–connecdenn peptide motif domain was significantly greater than that of GFP alone as determined by an ANOVA test followed by a Tukey's *post hoc* test ($p < 0.05$). A representative image of neurons transfected with GFP–connecdenn peptide motif domain shows that some GFP–connecdenn-positive boutons had reduced KCl-induced FM4-64 uptake compared with nontransfected neighboring processes, whereas others had no detectable uptake (Fig. 2.8*A*). Thus, overexpression of the peptide motif domain of connecdenn is sufficient to disrupt the endocytic machinery required for CME of SVs.

Because the overexpression experiments suggested a role for connecdenn in SV endocytosis, we sought to examine the effect of knocking down the endogenous protein. Lentiviral delivery of shRNA is an emerging approach to perform loss-of-function experiments in nondividing cells such as hippocampal neurons [148]. We thus generated FIV vectors driving the expression of GFP alone or the parallel expression of GFP and shRNA specific for connecdenn as described in Materials and Methods. Hippocampal cultures were transduced at 14 DIV, and the level of connecdenn was determined at 21 DIV. Connecdenn expression was strongly reduced in neurons transduced with connecdenn shRNA relative to control (Fig. 2.8*B*). Quantification of multiple blots revealed a 67% reduction, whereas the levels of other proteins, including NECAP 1, CLCa/b, actin, and Na^+/K^+-ATPase, were not affected (Fig. 2.8*B*,*C*). FM4-64 uptake was then assessed as described for the dominant-negative experiments using an MOI that led to 20% transduction rates. FM4-64 uptake in boutons from neurons transduced with GFP and connecdenn shRNA was 55.4 ± 4.5% of that seen in nontransduced boutons from the same fields (17 fields from three independent cultures), whereas for GFP alone, uptake was 82.4 ± 5.4% of that seen in nontransduced boutons (11 fields from three independent cultures). The effect of connecdenn shRNA expression on FM4-64 uptake was significantly greater than control transduction as determined by an ANOVA test followed by a Tukey's *post hoc* test ($p < 0.05$). A representative image of neurons transduced with GFP and connecdenn shRNA shows that GFP-positive boutons had reduced or undetectable KCl-induced FM4-64 uptake compared with nontransduced neighboring processes (Fig. 2.8*D*). Together with the observations that connecdenn is highly enriched on CCVs and binds to multiple components of the endocytic machinery, our data demonstrate that connecdenn is a new component of the endocytic machinery for SVs.

Discussion

CME is achieved using a network of enzymatic, mechanical, and scaffolding proteins held together by low-affinity protein–protein and protein–lipid interactions. Hallmark features of the endocytic machinery are the multiple modular protein domains that mediate recognition of small peptide motifs and/or specific phospholipid head groups, such that each single element is interconnected with multiple other components of the machinery. This creates a Velcro-like arrangement in which multiple low-affinity interactions lead to a stable overall structure. This also allows for a dynamic situation in which minor changes in interaction affinities can lead to rapid assembly and disassembly of the machinery. Such a process would be highly cooperative, and, in fact, assembly of the clathrin coat proceeds with a Hill coefficient >6[149].

One of the most important interaction hubs within the endocytic network is AP-2, which participates in clathrin coat assembly and is recruited in a phosphatidylinositol(4,5)P_2-dependent manner to the plasma membrane in which its timely recognition of cargo prevents catastrophic disassembly of CCPs [150, 151]. In nerve terminals, AP-2 in a complex with stonin 2 interacts with phosphatidylinositol(4,5)P_2 and synaptotagmin, allowing for coats to nucleate on SV membranes after SV fusion with the plasma membrane [152, 153]. AP-2 also serves as a scaffolding platform for a battery of endocytic accessory proteins, including epsin, huntingtin interacting protein 1, AP180, amphiphysin, disabled 2, autosomal recessive hypercholesteremia gene product, and NECAP 1 [154, 155]. A common feature of these proteins is the presence of N-terminal globular domains, including E/ANTH (epsin/AP180 N-terminal homology) domains and BAR domains, which function in generating and/or sensing membrane curvature, and PTB (phosphotyrosine binding) domains, which serve as binding modules for endocytic cargo proteins [28, 156, 157]. Our data are the first to indicate that a DENN domain-containing protein is a component of the endocytic machinery. DENN domains are composed of uDENN and dDENN modules flanking a DENN module. These modules form a unit that is well conserved in diverse species, including humans, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* [158]. Although the function of the DENN

domain is unknown, it is noteworthy that several DENN domain-bearing proteins are implicated in vesicle trafficking including myotubularin-related proteins 5 and 13, Rab6-interacting protein, and DENN/MADD/Rab3GEP [158, 159]. Interestingly, DENN/MADD/Rab3GEP is a guanine nucleotide exchange factor for the SV protein Rab3, and knock-out of this protein leads to mice that have decreased numbers of SVs, suggesting a possible alteration in SV reformation [160]. Our data provide a direct link of a DENN domain protein to vesicle trafficking and will allow for a more detailed analysis of the role of DENN domains in membrane trafficking and CME in particular.

In addition to globular N-terminal domains, endocytic accessory proteins generally contain C-terminal peptide motif domains, which are presumably weakly structured and contain multiple sites for interactions with endocytic proteins, including clathrin and AP-2 [4, 7, 161]. Connecdenn shares this common topology. Following from the globular N-terminal domain, the tail is presumably poorly structured and harbors three sequences, FSDVF, DPF, and WETFE, that match three distinct motifs that target the platform and sandwich subdomains of the α-ear. In fact, all three motifs contribute to α-ear binding, although the WETFE is the predominant sequence, consistent with the observation that WXXF-acidic motifs generally have higher affinity than DPF/W or FXDXF motifs [134]. Because all three motifs are involved in binding, connecdenn may engage both α-ear subdomains, creating avidity effects that allow for a more stable interaction, consistent with the ability to coimmunoprecipitate connecdenn with AP-2 from brain extracts. Based on co-crystallization of the α-ear with a peptide containing a WXXF-acidic motif from synaptojanin, it was suggested that small, uncharged residues are preferred in the position following the invariant W attributable to their proximity to F740 in the α-ear [134]. Interestingly, NMR analysis demonstrates that the WXXF-acidic motif from connecdenn (WETFE-COO^-) interacts with α-ear in the same manner as that from NECAP (WVQF-COO^-). Because connecdenn contains a glutamic acid in this position, this indicates that acidic residues are also permissible. Thus, searches for WXXF-acidic motifs need not be limited by small hydrophobic amino acids following the W, broadening the potential scope of proteins that contain the motif. Connecdenn also carries multiple PXXP core sequences, and, in brain, it forms complexes with the endocytic SH3

domain-containing proteins endophilin A1 and intersectin. Although the precise role of connecdenn interactions with AP-2, intersectin, and endophilin remain unknown, disruption of these interactions through overexpression of the peptide motif domain leads to a significant decrease in SV endocytosis.

Although the architecture of connecdenn is similar to classical AP-2-binding accessory proteins, its biochemical association with CCVs is strikingly different. Most accessory proteins are not enriched on CCVs, suggesting that they function relatively early in the formation of a CCP [155]. Interestingly, it has been demonstrated recently that the ear domain of the β2-adaptin subunit of the AP-2 complex (β2-ear) plays an important role in the recruitment of accessory proteins [7, 12]. CHC interacts with the ß2-ear, as well as with the region that links the ß2-ear with the ß2-adaptin core [162] [163]. It is thought that proteins that are dependent on interactions with ß2-ear are lost from CCVs as clathrin assembles and competes with the accessory proteins for the ß2-ear-binding site [7, 12]. In this manner, CHC assembly controls the temporal organization of accessory protein function such that proteins that are required late in the endocytic process are recruited through direct or indirect interactions with the α-ear [164]. Because connecdenn interacts exclusively with the α-ear and is retained on the vesicles, it likely functions later in CCP formation. Moreover, connecdenn remains on CCVs after chemical stripping of clathrin coats. Thus, in addition to functioning in endocytosis, connecdenn could have a post-uncoating role in endocytic vesicle transport or in endosomal function. An example of such a dual function has been reported for clathrin assembly lymphoid myeloid leukemia protein (CALM), a homolog of AP180 that binds to AP-2 [165]. CALM function is required for CME of epidermal growth factor receptor [166] and the protein is enriched in synapses, consistent with a role in CME of SVs [167]. Interestingly, knocking down CALM leads to a failure of transferrin to accumulate in perinuclear recycling endosomes [165], suggesting that CALM knockdown disrupts endosome traffic or fusion.

Given that connecdenn is enriched in brain, is found in the presynaptic terminal, and is a component of the machinery for CME, it most likely functions in the CME of SVs. We thus sought to test for a role for connecdenn in SV endocytosis using a loss of function approach. Knocking down connecdenn by lentiviral delivery of shRNA led to a

significant decrease in CME of SVs as measured by FM4-64 dye uptake. Given the complexity and redundancy of the machinery for SV endocytosis, it is perhaps surprising that knockdown of a single component leads to a significant effect on the process. This would suggest that connecdenn is an important component of the machinery. After uncoating, SVs are reloaded with neurotransmitter and are made available for additional rounds of exocytosis, either directly or after additional sorting through an endosomal intermediate [168]. Because connecdenn is absent from SVs, there must be a mechanism to remove the protein from endocytic vesicles before the exocytosis step. Thus, any potential effect of connecdenn on exocytosis would result from indirect effects mediated by its involvement in endocytosis. In summary, our data reveal connecdenn as a novel component of the synaptic endocytosis machinery.

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MGSRIKQNPETTFEVYVEVAYPRTGGTLSDPEVQRQFPEDYSDQEVLQTL 50
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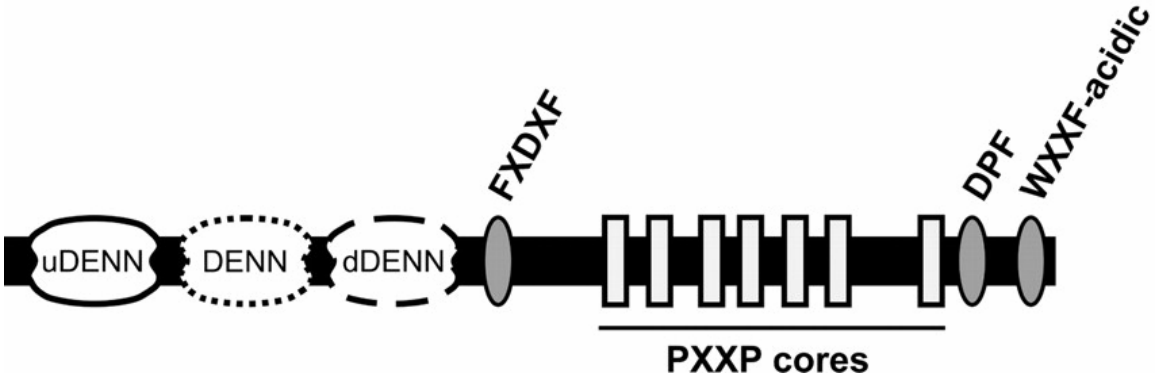
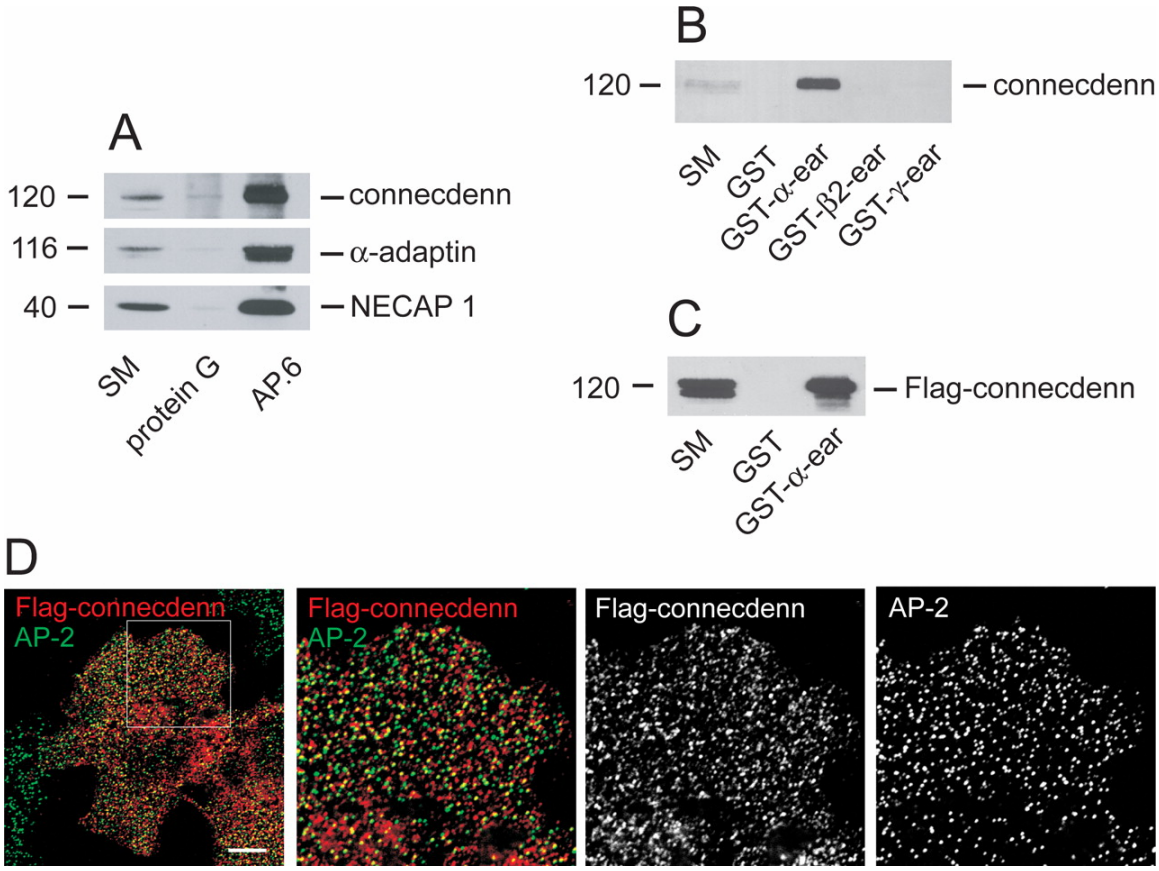


Figure 2.1. Identification of connecdenn. The amino acid sequence of mouse connecdenn (from gi31542027) and a domain/motif model are presented. The DENN domain is composed of uDENN, DENN, and dDENN modules indicated by solid, dotted, and dashed lines, respectively. Potential AP-2-binding motifs are indicated by ovals with dark shading, and proline-rich regions containing PXXP cores are indicated by boxes with light shading. The annotated mouse connecdenn has been entered into GenBank under the accession number DQ448594.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SM
GST
GST-α-ear
D
Flag-connecdenn
AP-2
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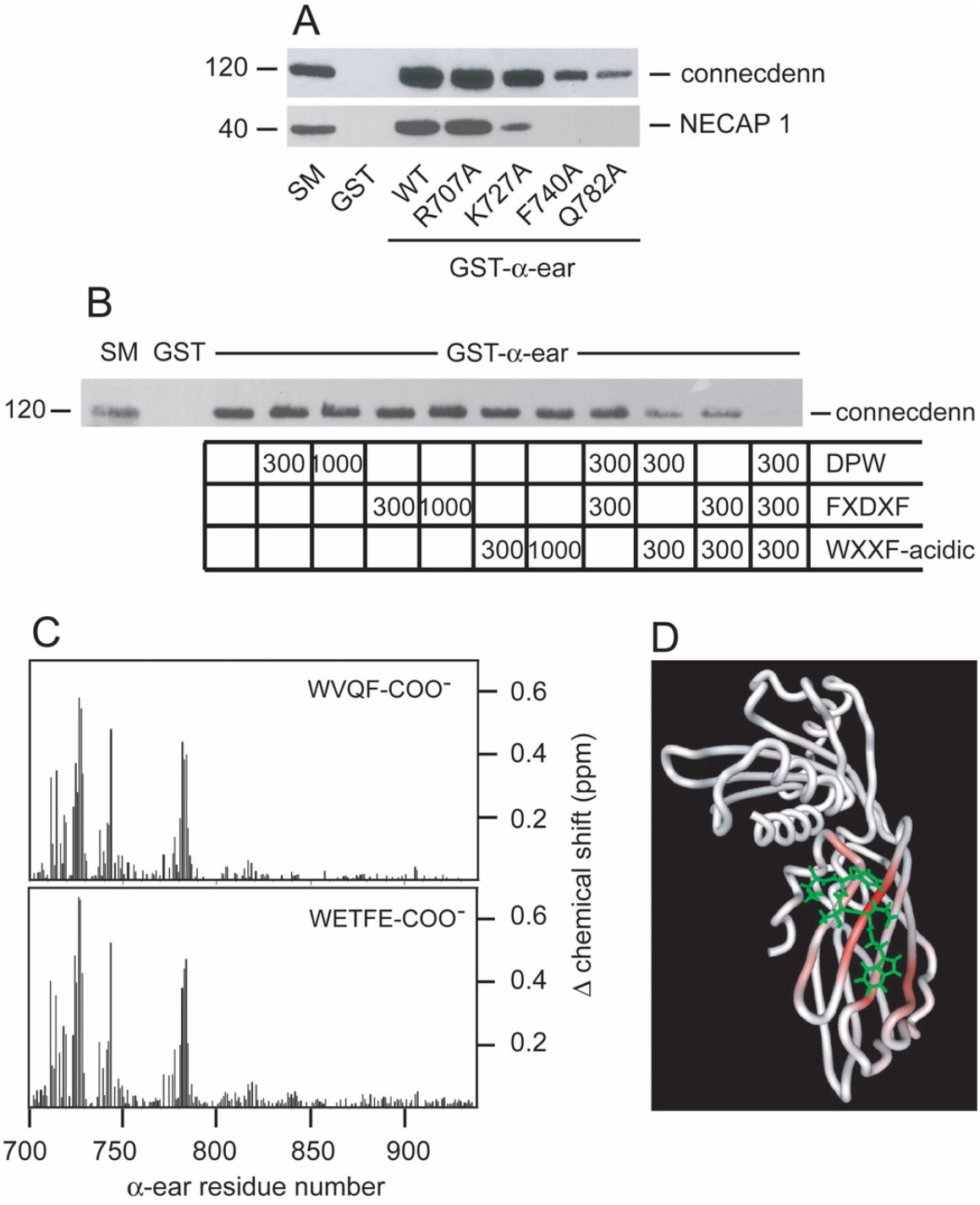
Flag-connecdenn
AP-2

Figure 2.2 Interaction of connecdenn with AP-2. ***A***, Soluble brain lysates were incubated with protein G beads alone or antibody AP.6 against the α-adaptin subunit of AP-2 and protein G beads. Proteins specifically bound to the beads were processed for Western blot with antibodies against the indicated proteins. ***B***, Soluble brain lysates were incubated with GST or GST fused to the ear domains of α-, β2-, and γ-adaptin, precoupled to glutathione-Sepharose beads. Proteins specifically bound to the beads were processed for Western blot with antibody against connecdenn. ***C***, Lysates from HEK-293 cells transfected with Flag-tagged connecdenn were incubated with GST or GST–α-ear precoupled to glutathione-Sepharose beads, and proteins specifically bound to the beads were processed for Western blot with antibody against the Flag-epitope tag. ***D***, COS-7 cells transfected at low levels with full-length Flag-tagged connecdenn and processed with a polyclonal Flag antibody (red channel) and a monoclonal antibody against AP-2 (AP.6, green channel). The blend of the two images is shown in the left panel, and a blend and individual images of the region indicated by the box is shown in the right three panels. Scale bar: lower-magnification image, 10 μm; higher-magnification images, 4.3 μm.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connecdenn
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NECAP 1
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GST
WT
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K727A
F740A
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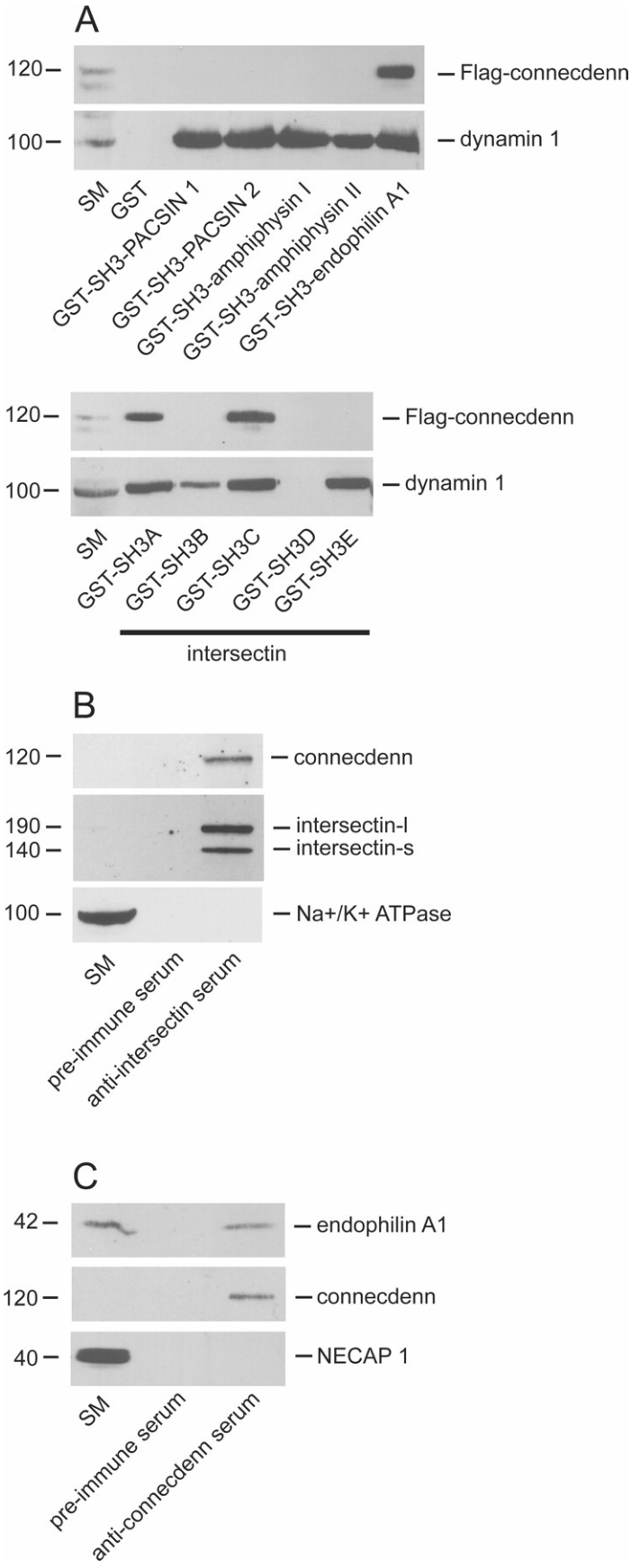
B
SM GST
GST-α-ear
120
connecdenn
300 1000 300 300 300 DPW
300 1000 300 300 300 FXDXF
300 1000 300 300 300 WXXF-acidic
C
WVQF-COO⁻
WETFE-COO⁻
0.6
0.4
0.2
Δ chemical shift (ppm)
700 750 800 850 900
α-ear residue number
D

Figure 2.3 Identification of the connecdenn-binding site on AP-2. ***A***, Soluble brain lysates were incubated with GST or GST fused to wild-type (WT) α-ear or a variety of α-ear point mutants as indicated, precoupled to glutathione-Sepharose beads. Proteins specifically bound to the beads were processed for Western blot with antibodies against the indicated proteins. ***B***, Soluble brain lysates were incubated with GST or GST–ear, precoupled to glutathione-Sepharose beads. Incubations were without or with a DPW peptide (CSDPWGSDPWG) from epsin 1, a FXDXF peptide (CSFFEDNFVPE) from amphiphysin1, or a WXXF-acidic peptide (CQAPQPSNWVQF-COO–) from NECAP 1 at the molar ratios of peptide to fusion protein indicated. Proteins specifically bound to the beads were processed for Western blot with antibody against connecdenn. For all above experiments, an aliquot of starting material (SM) equal to 1/10 of that added to the beads was run in parallel. ***C***, Magnitude of the amide chemical shift changes [{(1H shift)2 + (15N shift x 0.2)2}1/2 in parts per million (ppm)] of the α-ear during binding CQAPQPSNWVQF-COO– (WVQF-COO–, NECAP 1; top) and CVEQLRRQWETFE (WETFE-COO–, connecdenn; bottom). The residue numbers correspond to mouse -adaptin. ***D***, HADDOCK modeled structure of WETFE/α-ear complex. The backbone trace of the α-ear is colored according to the size of the amide chemical shift changes during binding of the connecdenn peptide.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Flag-connecdenn
dynamin 1
SM
GST
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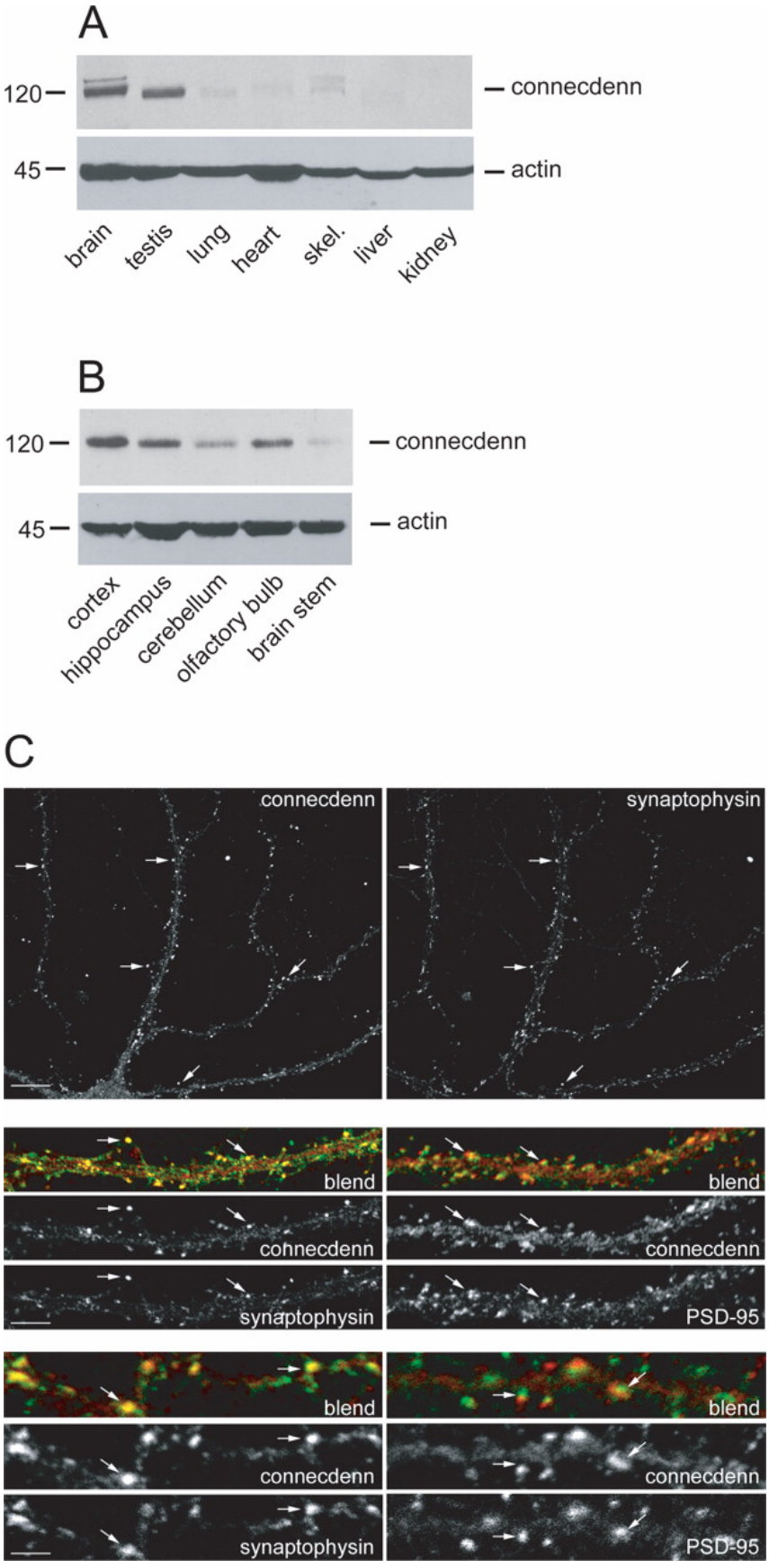
GST-SH3-amphiphysin II
GST-SH3-endophilin A1
120
100
Flag-connecdenn
dynamin 1
SM
GST-SH3A
GST-SH3B
GST-SH3C
GST-SH3D
GST-SH3E
intersectin
B
120
190
140
100
connecdenn
intersectin-l
intersectin-s
Na+/K+ ATPase
SM
pre-immune serum
anti-intersectin serum
C
42
120
40
endophilin A1
connecdenn
NECAP 1
SM
pre-immune serum
anti-connecdenn serum

Figure 2.4 Connecdenn binds to SH3 domains from endocytic proteins. ***A***, Soluble lysates from Flag–connecdenn-transfected HEK-293 cells were incubated with GST or GST fused to the SH3 domain of either amphiphysin I or II, PACSIN 1 or 2, endophilin A1, and the five individual SH3 domains of intersectin, precoupled to glutathione-Sepharose beads. Proteins specifically bound to the beads were processed for Western blot with antibodies against the indicated proteins. ***B***, Soluble brain lysates were incubated with anti-intersectin serum or preimmune serum from the same rabbit along with protein A-Sepharose beads. Proteins specifically bound to the beads were processed for Western blot with antibodies against the indicated proteins. ***C***, Soluble brain lysates were incubated with anti-connecdenn serum (3775) or preimmune serum from the same rabbit along with protein A-Sepharose beads. Proteins specifically bound to the beads were processed for Western blot with antibodies against the indicated proteins. For ***B*** and ***C***, Western blots with polyclonal antibodies were revealed with protein A-HRP. For all experiments, an aliquot of starting material (SM) equal to 1/10 of that added to the beads was run in parallel.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45
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brain
testis
lung
heart
skel.
liver
kidney
B
120
connecdenn
45
actin
cortex
hippocampus
cerebellum
olfactory bulb
brain stem
C
connecdenn
synaptophysin
blend
connecdenn
synaptophysin
blend
connecdenn
PSD-95
blend
connecdenn
synaptophysin
blend
connecdenn
PSD-95

Figure 2.5 Connecdenn is brain enriched and is detected at the synapse of hippocampal neurons in culture. Equal protein extracts from various rat tissues including skeletal muscle (skel.) (***A***) and rat brain regions (***B***) were blotted with antibodies against connecdenn and actin. ***C***, Rat hippocampal neurons at 21 DIV were processed for indirect immunofluorescence with a polyclonal antibody against connecdenn (3775) and monoclonal antibodies against either synaptophysin or PSD-95. For the bottom 12 panels, the blend of connecdenn (red) and synaptophysin or PSD-95 (green) or the individual images are shown. Scale bars: top two panels, 10 μm; middle six panels, 3.33 μm; bottom six panels, 2.33 μm. The percentage of connecdenn-positive punctate that colocalize with synaptophysin-positive punctae is 71% in ***C***, above the average of 45.0% for all cells examined.

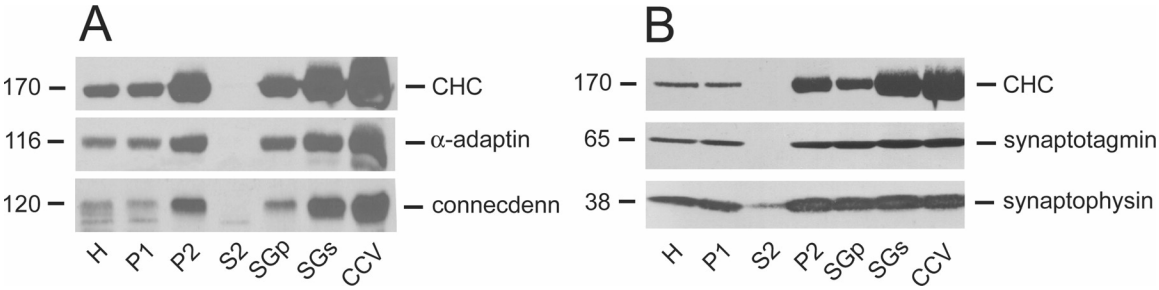
A
170
116
120
CHC
α-adaptin
connecdenn
H
P1
P2
S2
SGp
SGs
CCV
B
170
65
38
CHC
synaptotagmin
synaptophysin
H
P1
S2
P2
SGp
SGs
CCV

Figure 2.6 Connecdenn is enriched on CCVs. Equal protein aliquots of the successive fractions of a procedure leading to highly enriched CCVs were analyzed by Western blot with antibodies against CHC, α-adaptin, and connecdenn (***A***) and CHC, synaptotagmin, and synaptophysin (***B***). H, Homogenate; P, pellet; S, supernatant; SG, sucrose gradient.

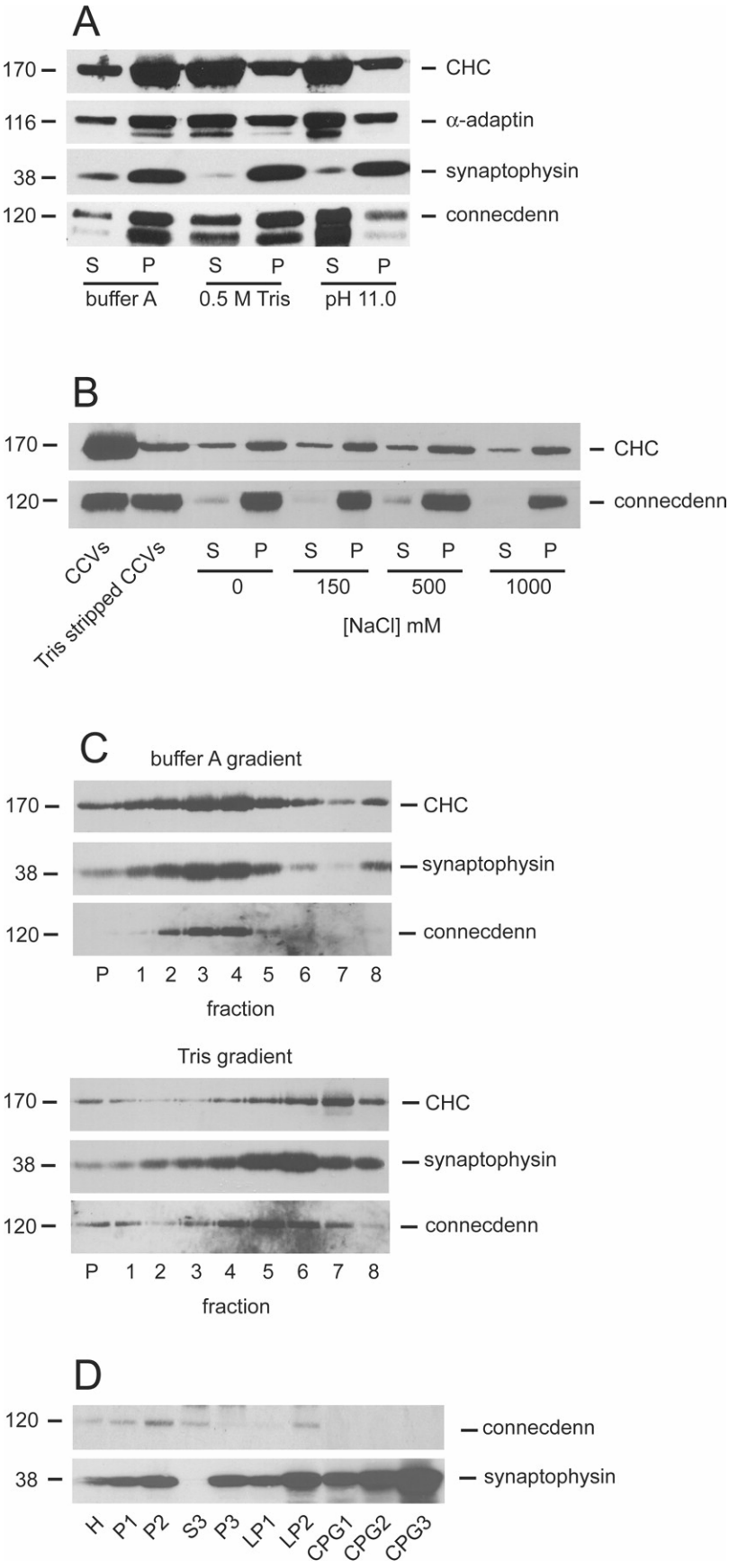
A
170
116
38
120
CHC
α-adaptin
synaptophysin
connecdenn
S P S P S P
buffer A
0.5 M Tris
pH 11.0
B
170
120
CHC
connecdenn
CCVs
Tris stripped CCVs
S P S P S P S P
0
150
500
1000
[NaCl] mM
C
buffer A gradient
170
38
120
CHC
synaptophysin
connecdenn
P 1 2 3 4 5 6 7 8
fraction
Tris gradient
170
38
120
CHC
synaptophysin
connecdenn
P 1 2 3 4 5 6 7 8
fraction
D
120
38
connecdenn
synaptophysin
H P1 P2 S3 P3 LP1 LP2 CPG1 CPG2 CPG3

Figure 2.7 Connecdenn is stably associated with CCV membranes. ***A***, CCVs isolated from rat brain were resuspended in buffer A, 0.5 M Tris buffer, or sodium carbonate buffer at pH 11.0. The samples were centrifuged at high g, and proteins in the supernatant (S) and pellet (P) fractions were analyzed by Western blot with antibodies against the indicated proteins. ***B***, CCVs were stripped of their coats with two successive rounds of incubation with 0.5 M Tris. The resulting vesicles were split in five aliquots, pelleted at high g, and resuspended in Laemmlli sample buffer (Tris-stripped CCVs) or in 10 mM HEPES, pH 7.4, containing 0, 150, 500, or 1000 mM NaCl. Samples were again centrifuged, and the pellet (P) and supernatant (S) fractions were analyzed by Western blot with antibodies against the indicated proteins. ***C***, CCVs in buffer A and CCVs extracted with Tris buffer were loaded on the top of linear 20–50% sucrose gradients prepared in buffer A and Tris buffer, respectively, and were centrifuged at 145,000 x g for 1.5 h. The gradients were fractionated from the bottom, and equal volume aliquots of each fraction along with the pellet (P) were analyzed by Western blot with the indicated antibodies. ***D***, Equal protein aliquots of the successive fractions of a procedure leading to highly enriched SVs were analyzed by Western blot with the indicated antibodies. H, Homogenate; P, pellet; S, supernatant; LP, lysed pellet; LS, lysed supernatant; CPG, controlled pore glass. CPG3 contains purified SVs.

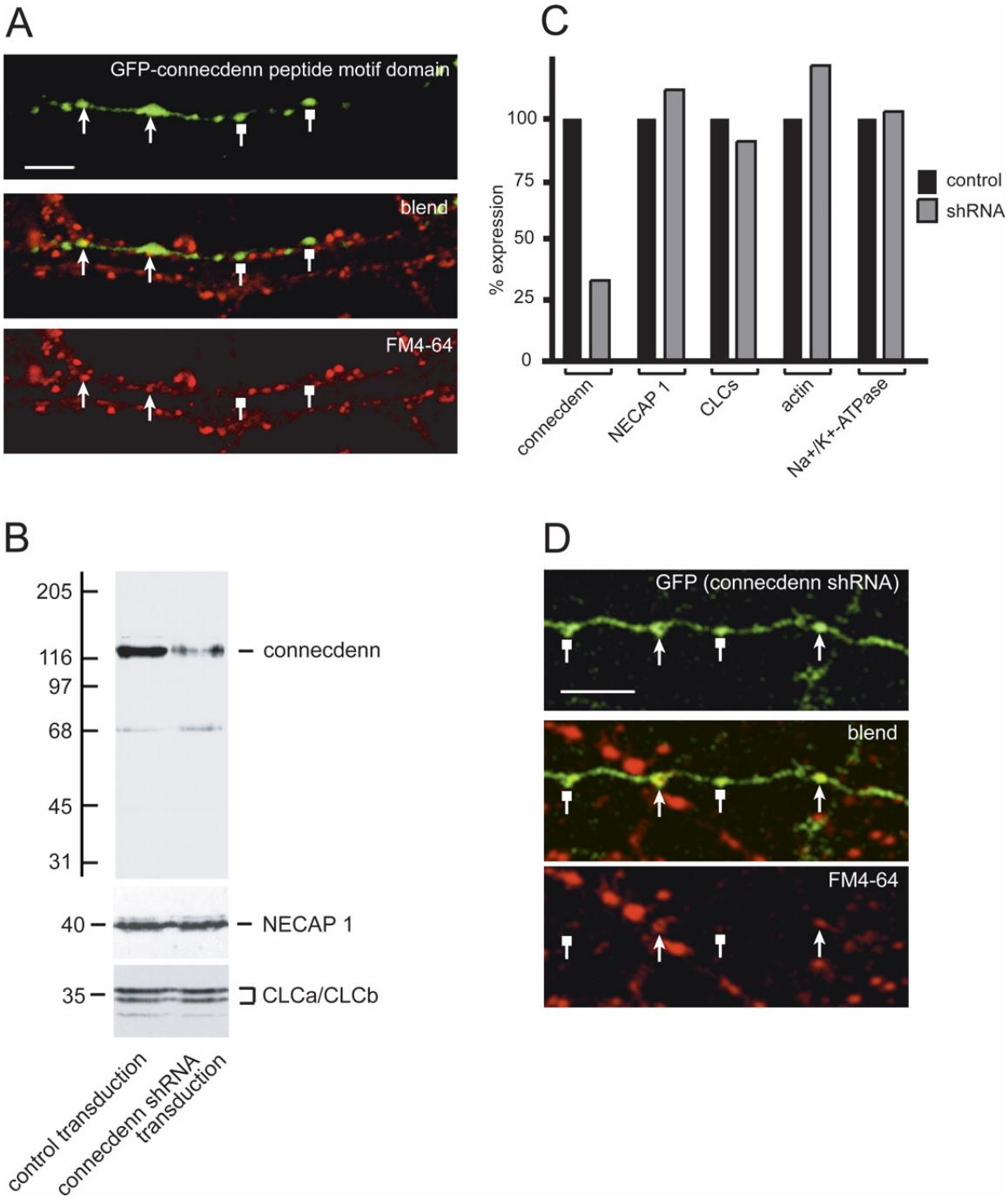
A
GFP-connecdenn peptide motif domain
blend
FM4-64
C
% expression
100
75
50
25
0
control
shRNA
connecdenn
NECAP 1
CLCs
actin
Na+/K+-ATPase
B
205
116
97
68
45
31
connecdenn
40
NECAP 1
35
CLCa/CLCb
control transduction
connecdenn shRNA transduction
D
GFP (connecdenn shRNA)
blend
FM4-64

Figure 2.8 Disruption of connecdenn function reduces CME of SVs in neurons. ***A***, An axon labeled with GFP-connecdenn peptide motif domain is seen to run along a dendrite from a nonexpressing cell. KCl-induced endocytosis of FM4-64 is indicated in red. Note the punctate staining pattern, indicative of FM dye uptake into nonlabeled axons. Note the reduced (arrowheads) or lack of (square heads) uptake in varicosities of the axon expressing GFP–connecdenn peptide motif domain. Scale bars, 5 μm. ***B***, Hippocampal neurons at 14 DIV were transduced with FIV expressing GFP alone (control transduction) or GFP and an shRNA specific for connecdenn. At 21 DIV, protein extracts were made, and equal protein aliquots were analyzed by Western blot with antibodies against NECAP 1 and CLCa/b as well as actin and Na^+/K^+-ATPase (data not shown). ***C***, Blots were scanned, and the relative amounts of the indicated proteins in control versus shRNA transduced neurons was plotted. ***D***, A representative image of an axon from a neuron transduced with an FIV virus driving expression of GFP and an shRNA specific for connecdenn. The GFP-labeled axon is seen to cross an axon from a nonexpressing cell. KCl-induced endocytosis of FM4-64 is indicated in red. Note the punctate staining pattern, indicative of FM dye uptake into nonlabeled axons. Note the reduced (arrowheads) or lack of (square heads) uptake in varicosities of the axon expressing GFP. Scale bars, 5 μm.

Ackowledgements

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Preface to chapter 3 – Function of connecdenn in non-neuronal cells

The characterization of connecdenn as a component of CCVs is supported by biochemical analyses. Connecdenn enriches similarly to AP-2 and clathrin on brain CCVs. Connecdenn also binds through C-terminal amino acid peptide motifs to the CME adaptor complex AP-2, and the accessory proteins endophilinA1 and intersectin1. The importance of these interactions is supported by the observed block in synaptic vesicle membrane retrieval when the C-terminal region of connecdenn is over-expressed in cultured dissociated hippocampal neurons. Furthermore, knockdown of connecdenn also produced a block in SV membrane re-uptake arguing for connecdenn being an important regulator of CME. Thus, chapter 2 presented data confirming a functional role of connecdenn in CME, mainly via its C-terminal region. However, intriguing biochemical data shows that connecdenn remains stably associated to CCVs after clathrin-coat removal, suggesting a post-endocytic role.

Recently, a study in *C. elegans* has revealed a role for the connecdenn orthologue RME-4 both in CME and downstream on endocytic vesicles through the Rab35 orthologue RME-5 [169]. Interestingly, the study showed similar effects following ablation of either connecdenn or Rab35 on trafficking of early endocytic vesicles. Furthermore, the genetic linkage was further supported by physical interaction in a GDP-dependent manner [169]. This suggested that connecdenn may be a GEF for Rab35. Connecdenn, at its N-terminus encodes an evolutionary conserved tri-partite DENN domain. The DENN domain itself has not been characterized yet but has been proposed to function as a GEF based on studies with the Rab3 GEF protein DENN/MADD [107, 158]. Intrigued by this finding, we set out to characterize the biochemical and cellular function of the interaction between connecdenn and Rab35.

Chapter 3: The connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes

Abstract

DENN domains are an evolutionary ancient protein module conserved from yeast to humans. Mutations in the module cause developmental defects in plants and human diseases, yet the function of this common domain remains unknown. We now demonstrate that the connecdenn DENN domain functions as a guanine nucleotide exchange factor for Rab35 to regulate endosomal trafficking. We also show that Rab35 controls early endosomal morphology by regulating Rab5, a central Rab for cargo trafficking along the endocytic pathway. Rab35 has been suggested to mediate fast transferrin recycling from early endosomes, however, our loss-of-function studies demonstrate that Rab35 is not involved in transferrin recycling, leaving the Rab controlling this route yet to be identified. In contrast, the loss of Rab35 activity inhibits MHCI recycling and prevents the early endosomal recruitment of EHD1, a common component of endosomal recycling tubules. Our data are the first to reveal an enzymatic activity for a DENN domain and demonstrate that distinct Rab GTPases can recruit common protein machinery to various sites within the endosomal network to establish cargo-selective recycling pathways.

Introduction

Cargo molecules enter eukaryotic cells in endocytic carriers formed through clathrin-dependent and -independent mechanisms. The carriers deliver cargo to the endosomal system, which is divided into early, late, and recycling endosomes, in part based on their specific lipid and protein composition. From early endosomes, cargo is sorted for recycling back to the plasma membrane, either directly or following delivery to perinuclear recycling endosomes. Cargo destined for degradation remains in early endosomes and is eventually transferred to late endosomes/lysosomes.

The directional flow of cargo and the functional compartmentalization of the endosomal system depends on the actions of members of the Rab family of small GTPases. Like other Ras-like GTPases, Rabs are stimulated by guanine nucleotide exchange factors (GEFs) and repressed by GTPase activating proteins (GAPs) to oscillate between an active GTP-bound and an inactive GDP-bound state. Once turned on, Rabs recruit effector proteins that function in multiple aspects of endosomal trafficking including the formation of transport carriers, the tethering and fusion of vesicles, and vesicle transport along microtubules. To date, only a few Rabs have been matched to their GEFs and GAPs, hampering a better understanding of their regulation and thus, their function within the endosomal system.

Currently, Rab5 is the best understood endosomal Rab [170]. Rab5 is found in association with clathrin-coated vesicles (CCVs) where it regulates coat disassembly following fission [37]. It then couples the resulting endocytic carriers to motor proteins for inward transport along microtubules and along with PtdIns(3)P, recruits EEA1, a tethering factor that allows for the homotypic fusion of the nascent vesicles and with early endosomes [48, 69, 70]. Rab5 then recruits the HOPS complex, which in turn recruits and activates Rab7 to allow for retromer-mediated transport to the TGN and for conversion of Rab5-positive early endosomes into Rab5-negative late endosomes for cargo degradation [81, 171]. Rab5 and its effector proteins thus regulate a remarkable array of functions in endosomal trafficking, highlighting the need to adequately regulate its activity through a variety of known GEFs and GAPs. In addition to this complexity,

multiple Rabs can co-exist on a single endosome to increase the variety of sorting decisions available on the organelle [49, 172-174].

Rab35 is a less well characterized Rab that also functions in endosomal trafficking [169, 172, 175, 176]. Rab35 localizes to the plasma membrane and to internal vesicles and tubules and is necessary for endosomal secretion during immunological synapse formation and for the stabilization and successful abscission of the cytokinesis bridge during cytokinesis [172, 175]. Moreover, Rab35 is linked to actin dynamics during neurite outgrowth in PC12 cells and to the recycling of the clathrin-dependent cargo transferrin and the clathrin-independent cargo major histocompatibility complex class I (MHCI) and class II (MHCII) [177], [172, 178]. Recycling of MHCII involves the formation of Rab35/EHD1-positives tubules at early endosomes [176]. At the recycling endosomes, Rab11/EHD1-positive tubules recycle transferrin while MHCI and β1-integrin are sorted into Rab22/EHD1-positive tubules [50, 177, 179, 180]. EHD1/Rme-1 is the founding member of the EHD protein family that in mammals comprises four members, EHD1-4 [181]. EHD family members form homo- and heterooligomers and their nucleotide- and lipid-binding properties control their intracellular localization and their function in the formation of intracellular transport carriers [55, 182].

We recently identified connecdenn as a component of the proteineous coat of CCVs and demonstrated that connecdenn knockdown (KD) reduces synaptic FM4-64 uptake in cultured hippocampal neurons [183]. Interestingly, connecdenn remains stably associated with the membrane fraction of CCVs under conditions that efficiently remove the clathrin coat proteins, suggesting a post-uncoating function [184]. The connecdenn N-terminus harbors a Differentially Expressed in Neoplastic versus Normal cells (DENN) domain, a tripartite domain formed by upstream DENN (uDENN), DENN, and downstream DENN (dDENN) modules. DENN domains are found in 16 human genes including myotubularin related (MTMR) 5 and 13, DENN/MADD/Rab3GEP, suppressor of tumorgenicity 5, and connecdenn. Mutations in DENN domains and DENN domain proteins cause human diseases [185-189], however, the function of this domain remains unknown. Here we show that the connecdenn DENN domain serves as a lipid-binding module and is both necessary and sufficient for the stable membrane association of connecdenn with

uncoated vesicles. Importantly, the DENN domain binds Rab35 and functions as specific GEF for this GTPase. Loss-of-function studies indicate that connecdenn-controlled Rab35 activation imposes a negative regulation on Rab5 activity necessary for early endosome morphology and intracellular positioning. Rab35 activation is also critical for recruitment of EHD1 to early endosomal membranes and to facilitate MHCI recycling but has no effect on transferrin and β1-integrin trafficking. Our data thus highlight that distinct Rab GTPases can recruit a common protein machinery to various sites within the endosomal network in order to establish distinct endosomal recycling pathways that are utilized by a subset of cargo trafficking through the endosomal network.

Results

In our initial connecdenn study, we discovered an exceptionally stable association of connecdenn with the membrane fraction of CCVs following their uncoating ([183]; Suppl. Fig. 3.1A), suggesting that connecdenn could have a post-uncoating role. To identify the molecular determinant conveying membrane stability, we tested Flag-tagged variants of full-length connecdenn, the C-terminal region lacking the DENN domain, and the isolated DENN domain for their ability to target to CCVs in transfected HEK 293-T cells (Fig. 3.1A). The ectopically expressed full-length protein enriched on isolated CCVs and remained on the stripped vesicles similar to endogenous connecdenn (Fig. 3.1B/C). The C-terminal region also enriched on CCVs (Fig. 3.1B), likely through interactions AP-2, a coat protein that binds directly to connecdenn [183]. However, the C-terminal region of connecdenn was readily extracted from purified vesicles, suggesting that the DENN domain, which by itself did not target to CCVs, is necessary for connecdenn to resist vesicle uncoating (Fig. 3.1B/C). To verify this, we fused the connecdenn DENN domain to the C-terminal region of NECAP 1, a CCV coat protein that targets to CCVs through motifs in its C-terminal region but that efficiently strips off the vesicles together with clathrin ([121], Fig. 3.1D). Importantly, the chimeric DENN/NECAP 1-CT protein not only targeted to CCVs but remained on the vesicles following coat removal (Fig. 3.1D), confirming that the DENN domain is necessary and sufficient for the stable vesicle interaction observed for connecdenn.

To better understand this association, we tested the isolated connecdenn DENN domain for lipid binding using sedimentation assays. The DENN domain interacted with all lipids tested with binding varying up to 2-fold over the binding seen with pure phosphatidylcholine liposomes (Fig. 3.1E). In respect to phosphatidylinositol-phosphates (PtdInsP), which help create the specificity of organellar membranes, the DENN domain showed a slight preference for PtdIns(3)P (Fig. 3.1E). The lipid binding profile of the connecdenn DENN domain is reminiscent of that for the PTEN domain of auxilin. This module is required for the burst of auxilin recruitment to the forming CCV that occurs just prior to scission and is required for auxilin function during post-scission vesicle

uncoating [38, 40]. The forming CCV progresses from a PtdIns(4,5)P2 to a PtdIns(3)P composition at later stages. Connecdenn may thus be initially recruited through interactions with coat proteins with the DENN domain allowing for increased stability during the transition from PtdIns(4,5)P2 to PtdIns(3)P, and anchoring to the uncoated vesicle for a subsequent post-endocytosis function.

An independent lead on the post-uncoating function of connecdenn was provided by a recent study in *C. elegans* [169]. RME-5, the *C. elegans* ortholog of Rab35, was shown to depend on RME-4, the *C. elegans* ortholog of connecdenn for recruitment into CCVs to reach the endosomal system and impairment of either protein leads to defects in yolk uptake and yolk receptor recycling in oocytes [169]. As RME-4 only binds to the inactive, GDP-bound form of RME-5, the mechanism of RME-5 activation and function during yolk receptor recycling remains unclear [169]. To clarify the relationship of connecdenn and Rab35, we first sought to test if Rab35, like connecdenn is a component of CCVs. Rab35 is indeed present on CCVs and has a similar pattern in CCV fractions as Rab5, which regulates CCV formation and endosomal trafficking (Fig. 3.2A). Neither of the Rabs, however, show the same degree of enrichment on CCVs as AP-2 and connecdenn (Fig. 3.2A). The absence of the early endosomal maker EEA1 from the CCV fraction shows that the presence of Rab35 and Rab5 in the CCVs is not due to an early endosomal contamination (Fig. 3.2A). The CCV association of Rab35 was further verified by its co-migration with CCVs on a continuous sucrose gradient (Fig. 3.2B). In COS-7 cells, a pool of Rab35 localizes to forming CCVs at the plasma membrane, consistent with its biochemical association with the organelle (Fig. 3.2C).

To address the molecular mechanism underlying the interaction of connecdenn with Rab35, we tested for the ability of GST-Rab35 to affinity-select Flag-tagged full-length connecdenn, the C-terminal region, or the isolated DENN domain from HEK 293-T cell lysates. Full-length connecdenn interacts with Rab35 (Fig. 3.3A). Importantly, the isolated DENN domain is fully responsible for the Rab35 binding and no interaction is seen with the C-terminus alone (Fig. 3.3A). Binding increased when the buffer was supplemented with EDTA (Fig. 3.3A), a chelating agent for bivalent ions such as $Mg2^{+}$,

which are necessary for the active conformation of nucleotide-tri-phosphates. This suggests that the DENN domain binding to Rab35 is sensitive to the nucleotide state of the GTPase. Consistently, purified GST-DENN domain affinity-selected wild-type Rab35 but showed stronger binding to Rab35 S22N (compare ratios of SM to bound Rab35), a mutation that strongly reduces nucleotide affinity analogous to Ras S17N. No binding was observed to Rab35 Q67L, a mutation analogous to Ras Q61L that impairs GTP hydrolysis and keeps the protein in a GTP-bound state (Fig. 3.3B). Flag-tagged connecdenn co-localized with Rab35 S22N in punctate structures while little colocalization was observed with the active variant Rab35 Q67L (Fig. 3.3C). Additional affinity-selection studies showed no binding of the connecdenn DENN domain to the endosomal GTPases Rab4, Rab5, and Rab11 under nucleotide-free conditions (Fig. 3.3D). Together, these data reveal the specific interaction of the connecdenn DENN domain with Rab35.

The preference for the inactive, GDP-loaded form of the GTPase is a signature of GEFs and suggests that connecdenn, through its DENN domain could function as a GEF for Rab35. We thus tested the DENN domain for GEF activity toward Rab35 and indeed, the isolated and purified domain efficiently facilitates the exchange of GTP for GDP on Rab35 with no exchange activity for Rab3 (Fig. 3.3E). The isolated DH/PH domain of intersectin, a potent GEF for the small GTPase Cdc42 [139], does not promote GTP-loading of Rab35 (Fig. 3.3E). The connecdenn DENN domain thus allows a robust and specific activation of Rab35 with a comparable exchange activity as that observed for the GEF domain of Rabex-5 towards Rab5 [190]. The ability of the DENN domain to retain connecdenn on vesicles beyond clathrin coat removal and to function as a Rab35 GEF could provide a means to activate Rab35 function while the endocytic vesicle enters the endosomal system.

To analyze the functional consequences of DENN domain-mediated Rab35 activation, we performed loss of function studies in COS-7 cells, a line with high levels of endogenous connecdenn (Suppl. Fig. 3.1B). Lentiviral delivery of GFP reporter constructs encoding microRNAs (miRNAs) for KD of Rab35 or its GEF connecdenn led

to an efficient connecdenn KD and to a significant decrease in Rab35 levels (Fig. 3.4A). Upon quantification, we found that connecdenn levels were reduced to approximately 2 and 9% of control using miRNAs CD nt248 and CD nt275, respectively, and Rab35 levels were reduced by 59 and 54% using miRNAs Rab35 nt63 and Rab35 nt419, respectively (Fig. 3.4A). Interestingly, connecdenn levels dropped in Rab35 KD conditions, suggesting that Rab35 regulates the stability of connecdenn through a currently unknown mechanism. KD of either connecdenn or Rab35 had no apparent influence on the levels of clathrin heavy chain, EEA1, and Rab11 (Fig. 3.4A).

Depletion of Rab35 led to a striking enlargement of Rab5- and EEA1-positive early endosomes, which in most cases clustered in the peri-nuclear region (Fig. 3.4B, Suppl. Fig. 3.2). In contrast, there was no obvious influence of Rab35 KD on distribution of the clathrin adaptors AP-1 and AP-2 or on the recycling endosome protein Rab11 (Fig. 3.4B, Suppl. Fig. 3.2). Importantly, the phenotype observed following Rab35 KD was identical to that observed when connecdenn was knocked down (Fig. 3.4B, Suppl. Fig. 3.2), indicating that connecdenn through its DENN domain functions as a principal regulator of Rab35 activity.

The presence of Rab5 and PtdIns(3)P on endocytic vesicles allows them to recruit EEA1, a tethering factor necessary for homotypic fusion of endocytic vesicles and for their fusion with early endosomes. The early endosomal compartment constantly receives newly endocytosed material from the plasma membrane and subsequently relays cargo into the recycling and degradative routes. An enlargement of this compartment could thus be caused by excessive fusion, a block in exit routes, or both. KD of connecdenn or Rab35 led to an approximate 6-fold up regulation in Rab5 levels (Fig. 3.4A) and while the mechanism of this up regulation is unknown, it suggests that the observed enlargement of early endosomes can be attributed, at least in part, to enhanced early endosomal fusion. This in turn would suggest that Rab35 is a negative regulator of Rab5-mediated fusion. To test this possibility, we over-expressed Flag-tagged Rab35 variants that are either constitutively active (Q67L) or inactive (S22N) and analyzed the effect of those mutants on the expression pattern of GFP-tagged wild-type Rab5. In cells over

expressing the constitutively active Rab35 variant, the pattern of GFP-Rab5 was similar to that observed in cells expressing GFP-Rab5 alone (Fig. 3.4C). In contrast, expression of the inactive Rab35 variant caused the appearance of enlarged GFP-Rab5 endosomes, equivalent to the structures seen in cells expressing the constitutively active form of Rab5, GFP-Rab5 Q79L alone (Fig. 3.4C). Thus, inactivation of Rab35 through either direct KD, or KD of its GEF connecdenn, or expression of an inactive Rab35 variant, relieves a negative regulation of Rab5 activity, causing increased or prolonged Rab5 activation and increased early endosomal size. A prolonged activity of Rab5 could also explain the clustering of early endosomes in the perinuclear region seen in the Rab35 and connecdenn KD cells and the Rab5 Q79L expressing cells (Fig. 3.4B/C, Suppl. Fig. 3.2). Since Rab5 regulates the inward transport of endosomes by coupling them to motor proteins, an upregulation of Rab5 could lead to a misplacement of early endosomes.

Rab35 has been linked to the recycling of clathrin-dependent and -independent cargo alike. A recent study demonstrated that over expression of dominant-negative Rab35 S22N impaired the recycling of the clathrin-dependent cargo transferrin at early time points, suggesting a function of Rab35 in fast transferrin recycling from early endosomes back to the plasma membrane [172]. We thus analyzed the effect of connecdenn or Rab35 KD on transferrin trafficking by flow cytometry. To our surprise, we failed to detect significant differences in the kinetics of transferrin uptake and recycling (Fig. 3.5A/B). This reveals that transferrin delivery to and exit from early endosomes in not impaired under KD conditions. We also used flow cytometry to test for kinetic defects following over-expression of GFP alone or GFP-tagged Rab35 variants in COS-7 and HeLa cells but again, we did not observe significant kinetic defects for transferrin recycling rates in either cell line (Suppl. Fig. 3.3 A/B). Together, our data demonstrate that connecdenn and Rab35 do not regulate transferrin recycling.

Clathrin-independent cargo has recently been shown to follow at least two distinct itineraries upon internalization. EHD1-positive tubules recycle MHCII from EEA1-positive early endosomes and they also recycle MHCI and β1-integrin from recycling endosomes [176, 191]. When testing connecdenn or Rab35 KD for an effect on the

trafficking of MHCI, we observed that following a 20 min pulse, labeled MHCI localized in part to EEA1-positive early endosomes in control and KD cells (Fig. 3.6A, Suppl. Fig. 3.4). In KD cells, MHCI co-localized with the enlarged endosomes that had relocalized to the perinuclear region (Fig. 3.6A, Suppl. Fig. 3.4). Along with the observations on transferrin recycling, these data demonstrate that the change in size and localization of the EEA1 endosomes in the KD cells does not affect their ability to receive cargo. However, following a 20 min chase, significantly higher amounts of labeled MHCI were retained in the KD cells when compared to control (Fig. 3.6B.), revealing that the DENN-domain mediated activation of Rab35 is necessary for efficient trafficking of MHCI following its delivery to EEA1-positive early endosomes. In control cells following a 20 min pulse, MHCI is readily detected in recycling tubules decorated with EHD1 (Fig. 3.7A). Moreover, a large number of EHD1-positive tubules originate at EEA1-positive early endosomes under control conditions (Fig. 3.7B, Suppl. Fig. 3.5). Upon KD of connecdenn or Rab35, EHD1 redistributes into the cytosol and tubule formation overall and at EEA1 endosomes is reduced (Fig. 3.7B, Suppl. Fig. 3.5). Our observations thus suggest that connecdenn-activated Rab35 controls the formation of EHD1-positive recycling tubules from early endosomes similar to the function attributed to Rab11 and Rab22 at recycling endosomes. Interestingly, this trafficking route appears to be cargo-specific. β1-integrin also uses the canonical MHCI transport pathway from early to recycling endosomes for Rab22/EHD1-mediated recycling to the plasma membrane. Using flow cytometry, we did not detect any significant differences in β1-intergin recycling between connecdenn or Rab35 KD and control cells (Suppl. Fig. 3.6). This demonstrates that Rab35 and its GEF connecdenn provide a means to promote the recycling of a specific subset of cargo from early endosomes.

Discussion

DENN domains are a tripartite unit found in multiple protein products encoded by 16 human genes including myotubularin-related proteins (MTMR) 5 and 13, Rab6-interacting protein 1 (Rab6IP1), suppressor of tumorgenicity 5, DENN/MADD/Rab3GEP, connecdenn, and a number of uncharacterized proteins. The presence of a single DENN domain protein in *Schizosaccharomyces pombe* indicates that the DENN domain is an evolutionary ancient module. Mutations in DENN domains and their parent proteins cause human diseases [185-189]. Notably, Charcot-Marie-Tooth 4B2 neuropathy is caused by a deletion of the uDENN module in MTMR 13 [188]. Even in *Arabidopsis thaliana*, a point mutation in the DENN domain of the protein SCD1 impairs exocytic vesicle trafficking crucial for cytokinesis and polarized cell expansion and causes sterility [192]. Thus, DENN domain proteins mediate crucial functions throughout evolution and yet, only minimal information is available regarding this important protein module.

In several cases, DENN domain proteins have been linked to the function of Rab GTPases but no common mode of interaction can be gleaned. Rab6IP1 binds to Rab6 and Rab11 in their GTP-bound form but also binds to GDP-bound Rab6 [193]. MADD/DENN selectively binds GTP-bound Rab3 and in this case, effector binding depends on the uDENN module [73]. However, MADD/DENN also functions as a GEF for Rab3, demonstrating that DENN domain proteins can not be classified as Rab effectors per se [107]. Notably, the enzymatic activity in DENN/MADD has only been observed for full-length protein and it has been argued that the GEF activity of MADD/DENN resides outside of the DENN domain [194]. Importantly, we now demonstrate that the purified connecdenn DENN domain has a intrinsic GEF activity specific for Rab35. Our study is thus the first to assign an enzymatic activity to the DENN domain.

Our findings beg an obvious question: Are DENN domains in general GEFs for Rabs? Based on what little information is available in the literature regarding DENN domain

proteins, the prediction would be that this enzymatic activity is specific to the connecdenn DENN domain for two reasons. First, Rab6IP1 is considered a Rab effector and while MADD/DENN is a GEF for Rab3, the DENN domain itself serves an effector function. Second, non-DENN domain proteins and protein complexes have been shown to function as GEFs for Rabs, demonstrating that other protein modules exhibit this enzymatic activity, most often in a Rab-specific manner [107, 195-208]. However, as the majority of DENN domain-bearing proteins remain unstudied to date, the available data pool is insufficient to warrant any predictions regarding a common function of the DENN domain. It will be interesting to see if other DENN domains can function as GEFs towards Rabs or even other small GTPases.

Our studies also demonstrate that the DENN domain functions as lipid-binding module with a general preference for negatively charged lipids. In face of a pI of 5.15 for the DENN domain, this may at first seem surprising. However, the F-BAR domain of CIP4 and the N-BAR domain of endophilin A1 have pIs of 5.8 and 5.6, respectively, and are known to use positively charged clusters on their surface for binding to PtdInsP-containing membranes [209]. Due to the lack of structural information regarding the DENN domain, we can only speculate that a similar mechanism of membrane interaction might apply here and could contribute to the exceptional stability of connecdenn on CCV membranes following their uncoating. The DENN domain remains on vesicle membranes in the presence of up to 1 M NaCl and this quality suggests a contribution of hydrophobic interactions to the membrane stability [183].

The uDENN, DENN, and dDENN modules are always found conjoined as a tripartite DENN domain, suggesting that they form a multi-functional unit and that all DENN domain properties will be utilized, either sequentially or concomitantly, during endocytic carrier formation and transport. In conjunction with data presented here, we propose a model for connecdenn function in which the protein is recruited to forming CCVs, in part through its interaction with AP-2 while the DENN domain functions as platform for Rab35 recruitment to the forming vesicle. Consistently, connecdenn KD leads to a lack of recruitment of Rab35 to clathrin-coated pits at the plasma membrane (data not shown,

[169]). Transition of the endocytic carrier from PtdIns(4,5)P2 to PtdIns(3)P, the PtdInsP to which the connecdenn DENN domain shows the highest affinity, then triggers connecdenn to switch its mode of interaction from the C-terminal interaction with the CCV coat to the DENN domain interacting with the vesicle membrane. This switch and/or the subsequent uncoating of the vesicle could serve as a signal for the DENN domain to exert its GEF activity towards Rab35. Connecdenn would then dissociate from the uncoated vesicle before the carrier fuses with EEA1-positive endosomes and in fact, we only observe minimal co-localization of connecdenn and EEA1 (Suppl. Fig. 3.1C). Together, this provides a mechanism to target activated Rab35 to early endosomes for subsequent function.

The proposed model of connecdenn function allows for spatio-temporal control of Rab35 geared towards GTPase activation during vesicle fission/uncoating and initial transport of the endocytic carrier. This would be consistent with the idea that one function of Rab35 is to negatively regulate the activity of Rab5. Rab35 and Rab5 are both localized to the plasma membrane and could thus simultaneously enter into forming vesicles, perfectly positioning Rab35 for the regulation of Rab5 activity levels throughout transport towards and on early endosomes. Rab5 controls numerous aspects of the early endosomal life cycle including homotypic fusion of endocytic carriers and their fusion with early endosomes, while also regulating their transport towards the cell center along cytoskeletal tracks. Consistent with the idea of increased Rab5 activity in connecdenn and Rab35 KD cells, we observed enlarged early endosomes that trafficked further into the cell and clustered in the perinuclear region. Studies on the relationship between Rab5 and Rab7 revealed that one Rab5 effector is a Rab7 GEF that activates Rab7 to allow for the transition from early to late endosomes [171, 208]. Activated Rab35 could control Rab5 activation in a similar manner, however, in this case, the Rab35 effector would either interfere with the recruitment or activation of a Rab5 GEF or function as a GAP for Rab5. Consistent with the latter, KD of the Rab5 GAP RabGAP-5 causes enlarged early endosomes that concentrate in the cell center [210], reminiscent of the phenotype we observed following connecdenn or Rab35 KD.

Over expression studies have linked Rab35 to the fast transferrin recycling pathway from early endosomes to the plasma membrane, a function that had previously been assigned to Rab4 [172, 211, 212]. However, Deneka et al. (2003) showed that KD of Rab4 results in enhanced transferrin recycling and our studies reveal that Rab35 KD does not affect transferrin trafficking. It thus appears that fast transferrin recycling from early endosomes to the plasma membrane does not depend on either Rab and that the protein machinery for this transport pathway is yet to be defined.

KD of Rab35 or its GEF connecdenn did, however, impair recycling of MHCI. MHCI is internalized in a clathrin-independent, Arf6-dependent pathway that merges with EEA1-positive endosomes that also contain clathrin-dependent cargo [179]. From there, MHCI is either delivered to late endosomes for subsequent degradation or traffics to the perinuclear recycling endosome, from where it recycles back to the plasma membrane in transferrin-negative, Rab22-positive tubular recycling endosomes [191]. In addition, these tubules contain Rab11 and EHD1, which also regulate the recycling of clathrin-dependent cargo from recycling endosomes [177, 191, 213, 214]. Clathrin-independent and clathrin-dependent cargo thus utilize in part common machineries to recycle from recycling endosomes to the plasma membrane [191]. Weigert et al. (2004) [191] further describe MHCI-positive tubules that originate from transferrin-positive endosomal structures but the protein machinery involved in their formation remained undefined. Our studies demonstrate that Rab35 activation is crucial for the recruitment of EHD1 to EEA1-positive endosomes and for efficient MHCI recycling. Together, these data suggest that MHCI recycling from distinct intracellular locations is likely mediated by common protein machinery but that the spatial organization of this machinery is determined by pathway-specific Rab GTPases. Moreover, these pathways seem, at least in part, to also be cargo-specific. β1-integrin has been shown to follow the MHCI trafficking route from the plasma membrane to the recycling endosomes and back [215-217], however, KD of Rab35 or its GEF connecdenn does not impair the trafficking of this cargo, suggesting that β1-integrin, like transferrin, does not enter into the Rab35-specific trafficking route. In contrast, MHCII was shown to recycle from early endosomes in a Rab35/EHD1-dependent manner and it will be interesting to see if MHC complexes are the only Rab35-

specific cargo or if any of the recently described clathrin-independent cargo will follow their path [176, 218]. Furthermore, one of the most robust recycling pathways known is the recycling of synaptic vesicles following neurotransmitter release. Given the function of connecdenn in recycling, an impairment of synaptic vesicle reformation could be the cause of reduced synaptic FM4-64 uptake observed in connecdenn KD neurons [176].

It appears that specific cargo can enter into distinct fast recycling pathways emerging from early endosomes and that the Rab35 pathway is permissive for MHCI and MHCII, while excluding transferrin and β1-integrin. Future studies will show if specific Rab GTPases create trafficking routes along the endosomal pathway that a number of cargo may choose to enter (e.g. dependent on the presence of sorting tags such as phosphorylation or ubiquitination) or if most cargo follow common default routes and the large number of Rabs in the human genome reflect the need to direct a select subset of cargo through deviating routes. This still leaves the question as to why the regulatory machinery for early endosomal recycling of a clathrin-independent cargo would be recruited through clathrin-mediated endocytosis. One possible benefit of this arrangement could be the spatio-temporal regulation of cargo recycling. Early endosomes receive material from various endocytic pathways and are thus the first location where all components required for MHCI recycling would come together. In the canonical pathway of antigen presentation by MHCI, the complex is loaded with peptides in the lumen of the ER and sent to the plasma membrane for antigen presentation (review in Wearsch and Cresswell, 2008 [219]). From there, the complex continuously cycles between endosomes and the plasma membrane until it is sorted for degradation. However, the recently described process of cross-presentation involves MHCI loading within early endosomes [219-222]. Concurrent delivery of the MHCI recycling machinery to early endosomes could thus provide a means to promote cross-presentation and to assure an efficient and comprehensive antigen presentation on the cell surface.

Experimental Procedures

Antibodies and reagents

Mouse monoclonal antibodies against CHC (clone 23), α-adaptin (clone 8), and EEA1 (clone 14) were from BD Transductions (Lexington, KY, USA), against Flag (M2) from Sigma (St-Louis, MI, USA), and against MHCI (W6/32) and β1-integrin (TS2/16) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against GFP (A6455) and Rab11 (71-5300) were from Invitrogen (Carlsbad, CA, USA), against, c-myc (C-3959) from Sigma, against Rab5 (Ab18211) from Abcam (Cambridge, MA, USA), and against EEA1 (C45B10) from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibodies against CLCs and connecdenn (3776) have been described previously [183, 223]. Polyclonal antibodies against Rab35 were raised in rabbits against GST-tagged full-length human Rab35. AlexaFluor 633-conjugated human transferrin (T-23362) was purchased from Invitrogen and Cy3-conjugated human transferrin (009-160-050) was from Jackson ImmunoResearch (West Grove, PA, USA).

Subcellular fractionation

CCVs were purified from adult rat brain or cell lines using buffer A (100 mM MES, pH 6.5, containing 1 mM EGTA and 5 mM $MgCl_2$) as described previously [129]. For the extraction of coat proteins, 50-100 µg aliquots of CCVs were centrifuged at 200,000xg for 15 min. The pellets were resuspended in 100 µl of buffer A or 0.5M Tris pH 8.0 EDTA (1:1 buffer A and 1M Tris pH 9.0) and incubated for 30 min on ice. The samples were centrifuged at 200,000 xg and the resuspended pellets were analyzed in parallel with the supernatant fraction. To test for the association of Rab35 with purified CCVs, CCVs were separated on a linear 20–50% sucrose gradient prepared in buffer A by centrifugation in a Sorvall (Newtown, CT) AH629 rotor at 145,000 xg for 1.5 h. The gradients were fractionated from the bottom, and gradient fractions were analyzed by Western blot.

Cell culture

293-T and COS-7 cells were maintained in DMEM High Glucose (Invitrogen, Grand Island, cat. no. 11995) containing 10% heat-inactivated FBS (PAA Laboratories Inc., Etobicoke), 100 U/ml penicillin, and 100 μg/ml streptomycin (both Invitrogen).

Purification and cleavage of fusion proteins

Bacterial GST fusion proteins were expressed in BL21. Cells were lysed by sonication in a PBS buffer containing protease inhibitors (0.83 mM benzamidine, 0.23 mM phenylmethylsulphonyl fluoride, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin) and 1% Triton X-100, incubated 15 min at 4ºC and spun at 205,000 xg for 30 min. The supernatant was incubated with pre-washed glutathione-Sepharose 4B beads (GE Healthcare, Chalfont St. Giles, United Kingdom) for 1 h at 4ºC and then washed with PBS. pEBG constructs for expression of GST-tagged fusion proteins in mammalian cells were transfected into HEK-293T cells using calcium phosphate. After 48 hours of expression, cells were scraped in PBS buffer purified as described above. For GDP/GTP exchange assays, the GST tag was removed by incubating the fusion proteins bound to glutathione Sepharose 4B, in thrombin cleavage buffer (50mM Tris, pH 8.0, 150mM NaCl, 5mM MgCl2, 1mM DTT), overnight at 4ºC using 5 units of thrombin (Sigma, St-Louis, USA) in 500 μl total volume. Thrombin was cleared with benzamidine Sepharose (GE Healthcare, Chalfont St. Giles, United Kingdom) and the supernatant containing the cleaved proteins was exchanged into experimental buffer and concentrated to a final volume of 150 μl using a Millipore Amicon Ultra – 15 centrifugal filter device (Millipore, Carrintwohill, Ireland). For GST affinity selection assays, all GST fusion proteins were expressed in bacteria with the exception of the connecdenn DENN domain, which was purified from 293-T cells. All proteins used in GDP/GTP exchange assays were expressed in HEK-293T cells except for GST-DH/PH, which was expressed in BL21.

GST affinity selection assays

Flag- and GFP-tagged proteins were expressed in 293-T cells and cells were lysed in HEPES buffer (10 mM HEPES-OH, pH 7.4, 100mM NaCl, 0.83 mM benzamidine, 0.23

mM phenylmethylsulphonyl fluoride, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin) in the absence or presence of 10mM EDTA as indicated. Triton X-100 was added to a final concentration of 1% and lysates were rocked for 5 min at 4°C before centrifugation at 205,000 xg to remove cell debris. Aliquots of the supernatant were incubated for 1 h at 4°C with GST fusion proteins pre-coupled to glutathione-Sepharose and then washed with the appropriate buffer. Samples were separated by SDS-PAGE and binding was detected by Western Blot.

GDP/GTP exchange assay

GST-tagged Rab3, Rab35, and connecdenn DENN domain were purified and cleaved as described above. Cleaved Rab3 and Rab35 were exchanged into GEF loading buffer (20mM Tris pH 7.5, 100mM NaCl) and the DENN domain was exchanged into GEF incubation buffer (20mM Tris pH 7.5, 100mM NaCl and 5 mM $MgCl_2$). 15 μM of purified GTPase were loaded with 40 μM GDP by incubation at 30ºC for 10 min in GEF loading buffer containing 5 mM EDTA. Loaded GDP was stabilized by the addition of 10 mM $MgCl_2$. Exchange reactions were carried out at room temperature in 130 μl total volume containing 1.5 μM loaded GTPase, 100 nM DENN domain, 0.5 mg/ml BSA, 5 μM GTPγS, 0.2 mCi/mmol [35S]GTPγS, and 0.5 mM DTT in GEF incubation buffer. At the indicated times, 15μl of the reaction were removed, added to 1ml of ice cold wash buffer (20 mM Tris pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$), passed through nitrocellulose filters, washed with 5 ml wash buffer and counted using a liquid scintillation counter (Beckmann Coulter LS6500 Scintillator).

Liposome preparation and lipid binding assay

Liposome binding assays were performed using liposome suspensions (1mg/ml) containing 90 mol % PtdChol and 10 mol % of each of the following phospholipids: PtdIns, PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2, PtdIns(3,4,5)P3 (Echelon Bioscience, Inc.), PtdSer, PtdChol (Avanti Polar Lipid Inc.). Lipid mixtures were resuspended in Lipid Buffer (25 mM HEPES pH 7.5, 150 mM KCl, 1 mM EDTA). To remove protein aggregates, purified proteins were centrifuged at 55,000 rpm for 45 min at 4ºC in a TL100 rotor (Beckman). Two micrograms of protein

were incubated with 100 μg liposomes for one hour at room temperature and centrifuged at 55,000 rpm for 1 hour at 4ºC. Protein inputs and pellets were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The intensity of proteins bands was measured using Odyssey Image software (LI-COR biosciences).

miRNA lentivirus production and KD

Target sequences for human connecdenn and Rab35 were designed using the Block-iT RNAi Designer (Invitrogen) (CD nt248 GATTCGGGTTCTGCCGCTTAT, CD nt275 GAGCGAAGAGCTGCTTCTGTA, Rab35 nt 63 GAGCAGTTTACTGTTGCGTTT, Rab35 nt 419 GGCAGATGGGCATCCAGTTGT) and subcloned into pcDNA6.2/GW-EmGFP-miR (Invitrogen) following manufacturer's instructions. The EmGFP-miR cassette was then amplified by PCR, subcloned into the pRRLsinPPT vector, and VSVG pseudotyped virus was produced in 293-T cells. Viral particles were concentrated by centrifugation and titered using 293-T cells. The control miR virus was described earlier [224]. For KD studies in COS-7 cells, cells were plated on the day of transduction. For transduction, the culture medium was replaced by DMEM High Glucose (Invitrogen) supplemented with 2% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 6 μg/ml polybrene (Sigma) and viruses were added at an MOI of 10. The next day media was replaced with fresh culture medium and the cells were cultivated until assays were performed five days after transduction.

Immunofluorescence

COS-7 cells were plated on poly-L-lysine-coated coverslips and transfected using Lipofectamine 2000 (Invitrogen) the next day. For transfection of KD cells, the incubation time with the Lipofectamine 2000/DNA mix was limited to one hour. Following over night incubation, cells were fixed in 4% PFA and processed for immunofluorescence following standard protocols. For the localization of marker proteins, KD cells were plated one to two days before analysis on poly-L-lysine-coated coverslips and processed for immunofluorescence on day five after transduction. For MHCI recycling, cells were incubated with 1 μg of antibody per 10^6 cells for 20 min at 37ºC in culture medium. The cells were chilled on ice and surface-bound antibody was

removed by acid wash, followed by a PBS wash, and the cells were fixed and processed as described above.

Endocytosis and recycling assays

For transferrin internalization assays, COS-7 cells transduced with control, connecdenn or Rab35 KD viruses were starved in DMEM High Glucose over night and analyzed on day five after transduction. The cells were chilled on ice for 30 min and then incubated with Alexa633-transferrin (200 μg/ml) in ice-cold DMEM on ice for one hour. Cells were washed with cold PBS and incubated in pre-warmed culture medium at 37ºC for the times indicated. At each time point, a sample of cells was chilled on ice, surface-bound transferrin was removed by acid wash (0.2M acetic acid, 0.5M NaCl), followed by a PBS wash. The cells were removed from the plate in 1 ml of PBS by pipetting, filtered through a cell strainer and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). For transferrin recycling assays using COS-7 KD cells or COS-7 and HeLa cells expressing GFP alone or GFP fusion proteins of Rab35 wt, Rab35 S22N or Rab35 Q67L for two to three days, cells were starved over night and incubated with Alexa633-transferrin for one hour at 37ºC. The cells were chilled on ice and surface-bound transferrin was removed by acid wash, followed by a PBS wash. The cells were then incubated in pre-warmed culture medium at 37ºC for the times indicated. At each time point, a sample of cells was chilled on ice, surface-bound transferrin was removed by acid wash and the cells were processed by flow cytometry as described above. Due to lower expression levels of GFP-Rab35 S22N, transfected cells were gated in the GFP channel such that all cells analyzed had a fluorescence intensity of 10 arbitrary units or more. For MHCI recycling assays, two sets of cells were incubated with 1 μg of antibody per 10^6 cells for 20 min at 37ºC in culture medium. The cells were chilled on ice and surface-bound antibody was removed by acid wash, followed by a PBS wash. The second set was further incubated in pre-warmed culture medium at 37ºC for 20 min, then chilled on ice and surface-bound antibody was removed by acid wash, followed by a PBS wash. Both sets were fixed in 2% PFA, permeabilized with 0.2% Triton X-100 in PBS and incubated with Alexa647-conjugated secondary antibodies for 30 min on ice. The cells were then washed with PBS, scraped off the plates and processed by flow cytometry as

described above. For β1-integrin recycling assays, two sets of cells were starved in DMEM High Glucose for 1 hour at 37ºC, then incubated with 1 μg of antibody per 10^6 cells for 2 hours at 37ºC in DMEM. The cells were chilled on ice and surface-bound antibody was removed by acid wash, followed by a PBS wash. The second set was further incubated in pre-warmed culture medium at 37ºC for 2 hours, then chilled on ice and surface-bound antibody was removed by acid wash, followed by a PBS wash. Both sets were fixed in 2% PFA, permeabilized with 0.2% Triton X-100 in PBS and incubated with Alexa647-conjugated secondary antibodies for 30 min on ice. The cells were then washed with PBS and processed by flow cytometry as described above. In each assay, 10,000 cells were analyzed for each time point and condition.

Expression constructs

Human Rab35 (NM_006861) was obtained from Invitrogen, human Rab3A (NM_002866), Rab4A (NM_004578), and Rab11A (NM_00663) were obtained from Origene (Rockville, MD, USA). The coding sequences were amplified by PCR and cloned into pGEX-6P1, pEBG, peGFP-C1, and pcDNA3-Flag [132]. Rab35 S22N and Q67L were produced by MEGA primer approach and cloned into pEGFP-C1, pcDNA3-Flag or -myc (generated as described in [132]), and pGEX-6P1. The constructs for the GST-tagged DH/PH domain of intersectin 1-l, Flag-tagged connecdenn and the C-terminal region of NECAP 1 were previously described [121, 139, 183], for the Flag-tagged C-terminal region of connecdenn, parts of the open reading frame encoding amino acids 372-1016 were cloned into pCMV-Tag2B. For expression of the DENN domain, the nucleotide sequence encoding amino acids 1-403 were amplified by PCR using an antisense oligo that introduced a restrictions site between the codon for amino acid 403 and the stop codon and cloned into pEBG and pcDNA3-Flag. The restriction site following the codon for amino acid 403 was then used to insert the region of NECAP 1 encoding amino acids 129-275 for generation of the chimera expression construct in pcDNA3-Flag. GFP-Rab5 wt, S22N, and Q67L expression constructs were a generous gift from Dr. Presley (McGill University, Canada) and the expression construct for myc-tagged EHD1 was a generous gift from Dr. Caplan (University of Nebraska, USA). All plasmid were verified by sequencing.

Oligo-nucleotides

CD AA1 F-BGLII	GCGCAGATCTATGGGCTCCAGGATCAAGCAAAAC
CD AA403 R-ECOR1	GCCCGAATTCTCACTGATGGTACAGTTTATCACTG
CD AA372 F-BGLII	GCGCAGATCTCTAGACCTTCTCAATTCCGGTGAAGGT
CD AA1016 R-BGLII	GCGCGAATTCTCACTCAAAGGTCTCCCACTGTCTGCG
CD NT248 S	TGCTGATAAGCGGCAGAACCCGAATCGTTTTGGCCACTGACTGACGATTCGGGCTGCCGCTTAT
CD NT248 AS	CCTGATAAGCGGCAGCCCGAATCGTCAGTCAGTGGCCAAAACGATTCGGGTTCTGCCGCTTATC
CD NT275 S	TGCTGTACAGAAGCAGCTCTTCGCTCGTTTTGGCCACTGACTGACGAGCGAAGCTGCTTCTGTA
CD NT275 AS	CCTGTACAGAAGCAGCTTCGCTCGTCAGTCAGTGGCCAAAACGAGCGAAGAGCTGCTTCTGTAC
PCDNA3-MYC S	AGCTTGCCACCATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTGG
PCDNA3-MYC AS	GATCCCAGGTCCTCCTCTGAGATCAGCTTCTGCATTGATGCCATGGTGGCA
RAB3 AA1 F-BAMH1	GCGCGGATCCATGGCATCCGCCACAGACTCG
RAB3 AA220 R-ECOR1	GCGCGAATTCTCAGCAGGCGCAGTCCTGGTG
RAB4 AA1 F-BAMH1	GCGCGGATCCATGTCGCAGACGGCCATGTC
RAB4 AA218 R-ECOR1	GCGCGAATTCCTAACAACCACACTCCTGAG
RAB11 AA1 F-BAMH1	GCGCGGATCCATGGGCACCCGCGACGACGAG
RAB11 AA216 R-ECOR1	GCGCGAATTCTTAGATGTTCTGACAGCACTG
RAB35 AA1 F-BAMH1	GCGCGGATCCATGGCCCGGGACTACGACCACC
RAB35 AA201 R-ECOR1	GCGCGAATTCTTATTAGCAGCAGCGTTTCTTTC
RAB35 NT63 S	TGCTGAAACGCAACAGTAAACTGCTCGTTTTGGCCACTGACTGACGAGCAGTTCTGTTGCGTTT
RAB35 NT63 AS	CCTGAAACGCAACAGAACTGCTCGTCAGTCAGTGGCCAAAACGAGCAGTTTACTGTTGCGTTTC
RAB35 NT419 S	TGCTGACAACTGGATGCCCATCTGCCGTTTTGGCCACTGACTGACGGCAGATGCATCCAGTTGT
RAB35 NT419 AS	CCTGACAACTGGATGCATCTGCCGTCAGTCAGTGGCCAAAACGGCAGATGGGCATCCAGTTGTC
RAB35 S22N R	AGTAAACTGTTCTTGCCCACAC
RAB35 Q67L R	AGCGCTCCAGCCCCGCTG

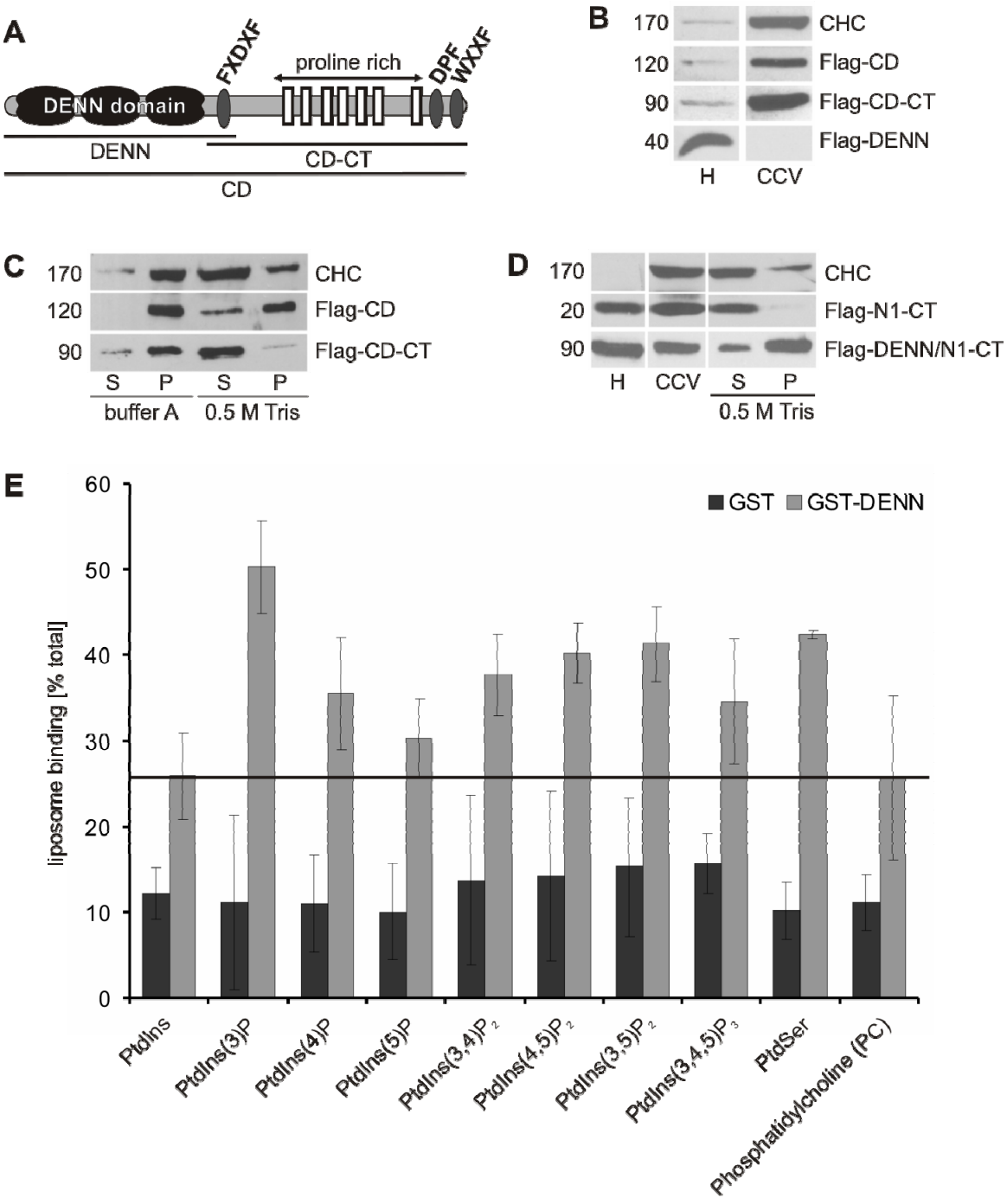

A
FXDXF
proline rich
DPF
WXXF
DENN domain
DENN
CD-CT
CD
B
170
120
90
40
CHC
Flag-CD
Flag-CD-CT
Flag-DENN
H
CCV
C
170
120
90
CHC
Flag-CD
Flag-CD-CT
S
P
S
P
buffer A
0.5 M Tris
D
170
20
90
CHC
Flag-N1-CT
Flag-DENN/N1-CT
H
CCV
S
P
0.5 M Tris
E
GST
GST-DENN
liposome binding [% total]
60
50
40
30
20
10
0
PtdIns
PtdIns(3)P
PtdIns(4)P
PtdIns(5)P
PtdIns(3,4)P2
PtdIns(4,5)P2
PtdIns(3,5)P2
PtdIns(3,4,5)P3
PtdSer
Phosphatidylcholine (PC)

Figure 3.1 – The connecdenn DENN domain is a lipid-binding modules and mediates a stable recruitment of connecdenn to the membrane fraction of CCVs. ***A.*** Schematic representation of connecdenn, indicating the N-terminal DENN domain and the peptide motifs in the C-terminal region for binding to AP-2 (FXDXF, DPF, WXXF) and SH3 domain-containing accessory proteins (proline-rich). Underlined regions represent the borders of constructs used in the study (DENN: DENN domain, amino acids 1-403, CD-CT: connecdenn C-terminal region, amino acids 372-1016, CD: full-length connecdenn, amino acids 1-1016). ***B.*** Equal protein amounts of homogenates (H) and purified CCVs from 293-T cells transfected with Flag-tagged full-length connecdenn (Flag-CD), the C-terminal region (Flag-CD-CT) or the DENN domain (Flag-DENN) were probed by Western blot with antibodies against clathrin heavy chain (CHC) and Flag. ***C.*** Equal amounts of purified CCVs from cells expressing Flag-tagged connecdenn (Flag-CD) or the C-terminal region (Flag-CD-CT) were incubated with buffer A or 0.5M Tris pH 8.0 (1:1 buffer A, 1M Tris pH 9.5) to strip the proteinous coat off the CCV membrane. The fractions were separated by centrifugation and equal volumes of resuspended pellets (P) and supernatants (S) were analyzed for protein partitioning by Western blot, using clathrin heavy chain (CHC) as control. ***D.*** Equal protein amounts of homogenates (H) and purified CCVs from 293-T cells transfected with the Flag-tagged C-terminal region of NECAP 1 (Flag-N1-CT) or a chimeric protein, where the connecdenn DENN domain had been fused to the NECAP 1 C-terminal region (Flag-DENN/N1-CT) were probed by Western blot with antibodies against clathrin heavy chain (CHC) and Flag. Purified CCVs were incubated with 0.5M Tris pH 8.0 to strip the proteinous coat off the CCV membrane as described above and supernatant (S) and pellet fractions (P) were analyzed in parallel. ***E.*** Purified GST or GST-DENN domain were incubated with liposome suspensions (1mg/ml) containing 90 mol% of PtdChol (PC) and 10 mol% of each of the indicated phospholipids. Bar graph represents the mean percentage (±SEM) of starting material retained on liposomes, N=4.

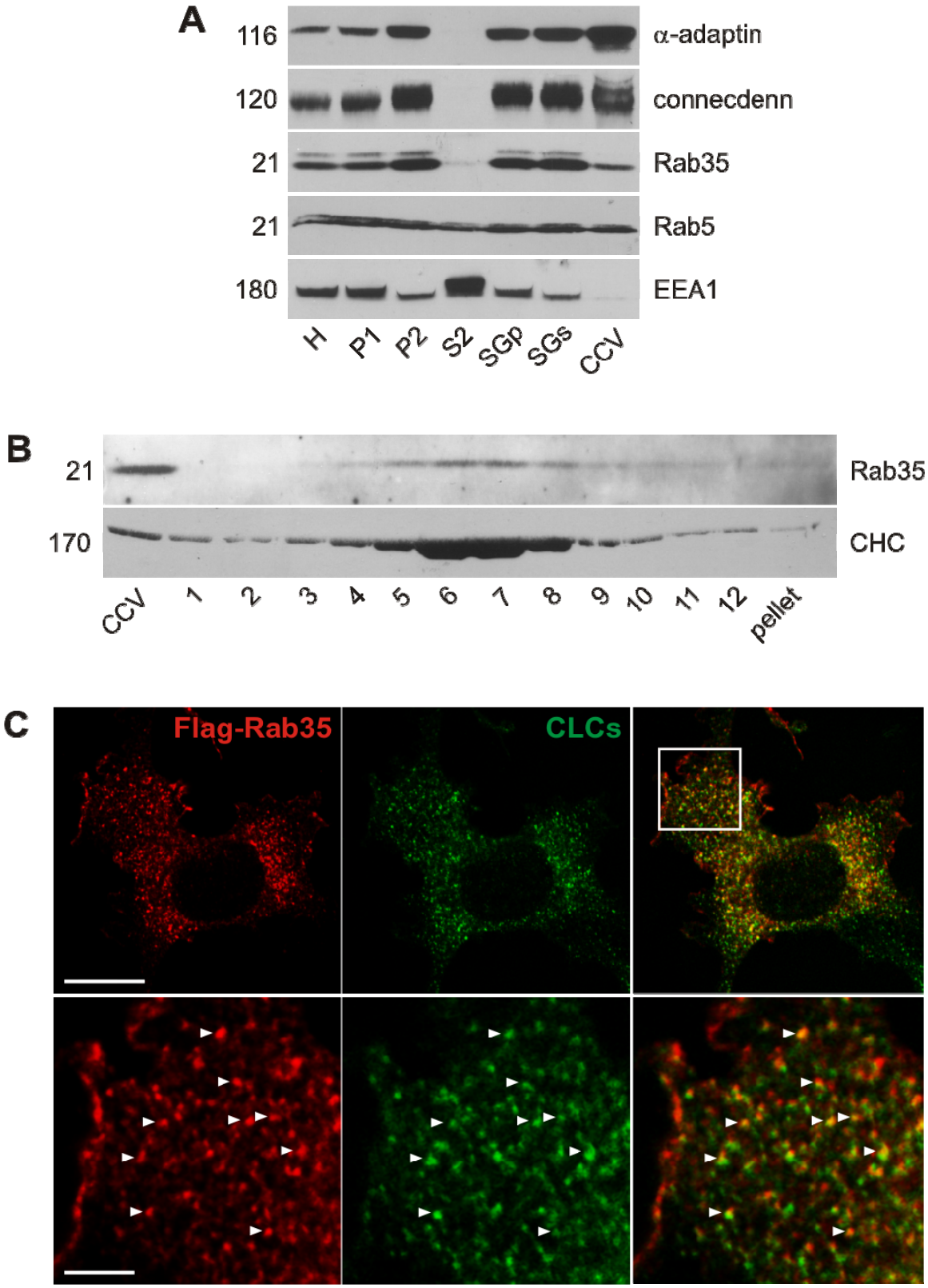

A
116
α-adaptin
120
connecdenn
21
Rab35
21
Rab5
180
EEA1
H
P1
P2
S2
SGp
SGs
CCV
B
21
Rab35
170
CHC
CCV
1
2
3
4
5
6
7
8
9
10
11
12
pellet
C
Flag-Rab35
CLCs

Figure 3.2 – Rab35 is associated with CCVs. The DENN domain of connecdenn binds Rab35 and functions as guanine nucleotide exchange factor. ***A.*** Equal protein amounts of various fractions obtained during the purification of CCVs from adult rat brain were analyzed by Western blot for the proteins indicated. ***B.*** Purified CCVs were separated on a continuous sucrose gradient and aliquots of the gradient fractions were analyzed by Western blot for clathrin heavy chain (CHC) and Rab35. ***C.*** COS-7 cells were transfected with Flag-Rab35 and localization of the ectopically expressed protein was compared to endogenous clathrin light chains (CLCs). The bottom row is a higher magnification of the area highlighted by the box. The bar represents 20 μm in the low and 5 μm in the high magnification.

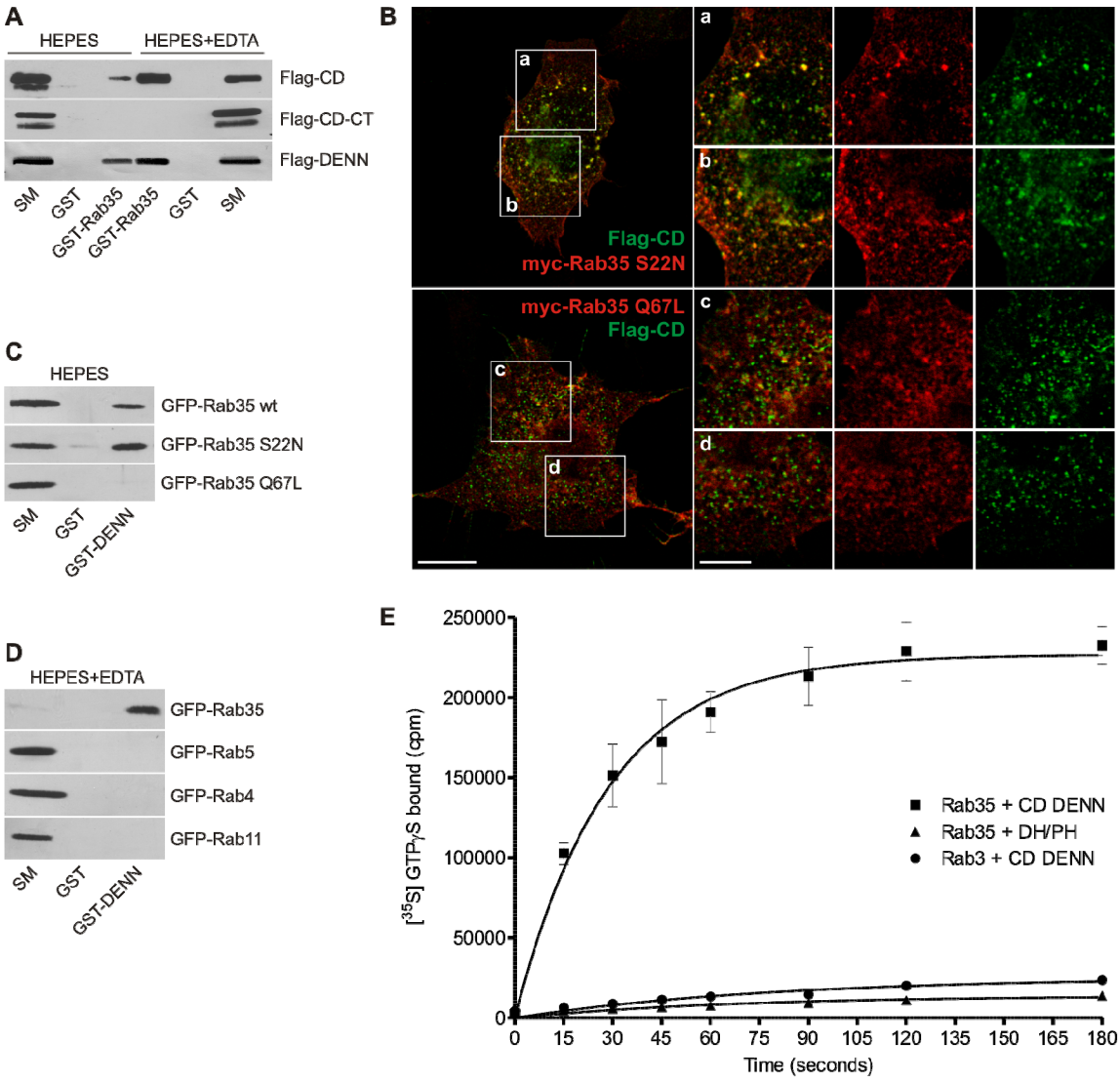

A
HEPES
HEPES+EDTA
Flag-CD
Flag-CD-CT
Flag-DENN
SM
GST
GST-Rab35
GST-Rab35
GST
SM
B
a
b
Flag-CD
myc-Rab35 S22N
myc-Rab35 Q67L
Flag-CD
c
d
C
HEPES
GFP-Rab35 wt
GFP-Rab35 S22N
GFP-Rab35 Q67L
SM
GST
GST-DENN
D
HEPES+EDTA
GFP-Rab35
GFP-Rab5
GFP-Rab4
GFP-Rab11
SM
GST
GST-DENN
E
250000
200000
150000
100000
50000
0
[35S] GTPγS bound (cpm)
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
Rab35 + CD DENN
Rab35 + DH/PH
Rab3 + CD DENN

Figure 3.3 – The DENN domain of connecdenn binds Rab35 and functions as guanine nucleotide exchange factor. ***A.*** Purified GST or GST-Rab35 were used to affinity-select Flag-tagged full-length connecdenn (CD), the C-terminal region (CD-CT) or the isolated DENN domain. Binding studies were performed in HEPES buffer in the absence (HEPES) or presence of 10 mM EDTA (HEPES+EDTA), which renders GTPases nucleotide-free and interaction was detected by Western blot. The starting material (SM) represents 10% of the material used in each condition. ***B.*** Purified GST and GST-DENN domain were used to affinity-select GFP-tagged Rab35 wild-type (wt), GFP-Rab35 S22N or GFP-Rab35 Q67L from lysates of transfected 293-T cells in HEPES buffer and binding was revealed by Western blot. The starting material (SM) represents 10% of the material used in each condition. ***C.*** COS-7 cells were transfected with Flag-tagged connecdenn (Flag-CD) and myc-tagged Rab35 S22N or Q67L and processed for immunofluorescence using antibodies directed against the tags. The regions highlighted by boxes a-d are shown in higher magnification as split channels and merge. The bar represents 20 μm for the low magnification and 5 μm for insets a-d. ***D.*** Purified GST and GST-DENN domain were used to affinity-select GFP-tagged Rab35, Rab5, Rab4, or Rab11 from lysates of transfected 293-T cells under nucleotide-free conditions (HEPES+EDTA) and binding was revealed by Western blot using GFP antibodies. The starting material (SM) represents 10% of the material used in each condition. ***E.*** To measure guanine nucleotide exchange activity, 1.5 μM GDP-loaded Rab3 or Rab35 were incubated with 100 nM connecdenn DENN domain or DH/PH domain of intersectin 1-l, a specific GEF for Cdc42, in the presence of 5 μM cold GTPγS and 0.2 mCi/mol 35S-GTPγS. At the time points indicated, an aliquot of the reaction was analyzed for nucleotide exchange as described in the materials and methods. The bar graph represents mean cpm ± standard deviation, N=3, the data was plotted using GraphPad Prism and the curve was fit by nonlinear regression one phase association.

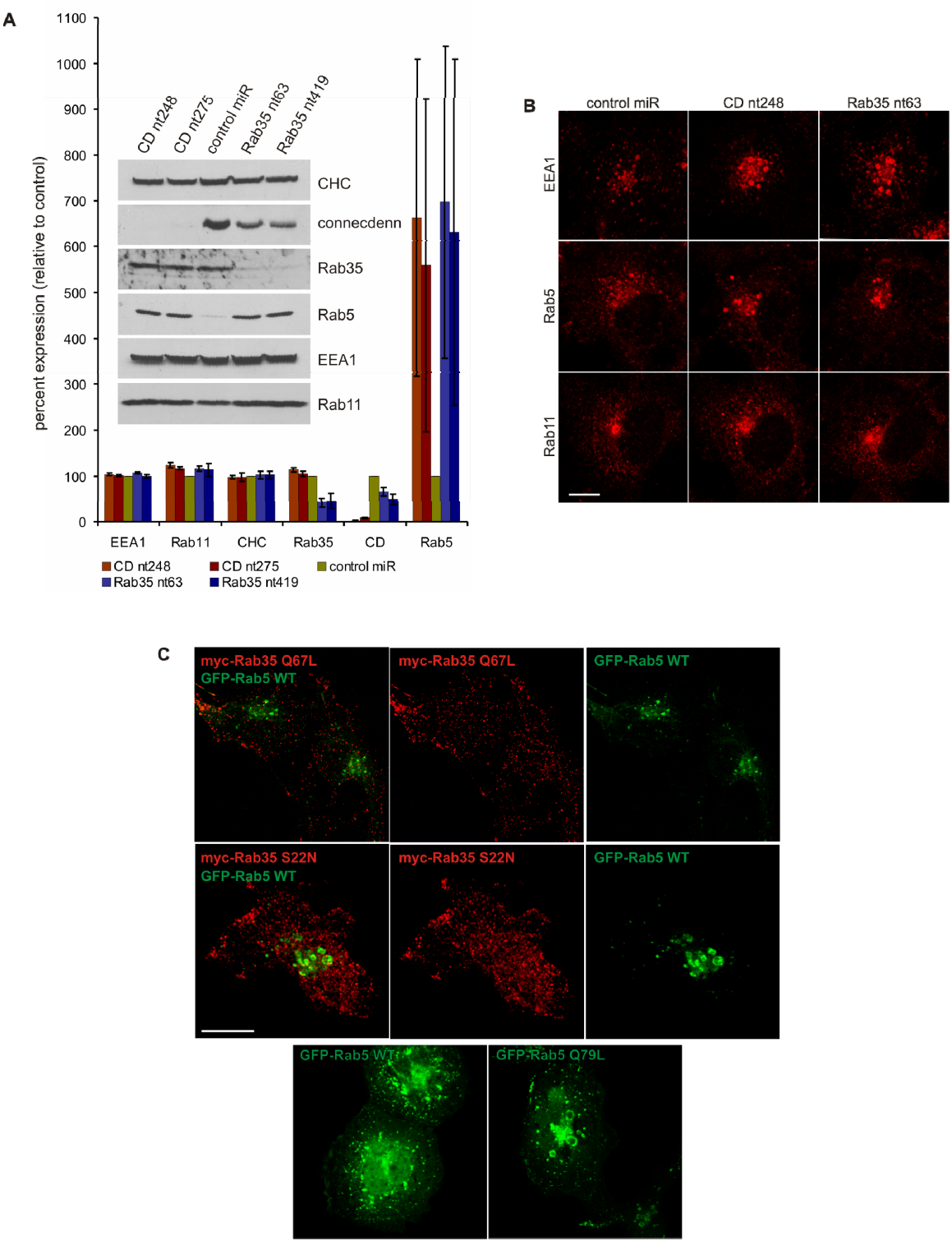
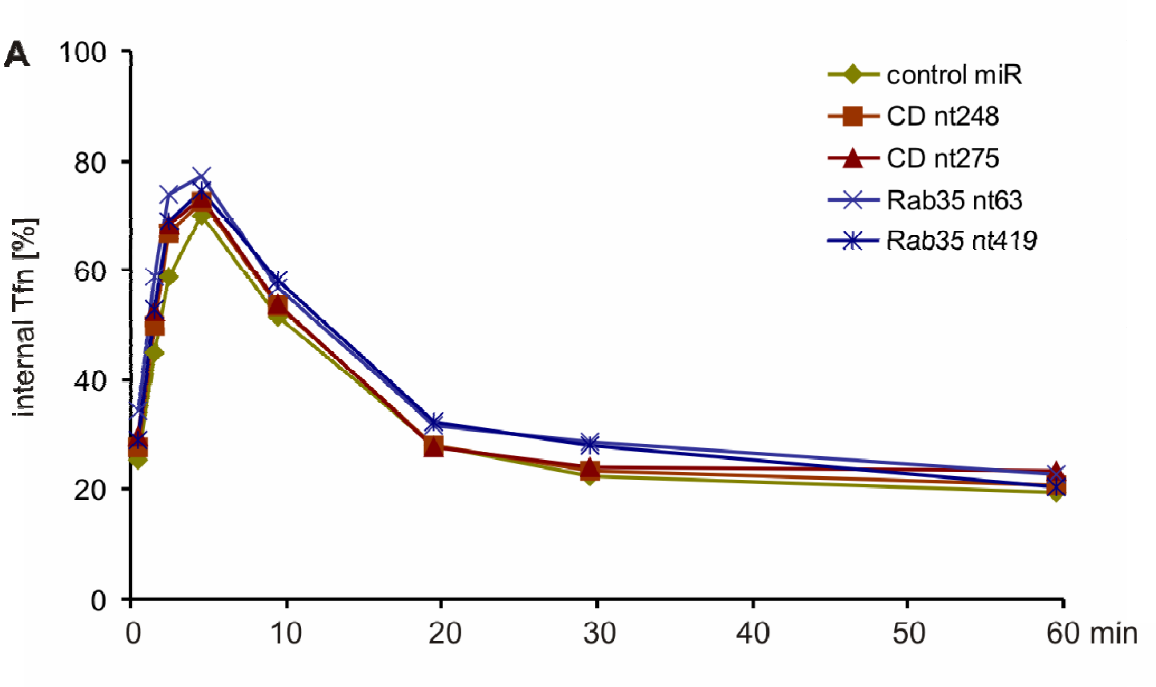

A
percent expression (relative to control)
1100
1000
900
800
700
600
500
400
300
200
100
0
CD nt248
CD nt275
control miR
Rab35 nt63
Rab35 nt419
CHC
connecdenn
Rab35
Rab5
EEA1
Rab11
EEA1
Rab11
CHC
Rab35
CD
Rab5
CD nt248
CD nt275
control miR
Rab35 nt63
Rab35 nt419
B
control miR
CD nt248
Rab35 nt63
EEA1
Rab5
Rab11
C
myc-Rab35 Q67L
GFP-Rab5 WT
myc-Rab35 Q67L
GFP-Rab5 WT
myc-Rab35 S22N
GFP-Rab5 WT
myc-Rab35 S22N
GFP-Rab5 WT
GFP-Rab5 WT
GFP-Rab5 Q79L

Figure 3.4 – KD of connecdenn or Rab35 causes perinuclear clustering and enlargement of early endosomes. ***A.*** COS-7 cells were transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) and expression levels of the indicated proteins were analyzed by Western Blot. For the bar graph, Western blots from four independent transductions were quantified by densitometry using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008). Expression levels are represented in percent (±STDEV), setting the control miRNA to 100%. ***B.*** COS-7 cells were transduced with lent viruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248) or Rab35 (Rab35 nt63) and processed for immunofluorescence to reveal the localization of endogenous markers of early endosomes (EEA1, Rab5) and the recycling endosome (Rab11). The bar represents 10 μm. ***C.*** COS-7 cells were transfected with GFP-Rab5 wt or GFP-Rab5 Q67L alone for reference, or co-transfected GFP-Rab5 and myc-Rab35 S22N, or GFP-Rab5 and myc-Rab35 Q67L and Flag-tagged proteins were detected by immunofluorescence to compare localization and effect of the Rab35 variants on the localization of Rab5. Split channels are shown for co-transfected cells. The bar represents 20 μm.


A
100
80
60
40
20
0
internal Tfn [%]
0
10
20
30
40
50
60 min
control miR
CD nt248
CD nt275
Rab35 nt63
Rab35 nt419

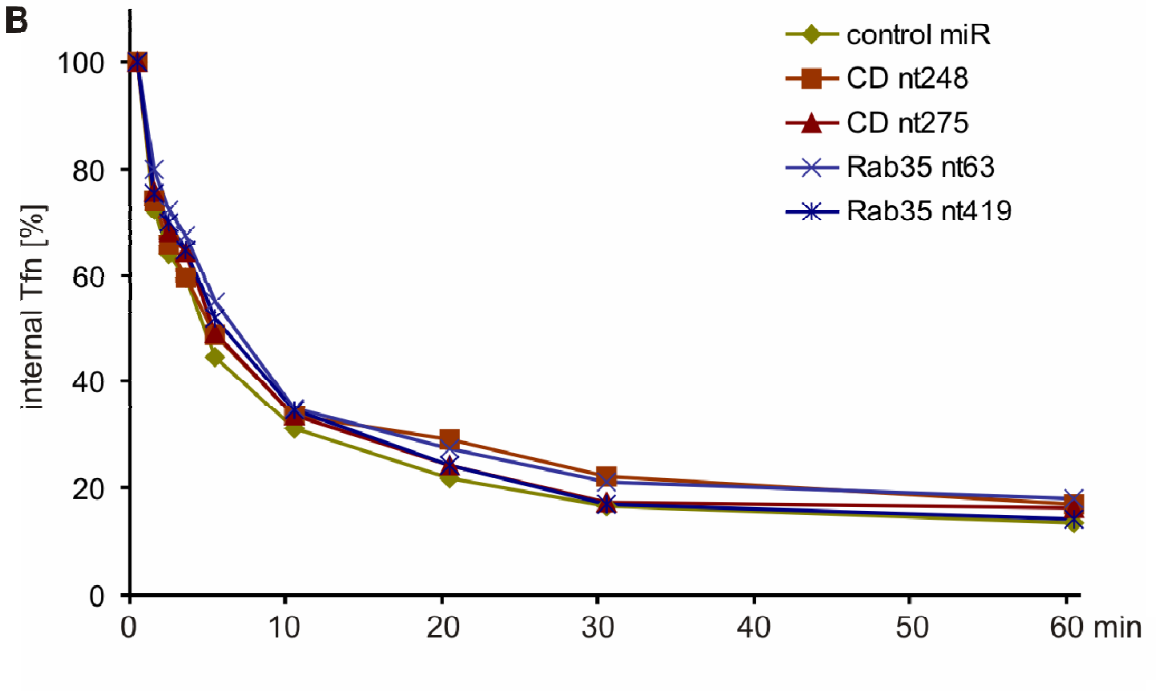

B
100
80
60
40
20
0
internal Tfn [%]
0
10
20
30
40
50
60 min
control miR
CD nt248
CD nt275
Rab35 nt63
Rab35 nt419

Figure 3.5 – Connecdenn and Rab35 do not control transferrin trafficking. ***A.*** COS-7 cells were transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419), surface-labeled on ice with AlexaFluor647-transferrin, shifted to 37ºC for the indicated times, and intracellular transferrin was measured by flow cytometry. The graph represent the mean of four independent experiments, plotted as percent of the initial surface label and statistical analysis by Repeated Measure Two-Way ANOVA followed by Bonferroni posttests revealed no significant differences between control and KD cells. ***B.*** COS-7 cells were transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419), continuously labeled with AlexaFluor647-transferrin at 37ºC for one hour, chased at 37ºC for the indicated times, and intracellular transferrin was measured by flow cytometry. The graph represent the mean of four independent experiments, plotted as percent of the initial internal label and statistical analysis by Repeated Measure Two-Way ANOVA followed by Bonferroni posttests revealed no significant differences between control and KD cells.

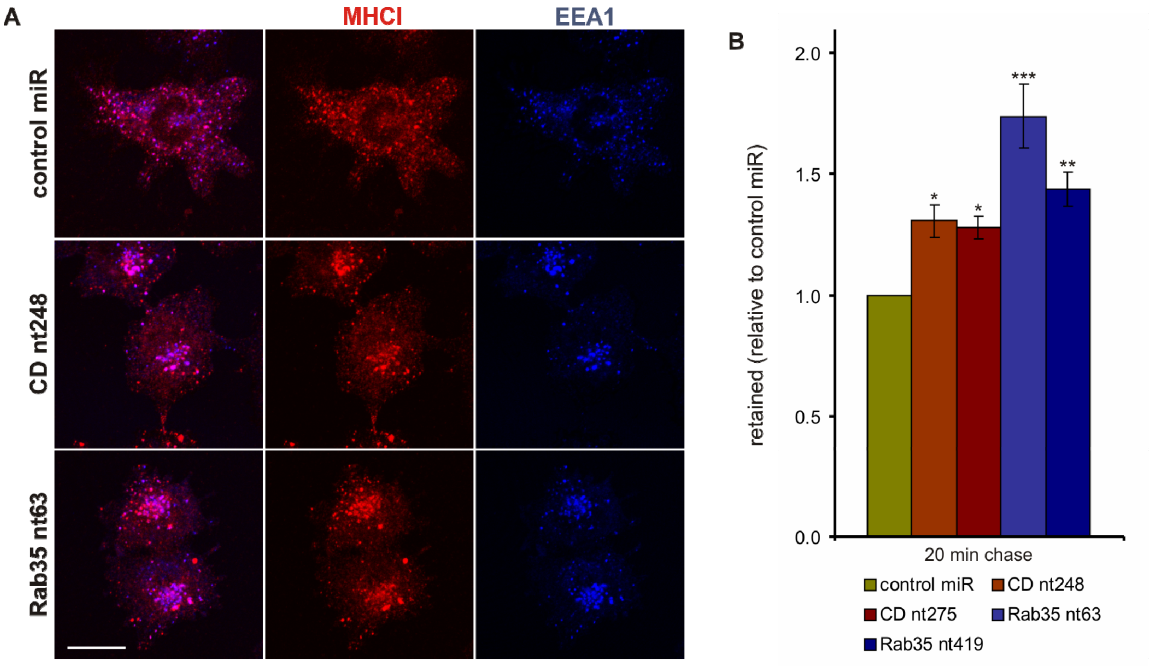

A
MHCI
EEA1
control miR
CD nt248
Rab35 nt63
B
retained (relative to control miR)
2.0
1.5
1.0
0.5
0.0
*
*
***
**
20 min chase
control miR
CD nt248
CD nt275
Rab35 nt63
Rab35 nt419

Figure 3.6 – Connecdenn and Rab35 KD affects MHCI trafficking. ***A.*** COS-7 cells transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248) or Rab35 (Rab35 nt63) were incubated for 20 min with antibodies against MHCI and processed by immunofluorescence to reveal the localization of the internalized antibodies and endogenous EEA1. The bar represents 20 μm. ***B.*** COS-7 cells transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were incubated for 20 min with antibodies against MHCI, chased for 20 min, and intracellular MHCI antibodies were detected by flow cytometry. The graph represents the mean fluorescence after the 20 min chase of four independent experiments, plotted as relative ratio (±SEM) with the control miRNA set to 1. Statistical analysis by Repeated Measure One-Way ANOVA followed by Dunnett's posttests revealed significant differences between control and KD cells (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

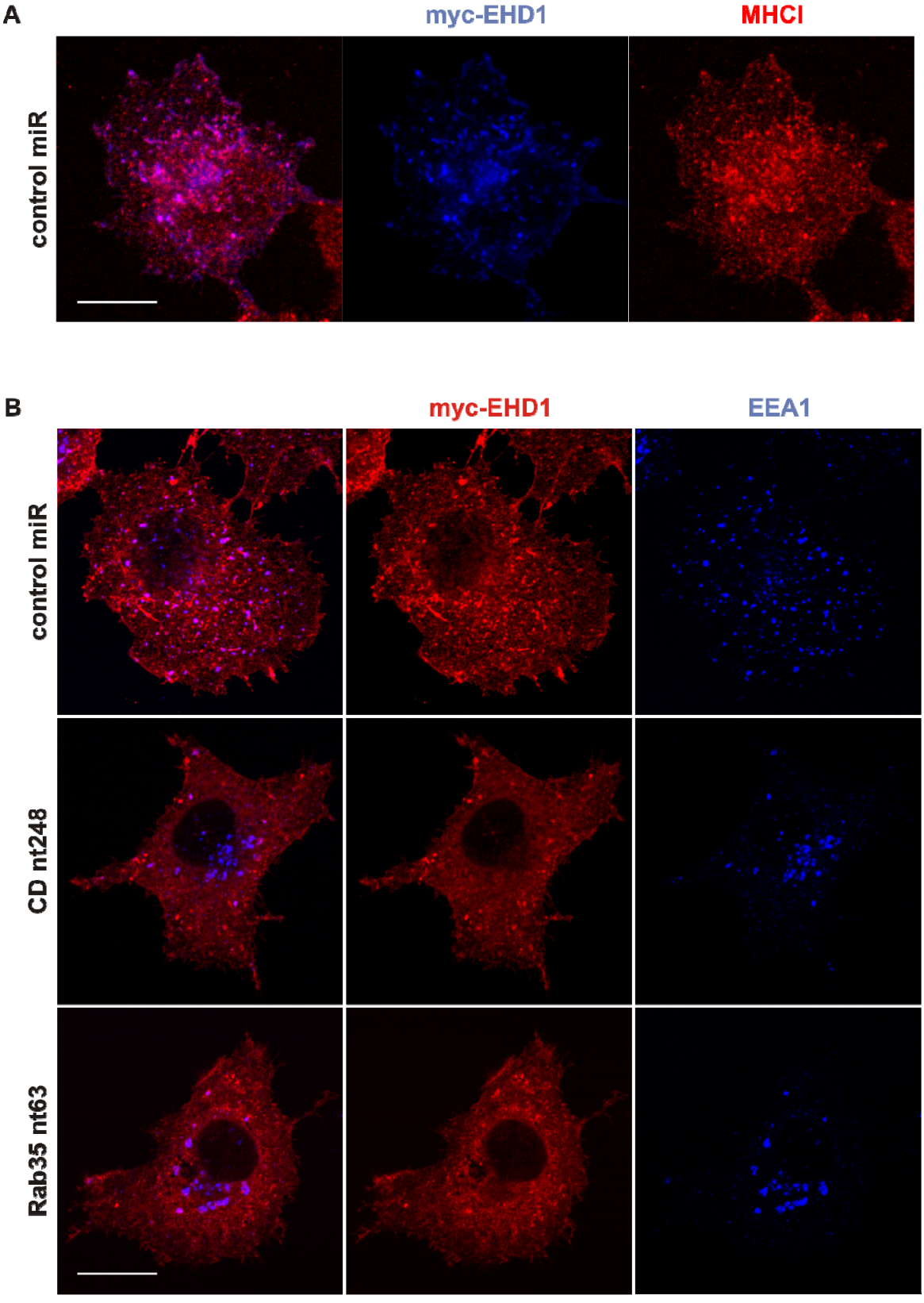
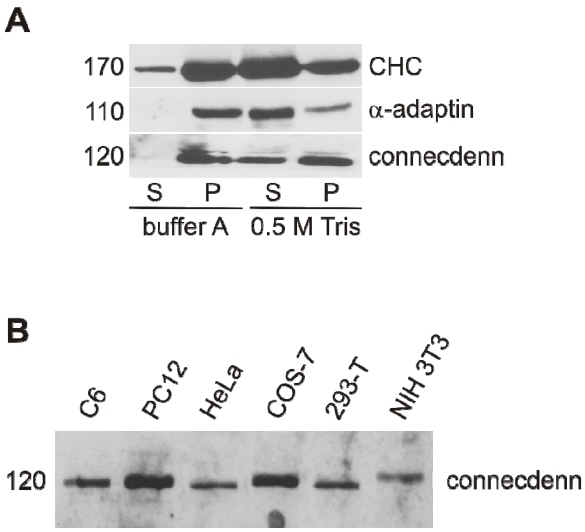
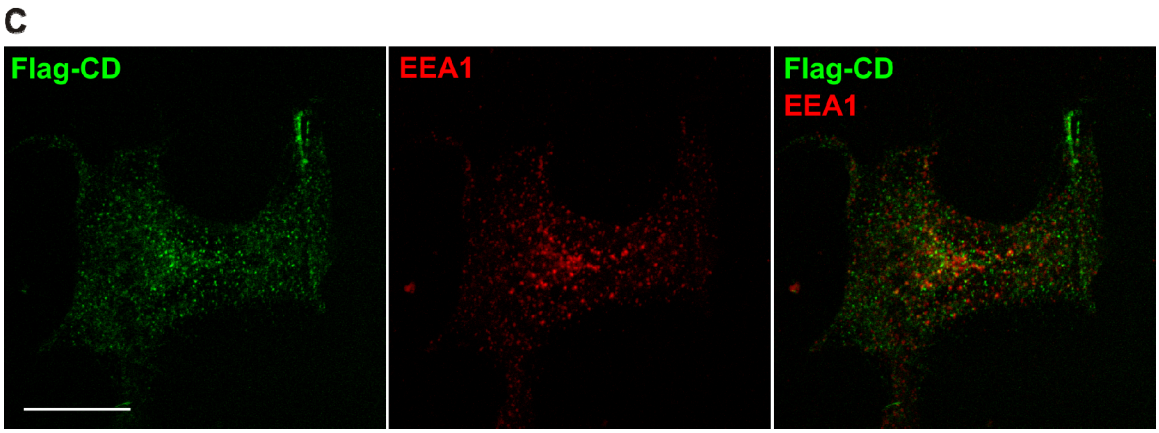
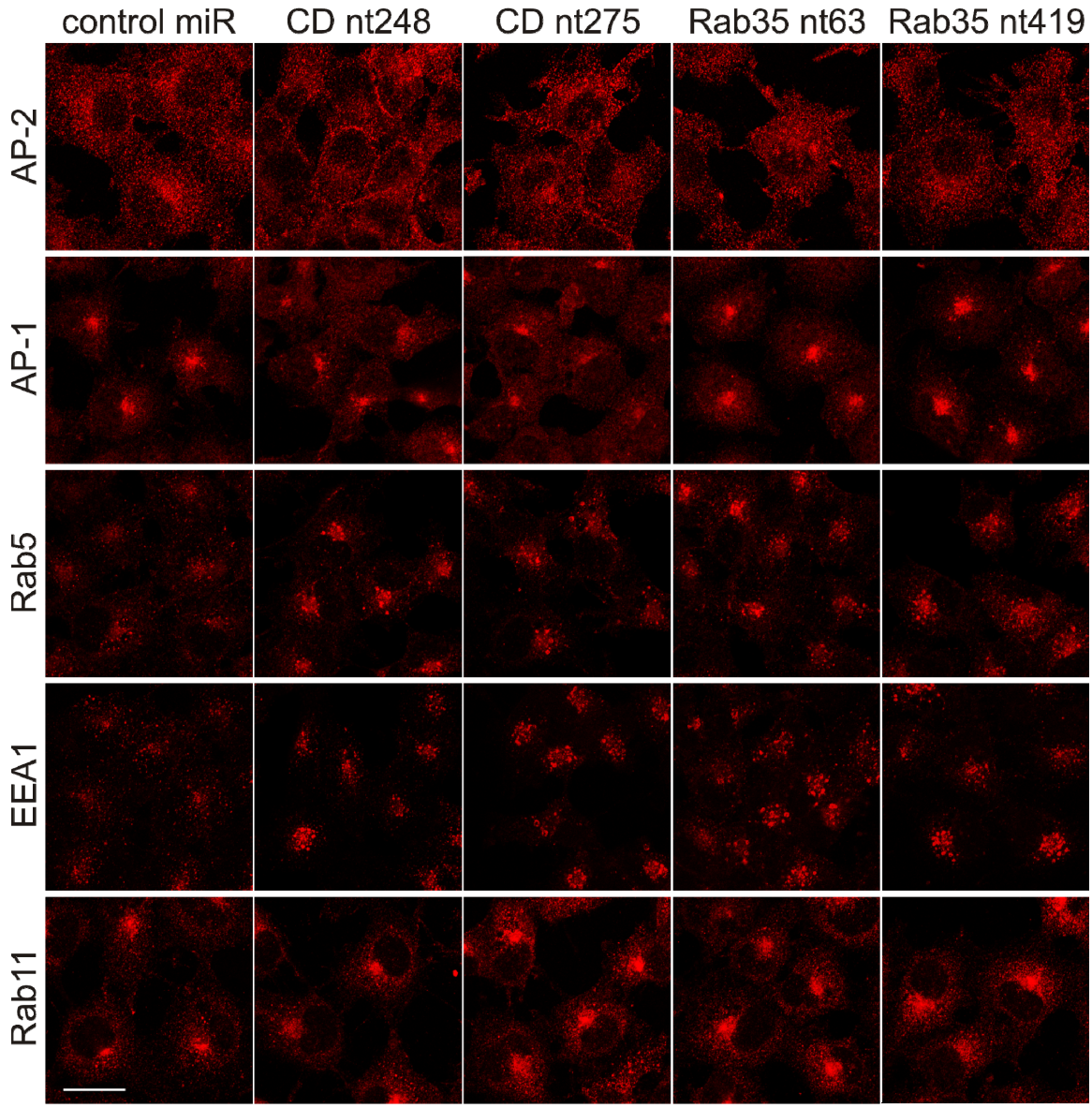

A
myc-EHD1
MHCI
control miR
B
myc-EHD1
EEA1
control miR
CD nt248
Rab35 nt63

Figure 3.7 – Connecdenn and Rab35 control recruitment of EHD1 to early endosomes. ***A.*** COS-7 cells transduced with control miRNA and transfected with myc-EHD1 were incubated with antibodies against MHCI for twenty minutes and processed by immunofluorescence. The bar represents 20 μm. ***B.*** COS-7 cells transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248) or Rab35 (Rab35 nt63) were transfected with myc-EHD1 and processed by immunofluorescence to reveal the localization EHD1 and endogenous EEA1. The bar represents 20 μm.

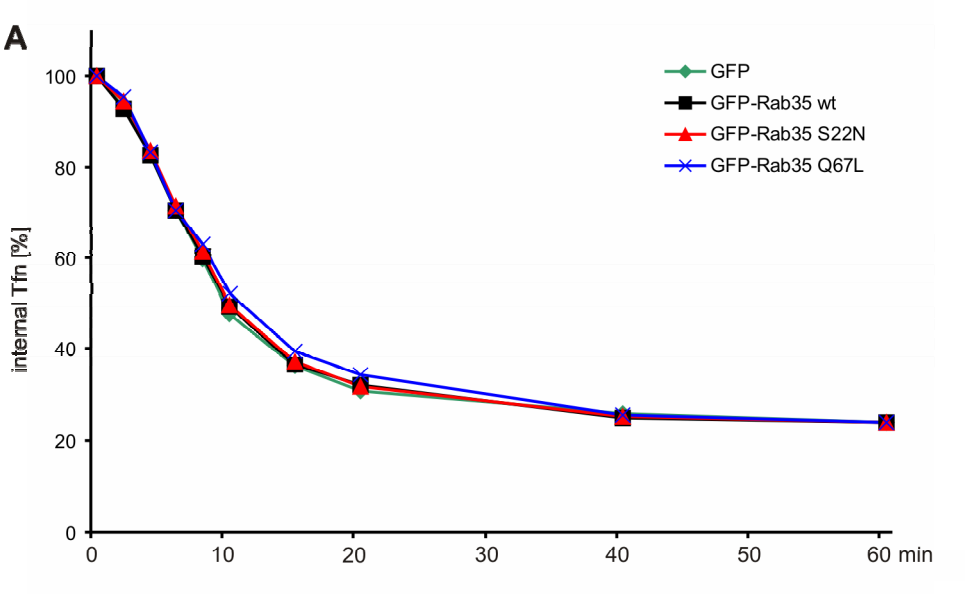


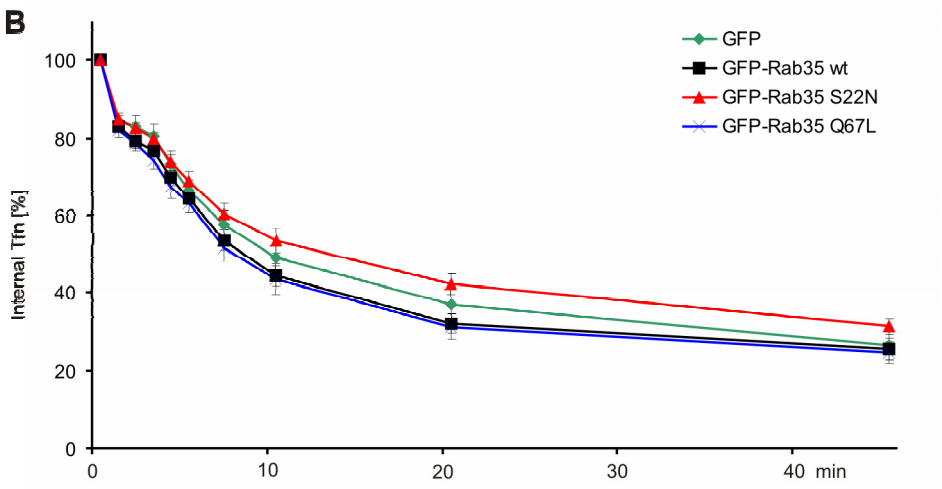


Supplementary figure 3.1 – Connecdenn is associated with CCV membrane. A. Equal amounts of purified rat brain CCVs were incubated with buffer A or 0.5M Tris pH 8.0 (1:1 buffer A, 1M Tris pH 9.5) to strip the proteinous coat off the CCV membrane. The fractions were separated by centrifugation and equal volumes of resuspended pellets (P) and supernatants (S) were analyzed for protein partitioning of clathrin heavy chain (CHC), AP-2 (α-adaptin), and connecdenn by Western blot. **B.** Equal amounts (200 μg) of lysates from various cell lines as indicated were analyzed by Western blot for connecdenn expression levels. **C.** COS-7 cells were transfected with Flag-tagged connecdenn and processed for immunofluorescence to compare the localization of connecdenn to endogenous EEA1. The bar represents 20 μm.

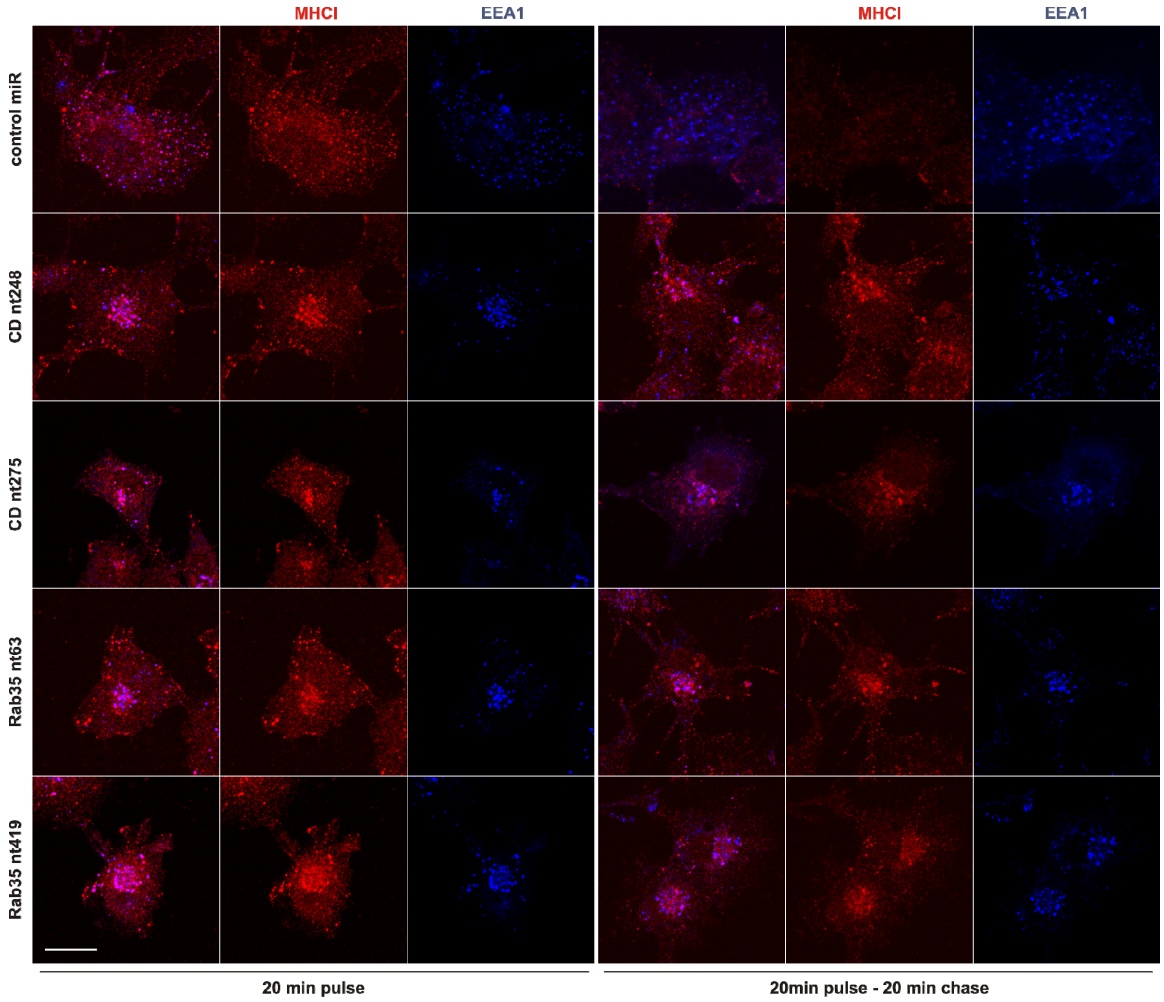


Supplementary figure 3.2 – KD of connecdenn or Rab35 affects early endosome morphology and localization. COS-7 cells were transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) and processed for immunofluorescence to reveal the localization of endogenous markers as indicated. The bar represents 20 μm.

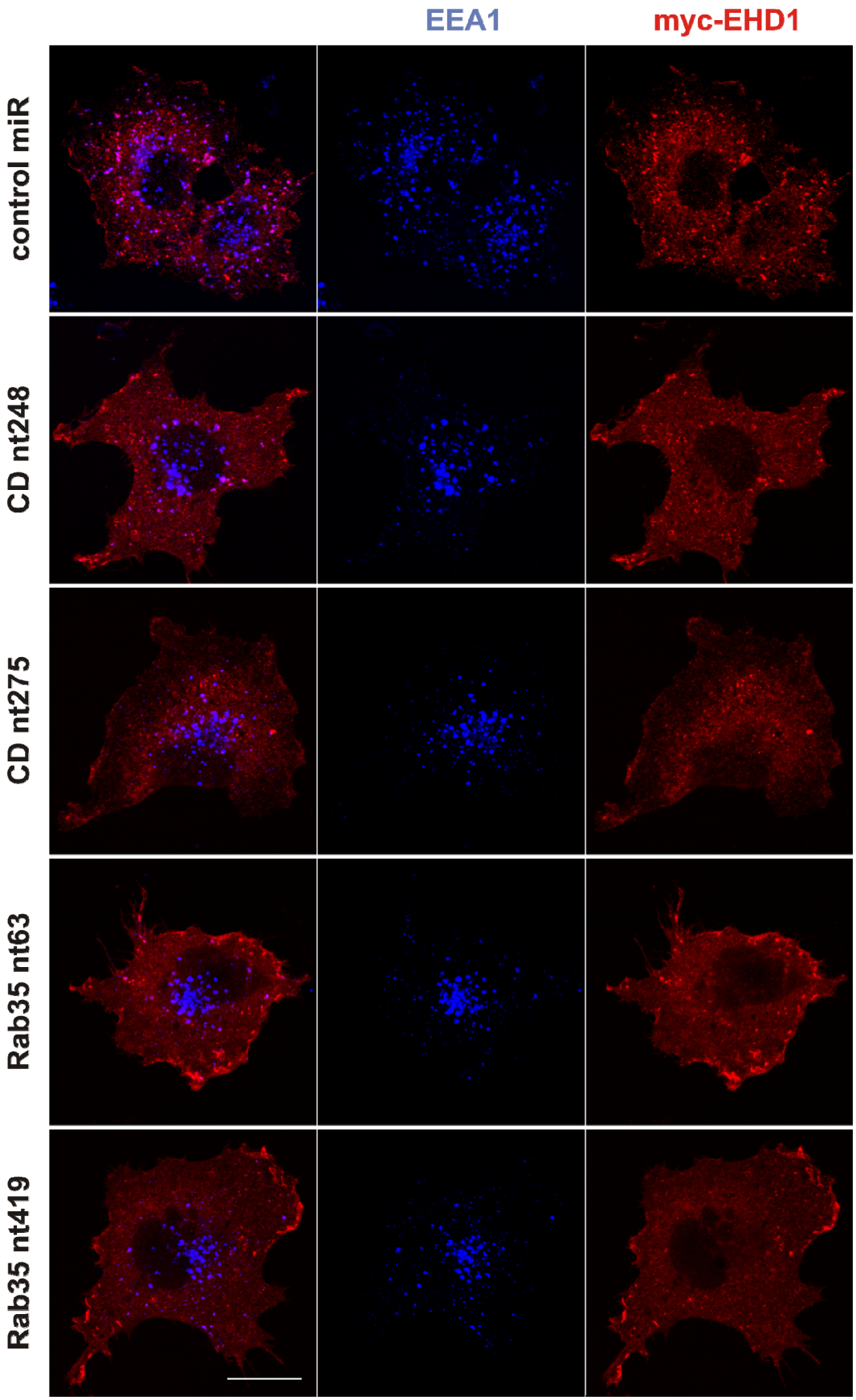




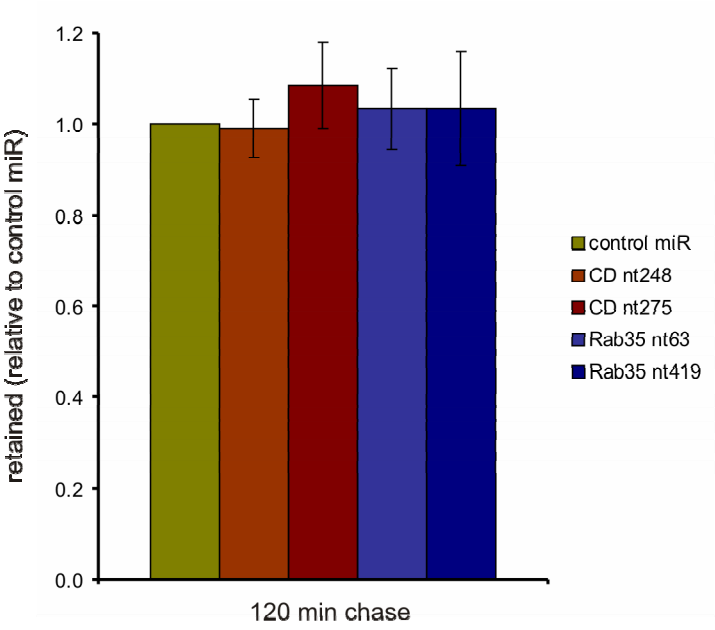
Supplementary figure 3.3 - Effect of connecdenn and Rab35 KD on transferrin uptake. HeLa (***A***) or COS-7 cells (***B***) were transfected with GFP alone or with GFP-tagged Rab35 wt, Rab35 S22N, or Rab35 Q67L, continuously labeled with AlexaFluor647-transferrin at 37ºC for one hour, chased at 37ºC for the indicated times, and intracellular transferrin was measured by flow cytometry. The graph represent the mean of four independent experiments, plotted as percent of the initial internal label and statistical analysis by Repeated Measure Two-Way ANOVA followed by Bonferroni posttests revealed no significant differences between control and KD cells.



Supplementary figure 3.4 – Connecdenn and Rab35 KD affects MHCI trafficking. COS-7 cells transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were incubated for 20 min with antibodies against MHCI and processed by immunofluorescence either directly or following a 20 min chase to reveal the localization of the intracellular antibodies and endogenous EEA1. The bar represents 20 μm.


EEA1
myc-EHD1
control miR
CD nt248
CD nt275
Rab35 nt63
Rab35 nt419

Supplementary figure 3.5 – Connecdenn and Rab35 control recruitment of EHD1 to early endosomes. COS-7 cells transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were transfected with myc-EHD1 and processed by immunofluorescence to reveal the localization EHD1 and endogenous EEA1. The bar represents 20 μm.



Supplementary figure 3.6 – KD of connecdenn or Rab35 does not affect β1-integrin trafficking. COS-7 cells transduced with lentiviruses for expression of a non-targeting control miRNA or miRNAs for KD of connecdenn (CD nt248 and CD nt275) or Rab35 (Rab35 nt63 and Rab35 nt419) were incubated for one hour with antibodies against β1-integrin and analyzed by flow cytometry either directly or following a two hour chase. The graph represent the mean fluorescence (±SEM) after the two hour chase of four independent experiments, plotted as relative ratio with the control miRNA set to 1. Statistical analysis by Repeated Measure One-Way ANOVA followed by Dunnett's posttests revealed no significant differences between control and KD cells.

Acknowledgements

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Chapter 4. General discussion and conclusions

In recent years, new technology has allowed us to make immense progress in understanding the mechanisms of intra-cellular trafficking pathways. Among these technologies, the development of organelle proteomics has allowed us to appreciate the complexity of the proteins that make up organelles such as CCVs and SVs. From these studies, new concepts have emerged that question old, and by now, dogmatic views such as those concerning the role of clathrin-light chain function in CME [6, 223]. Proteomics, together with advances in gene silencing technologies in cell lines and neurons, have given us the ability to examine the functions of the various proteins composing these organelles with unprecedented precision. I have applied these techniques, partly in collaboration with others, to reveal the biological function of connecdenn and demonstrated that its DENN domain functions as a novel GEF for Rab35.

When I joined the laboratory of Dr. Peter McPherson in the winter of 2003, I collaborated on a project to purify CCVs from adult rat brains, and to analyze their protein content by mass spectrometry [5]. I subsequently adapted this protocol to isolate CCVs from developing brain [129]. This study led to the identification of several uncharacterized proteins, one of which was connecdenn.

A. Identification of connecdenn as a CCV-associated protein functioning in the SV cycle

Connecdenn is a brain- and CCV-enriched protein that binds directly to the α-ear of AP-2, and the SH3 domains of endophilinA1 and intersectin1 (chapter1, [183]). In brain, AP-2 is the most abundant and important adaptor complex in CME and serves as a hub to recruit a long list of accessory proteins. EndophilinA1 and intersectin1 are both brain-enriched accessory proteins with important roles in endocytic trafficking [119, 120], but

neither binds AP-2 directly. EndophilinA1 contains a BAR domain that is essential for its function. The BAR domain is a sensor and generator of membrane curvature, allowing the membrane to invaginate [28]. EndophilinA1 contributes to SV endocytosis in clathrin-dependent and -independent pathways through several mechanisms. EndophilinA1 binds both to N-WASP and dynamin, which function in the fission of endocytic vesicles through the induction of actin polymerization and mechanical scission of the membrane, respectively [225]. Actin is present at high concentrations in the periactive zone of nerve terminals in close proximity to endocytic vesicles. Stimulation to induce SV exocytosis has been shown to promote growth of a filamentous actin matrix in the peri-active zone, while inhibition of actin polymerization prevents SV recycling [91, 92, 226-230]. EndophilinA1 also couples SV endocytosis to incoming action potentials by recruiting dynamin in a Ca^{2+}-dependent manner following membrane depolarization [231]. Finally, endophilinA1 recruits synaptojanin to the forming CCV to allow the removal of the clathrin coat after vesicle budding [232].

Studies of intersectin in *D. melanogaster* suggest that it may function as a scaffolding protein in the SV cycle at the neuromuscular junction, allowing for the enrichment of endocytic proteins in the peri-active zone [143, 144]. In mammals, the intersectin1 gene encodes a short (intersectin1-s) ubiquitously expressed and long isoform (intersectin1-l) that is brain-enriched. Intersectin1-s contains two N-terminal EH domains followed by 5 SH3 domains, while intersectin1-l contains an additional DH-PH domain C-terminal to the SH3 domains that exhibits GEF activity toward the small GTPase Cdc42, a core regulator of the actin cytoskeleton [139]. The activity of the DH-PH domain is inhibited by intra-molecular interactions with the N-terminal SH3 domains. The binding of other proteins to the SH3 domains relieves the intra-molecular inhibition, leading to Cdc42 activation [139]. However, contrary to *D. melanogaster*, there is no evidence of a function for intersectin in the SV cycle in mammalian systems. In fact, most studies of intersectin1 in hippocampal neurons have demonstrated a DH-PH domain-dependent function in dendrite development but not in SV recycling [224, 233, 234].

Consequently, because AP-2 and endophilinA1 are well established in SV endocytosis the function of connecdenn in the SV cycle is likely dependent on AP-2 and endophilinA1 but not intersectin1. The importance of connecdenn in the SV cycle is underlined by our studies in cultured hippocampal neurons showing that a modest 65% reduction in connecdenn expression levels leads to a 30% decrease in FM4-64 uptake. A 65% reduction is not considered to be a very strong knockdown, and often much stronger knockdowns are required to reveal functional phenotypes. This suggests that connecdenn may be a limiting factor in SV recycling and it will be interesting to re-address this phenotype using the miRNA lentivirus described in chapter2, which would allow for a more efficient knockdown of connecdenn.

A1. Possible effects of chronic knockdown of protein expression in hippocampal culture maturation

One drawback of RNAi treatments is the fact that while repression of translation likely occurs almost immediately, it takes at least a few days to turn over the protein already present. In a system that progressively develops and matures in culture, such as that of primary hippocampal neurons, the necessity of long-term RNAi treatments makes it hard to distinguish between acute knockdown effects that would directly inhibit the SV cycle to effects caused by chronic knockdown, which may lead to maturation defects that could interfere with synapse formation and strength or SV turnover.

Once a synapse has formed, the SV pool is maintained through biogenesis of new SVs and the recycling of pre-existing vesicles following their fusion with the plasma membrane. Synaptic vesicle recycling must occur following both spontaneous/activity-independent fusion and action potential-mediated SV fusion. Although the basic machinery is likely the same, namely CME, depolarization causes an increase in the rate of endocytosis and thus additional proteins have to be recruited to the CME machinery to meet the increase in endocytosis following stimulation-triggered depolarization of the the nerve terminal. SV recycling following action potentials occurs through at least two

mechanisms, depending on the frequency of the action potential. At low firing frequencies, SV membranes are retrieved through CME, which is thought to promote a direct but slow replenishment of SV pool [235]. At high firing frequencies (e.g. induced by KCl treatment), SV membrane is removed from the plasma membrane through an ill-defined uptake pathway called bulk endocytosis. This process occurs independently of clathrin and dynamin activity [94-99, 236]. It leads to the formation of large, intermediate endosomes from which SV precursors bud off to replenish the SV pool during high neuronal activity.

In cultured hippocampal neurons, synapse formation peaks between 10 and 14 days DIV [237]. We thus tried to minimize the effect of our knockdown studies on synapse formation by transducing the cells at 14 DIV, such that our analyses at 21 DIV would likely reflect a phenotype caused by a direct impairment of the SV cycle. Together with the fact that connecdenn is a component of CCVs enriched in the pre-synaptic compartment of the synapse, we believe that the reduction in FM4-64 uptake can be mainly attributed to a direct impairment of SV endocytosis or SV recycling and is likely not due to alternations in synaptic development.

A2. Analysis of connecdenn in the various pathways for SV recycling

In culture, the SV cycle in neurons is minimally activated by background network activity. If connecdenn functions in SV recycling, a chronic knockdown of connecdenn would lead to a reduction in the total number of SVs over time. In this scenario, upon depolarization, fewer SVs are available to fuse and therefore fewer vesicles are reformed, which would appear as a reduction in SV endocytosis. This begs the question of whether connecdenn is involved in SV recycling by directly regulating SV reformation through CME or whether connecdenn controls the reformation of SVs from endosomes. Several approaches would be required to investigate these questions. First, EM analyses of synapses from control and connecdenn knockdown neurons would provide information regarding synapse morphology and synaptic content, and would allow us to determine if

the total size of the synaptic vesicle pool is affected under knockdown conditions. This, however, would not tell us if the stimuli-dependent cycle is affected. To specifically test for the functionality of the activity-dependent SV cycle, we would need to analyze control and knockdown neurons using electrophysiological methods to precisely stimulate repeated rounds of action potentials to determine rates of recovery between bursts. Recovery of SVs can be measured using fluorescent probes, where a pH-sensitive GFP has been fused to the extracellular/lumenal region of SV transmembrane proteins (e.g., synaptophysin, synaptotagmin, VAMP-2, or vGlut). With these probes, fluorescence is only observed when the GFP moiety is exposed to the neutral pH of the extracellular medium and is quenched within the SV lumen, which has an acidic pH due to the activity of the H^+-pump. SV fusion and retrieval can thus be measured indirectly due to varying GFP fluorescence intensities [238]. Employing this technique with repeated delivery of action potentials would allow us to compare the efficiency of SV recovery in control versus connecdenn knockdown cells.

Given the fact that studies demonstrating a role for connecdenn in SV recycling were performed using KCl, which mimics high firing frequencies [239] and the process of bulk endocytosis, it is possible that connecdenn is also directly involved in bulk endocytosis. In order to differentiate between effects on CME versus bulk endocytosis, cells are usually differentially stimulated at low frequencies to induce 100 action potentials at 10Hz, or at high frequencies to induce 300 action potentials at 20 Hz, respectively [97]. Low frequency stimulation does not trigger bulk endocytosis, and thus allows for a direct measure of CME-mediated SV recycling. Using this technique, comparing low versus high frequency stimulation will enable to evaluate the precise role of connecdenn in SV recycling. In addition, measuring post-synaptic activity following either high frequency or low frequency stimulation would allow us to address the functionality of the synapse as a whole. Comparing data from pre-synaptic SV membrane recycling to post-synaptic responses will allow us to determine the pre- and post-synaptic contribution of connecdenn to in synaptic communication.

The function of connecdenn in SV recycling also depends on its interacting partners. To better understand the importance of the interactions of connecdenn with endophilinA1, intersectin1, and AP-2 during SV regeneration and post-synaptic responses, over-expression of binding-deficient forms of connecdenn should be analyzed in electro-physiology experiments similar to those described above. Ideally, these experiments would be conducted in a connecdenn knockdown background to reduce the effects of over-expression, and to verify the specificity of the knockdown. Altogether, these experiments would allow us to understand the specific SV recycling pathway(s) regulated by connecdenn and its binding partners, and its overall effects on synaptic transmission.

A3. Other potential neuronal functions of connecdenn

The defect in SV endocytosis upon connecdenn knockdown could also reflect changes in synaptic connectivity resulting from either axonal or dendritic developmental defects, which, in part also depend on CME. CME is a critical neuronal function that allows for regulation of membrane morphology and interactions with the extra-cellular environment. This is done by retrieval of plasma membrane and regulation of cell surface receptor expression. Notably, extra-cellular adhesion molecules and receptors that sense guidance molecules important in neurite outgrowth are continually endocytosed and recycled back to the tip of the neurite, allowing for directional growth [240-243]. In dendrites and axons, the maturation process requires membrane remodeling to form structures necessary for synaptic signaling [244, 245]. Fine tuning the connectivity between axon terminals and dendrites is important in this process, and this relies on the proper positioning of inter-cellular adhesion molecules. The interaction between inter-cellular adhesion molecules controls synaptic strength by responding to long term depression or potentiation, and the amount of available receptor at the cell surface is regulated by CME. In chapter 2, we showed that connecdenn does in fact localize to the post-synaptic compartment, where AP-2, intersectin11, and endophilinA1 are also known to function. The major role of intersectin1-l in hippocampal neurons is to regulate dendrite development. This was shown using dominant-negative intesectin1-l constructs to

demonstrate the role of the DH-PH domain in Cdc42-mediated activation of in EphrinA1-induced spine maturation [233, 234], and recently, by intersectin1 knockdown [224]. Furthermore, various endophilin isoforms including A1 have also been associated with spine maturation and stabilization [246-250]. As mentioned above, both intersectin1-l and endophilinA1 regulate N-WASP activity and thus actin cytoskeleton organization, which is crucial for dendrite development and control of synaptic strength. Connecdenn may thus coordinate intersectin1-l and endophilinA1 function during dendrite maturation.

Interestingly, knockdown of connecdenn in primary hippocampal neurons at early stages of development (DIV<7) severely disrupts neuronal maturation and produces extensive arborization of neurites (our unpublished results), which may be similar to what has been reported for the dominant-negative and knockdown effects of intersectin1-l on dendrite development [224, 233]. Since we did not observe these severe phenotypes when knocking down connecdenn at later developmental stages (DIV > 14), it may suggest two functions for connecdenn: first, in early neuronal maturation, and later, in SV recycling. However, we have not yet studied the impact of connecdenn knockdown on synapse formation under either condition, nor have we done an initial analysis using pre- and post-synaptic markers such as synaptotagmin and PSD95 to provide a possible role for connecdenn in the development of synapses. Moreover, a morphological analysis of dendritic spines and their maturation from filamentous to stubby mushroom spines would clarify the post-synaptic role of connecdenn. These studies could be performed in the absence or presence of EphrinA1, which induces dendrite maturation to determine if connecdenn functions in this process. This experiment should be complemented with rescue experiments using connecdenn mutants (described above) to further elucidate whether AP-2, intersectin1 and endophilinA1 interactions are required for dendrite development. It would also be interesting to determine if the interaction of connecdenn with intersectin1-l could relieve the inhibition of the GEF activity of the DH-PH domain toward Cdc42. In this case, connecdenn knockdown should mimic the defects in dendritic development observed in intersectin1-l knockdown neurons. These experiments along with electrophysiological and electron microscopy analyses of synapses will

greatly help us to understand the array of functions of connecdenn in hippocampal neurons.

B. Characterization of the DENN domain of connecdenn

The functions of connecdenn described thus far have all been related to interactions mediated by the connecdenn C-terminus. However, the 400 N-terminal amino acids of connecdenn encode a DENN domain and we have demonstrated that this protein module is a critical regulator of Rab35 function in the endocytic pathway. DENN domains were initially found in and termed after the protein **D**ifferentially **E**xpressed in **N**eoplastic versus **N**ormal cells (**DENN**). DENN is also referred to as Rab3-REP [107], MADD [251] and Aex-3 [74], however I will use the term MADD to refer to this protein to contrast to the DENN domain itself. The DENN domain is a tri-partite module consisting of the up-stream DENN (uDENN), central DENN (DENN), and down-stream DENN (dDENN) module. The modules are linked together with amino acids stretches of various lengths to produce a domain ranging from 350 to 750 amino acids in total length. The DENN domain is evolutionarily conserved and is found in one gene in yeast and in 18 genes (including 2 pseudo-genes) in humans. In most cases, the function of the protein products of these genes is unknown. The few studied DENN domain proteins, namely MADD [73, 74, 107], SBF1 [185], SBF2 [188], Rab6IP1 [193] and here, connecdenn, have all been linked to vesicle trafficking, but no direct information concerning the function of their DENN domains is available, with the exception of the MADD DENN domain. Specifically, it was recently demonstrated that the uDENN module of MADD binds GTP-loaded Rab3 [73]. MADD also binds to the microtubule motors KIF1Bbeta and KIF1A through its death domain, providing a link for Rab3 vesicles to traffic on microtubules [73]. Apart from this study, general DENN domain functions remain unknown. Moreover, no information is available as to whether the distinct modules function independently or as a functional unit.

B1. The DENN domain is a lipid binding module with Rab35-specific GEF activity

The stable association of connecdenn with CCVs following coat removal provided the first indication for a function for the DENN domain. This led to the finding that the DENN domain of connecdenn has a general hydrophobic affinity for lipids and phospholipids, as shown in the liposome sedimentation assays. Interestingly, the connecdenn DENN domain shows a slight preference for PI(3)P, an important phospholipid in endosomal function [252]. PI(3)P is synthesized late during CME [36]; since connecdenn associates with uncoated vesicles, it suggests that connecdenn may function late in CME or on early endocytic vesicles before they reach sorting endosomes. Furthermore, connecdenn does not localize with EEA1-labeled vesicles. This is in agreement with a recent study in *C. elegans* demonstrating an interaction between RME-4 and endosomal RME-5, the orthologs of connecdenn and Rab35, respectively [169]. This study provided crucial information that enabled us to establish that the DENN domain of connecdenn possesses robust Rab35-specific GEF activity. In fact when tested with the same experimental techniques and similar Rab/GEF concentrations, the DENN domain GEF activity is similar to that observed for the Rabex5 VPS9 domain toward Rab5, a well-described Rab5-specific GEF domain [190].

Given the spatial localization of connecdenn and the PI(3)P-binding properties of its DENN domain, it is tempting to speculate that the GEF activity could be regulated by PI(3)P. This regulation might happen in two possible ways. In the first scenario, connecdenn is recruited to the plasma membrane by AP-2 to a PI(4,5)P_2-enriched environment, which would not be compatible with DENN activation. As the vesicle buds off and PI(4,5)P_2 is converted into PI(3)P through the action of lipid phosphatases and kinases recruited by Rab5 [36], the DENN domain is then recruited to the membrane. This may lead to a conformational change leading to either activation or inhibition of its GEF activity. Because different cargo leads to the recruitment of different accessory proteins into the vesicle, the amount of PI(4,5)P_2 converted to PI(3)P may vary, leading to a direct link to the level of connecdenn GEF activity and thus Rab35 activity. This in

turn would regulate the fate of the endocytic vesicle and cargo being Rab5, Rab35 or Rab5/Rab35 regulated.

In an alternate scenario, PI(3)P does not directly regulate the GEF activity, but rather serves to recruit the DENN domain into the proximity of membrane-anchored Rab35, and thus localizes the enzyme to its substrate for efficient activation. The latter case is similar to that for the recruitment of auxilin/GAK via its PTEN domain to PI(3)P that is formed late in CME or on CCVs for subsequent clathrin coat removal[38, 39]. To distinguish these possibilities, the influence of PI(3)P on the GEF activity of the DENN domain should first be tested in *in vitro* GEF assays using a soluble short chain PI(3)P analogue. Alternatively, liposomes with defined compositions can be used to test whether lipid binding increases or decreases GEF activity; this assay would also represent a more physiological model. To address if components of the clathrin coat interfere with the GEF activity of the DENN domain, the assay could be done using purified CCVs or uncoated vesicles. We would expect that if the coat interferes with the GEF activity of the DENN domain, the exchange reaction would only occur on coat-stripped vesicles. Finally, addressing the function of each individual DENN module in lipid binding and GEF assays will provide information on the requirements for the DENN domain as a whole.

B2. Are all DENN domains GEFs for Rabs?

The importance of our discovery that the DENN domain of connecdenn functions as a GEF is highlighted by the fact that unlike GEFs for Rho, Ras, ARF and Ran GTPases, which have been extensively studied, only very little information is available regarding the mechanisms by which Rabs are activated [253]. Previous to our finding, only proteins containing Vps9 or Sec2 domains were known to function as Rab GEFs against the Rab5 subfamily, and Rab3 and Rab8, respectively [66]. Additionally, GEF activity has been shown for the DENN domain-containing protein MADD, which functions

against the Rab3 subfamily [107]; however, in this case, the activity has not been assigned to a defined sequence or domain within the protein.

As discussed in chapter 3, the available existing data on the DENN domain is too scarce to propose a general function of DENN domains as either GEFs and/or effectors. Furthermore, the DENN domain is much larger than the Vps9 or Sec2 domains, and thus has likely evolved additional functions or means for intra-molecular or co-factor-mediated regulation, such as that suggested above for PI(3)P binding. For example, the poorly defined Rab GEF Ric1p only shows GEF activity when in complex with RGp1 [205]. Moreover, in MADD, the GEF activity was dependent on the presence of the death domain, which is located outside the DENN domain [194]. On first glance, this would suggest that the death domain exhibits the GEF activity, but since this study used a cell-based assay, deletion of the death domain might have caused a mis-targeting of the protein, leading to a non-specific impairment of the enzymatic activity. The recent finding that the DENN domain of MADD functions as an effector also suggests that the GEF activity is localized outside the domain [73]; however, the fact that effector function was localized to the uDENN module, combined with structural predictions, suggests that not all DENN modules may be required for GEF activity and that the DENN domain serves multiple functions. Thus, it remains an open question whether, in general, DENN domains function as GEFs.

To reconcile the different studies, one possibility is that the enzymatic activity is located in the dDENN module, while the uDENN module functions as an effector. The dDENN module is predicted to have an α-helical globular fold similar to the DH domain, a GEF for Rho family GTPase member [158] and based on this structural prediction, it has been suggested that the dDENN module may possess GEF activity. Many structural studies have demonstrated that GEF domains in general are α-helical and provide a single α-helix to cause the nucleotide exchange reaction. Indeed, the two currently known Rab-GEF domains, Sec2 and VPS9, although completely different in overall folding, are both exclusively α-helical and use one α-helix for their activity [66, 196, 254, 255]. Thus, obtaining a crystal structure for the connecdenn DENN domain or a co-crystal with

Rab35 would provide a breakthrough in the understanding of the function of this protein module.

To determine if DENN domains function as GEFs in general, at least two approaches could be considered. First, combinations of Rabs and DENN domain proteins known to interact should be used to test for GEF activity of the DENN domains towards the respective Rabs. We have attempted this for the DENN domain of MADD together with Rab3 [107], and for the DENN domain of Rab6IP1 with Rab6 and Rab11 [193], but despite numerous approaches, we failed to express and purify these DENN domains. We can only speculate that this might be the result of improper domain borders. In fact, in our first trial to purify the DENN domain of connecdenn, we used the domain borders predicted in the NCBI domain database, which yielded very low expression and protein aggregation. Once we extended the border from amino acids 1-374 to 1-403 based on sequence conservation, protein expression and solubility increased dramatically, allowing us to perform our functional assays. We have also tried extended borders for the MADD and Rab6IP1 DENN domains, however, without improvement. Structural information on the DENN domain, once available, may be very useful to guide further expression trials. Information regarding the secondary structures of the connecdenn DENN domain obtained through the analysis of its crystal structure may then guide the prediction of appropriate borders to successfully express other DENN domains. Interestingly, two other connecdenn isoforms exist in the human genome, DENND1B and DENND1C, with connecdenn being DENND1A. Sequence alignments show conservation up to the end of our extended DENN domain construct. An alignment of connecdenn with the yeast DENN domain-containing protein SPCC297.05 suggests that the dDENN module might extend for another 20-30 amino acids. Indeed, another graduate student in the laboratory working on the DENN domain of DENND1B and DENND1C tested these borders and successfully purified the domain. Structural studies in this respect will determine if these extra amino acid carry important structural elements necessary for folding. Furthermore, it will be interesting to analyze a DENN domain – Rab35 co-crystal, which would help to understand the binding interfaces and resolve which, if not all, modules are involved in the interaction. This will allow conclusions whether the mechanism of GEF activity is

maintained among Rab GEF domains. Finally, since Rab35 and Rab1a/b are members of the same sub-family, it will also be interesting to determine if connecdenn functions as a GEF for either or both Rab1a and Rab1b.

Given the large number of Rabs and DENN domain proteins in the human genome, it appears unfeasible to test all combinations possible to determine if any other DENN domains exhibit GEF activity toward a Rab. However, the other Rab GEF domains VPS9 and Sec2 are also conserved in yeast, and since not all Rabs in yeast have yet been assigned to their respective GEF, other domains with GEF activity likely exist. Moreover, the yeast strain *S. pombe* contains only one gene encoding for a DENN domain-containing protein, and 7 genes encoding Rabs. Therefore, a worthwhile approach would be to test the DENN domain from the yeast protein for GEF activity towards the yeast Rabs. Should the activity be conserved throughout evolution, the identification of the target Rab would allow us to use sequence similarity to narrow down the sub-family of mammalian Rabs most promising for further testing. Importantly, this would also establish an evolutionarily conserved link between DENN domains and Rab GTPases.

C. Characterization of the Rab35-controlled trafficking pathways

Rab35 is a poorly described member of the Rab protein family involved in cell division during cytokinesis. This function was linked to fast membrane recycling using the transferrin receptor (TfnR) as a marker [172]. Subsequent studies suggested a similar role in the trafficking of the yolk receptor in *C.elegans* [169], the major histocompatibility complex II (MHCII) [176], and the T-cell receptor (TcR) [175]. The caveat for all of these studies is two-fold: the over-expression of mutant Rab35 forms to study specific trafficking functions and/or the lack of characterization of the intracellular localization. To determine the function of connecdenn *in vivo*, we analyzed the trafficking of TfnR, MHCI and β1-integrin in cells knocked down for connecdenn or Rab35. We observed a specific delay in the recycling of the MHCI receptor only, which we attribute to its

entrapment in enlarged sorting endosomes. Importantly, both the connecdenn and Rab35 phenotypes are identical, implying that in COS-7 cells, connecdenn is the main Rab35 regulator.

C1. Does connecdenn strictly function through Rab35?

In COS-7 cells, we observed virtually identical phenotypes following knockdown of connecdenn or Rab35. Depletion of either protein caused a specific inhibition of MHCI recycling. There were however, no defects in endocytosis of any of the cargo tested. This was initially rather surprising given that in neurons connecdenn knockdown reduced the endocytosis of SVs. However, as discussed above, this may very well have been indirect through alterations in SV recycling and turnover via endosomes over time.

Rab35 has not yet been studied in neurons but, like connecdenn, it is enriched in brain and is found in CCVs purified from brain (preliminary data, chapter 2). Furthermore, our proposed function for Rab35 in fast recycling from endosomes could provide a mechanism for reformation of SV precursors from endosomal intermediates following bulk endocytosis. In any case, connecdenn and Rab35 could still function together during SV recycling. Consequently, all future experiments to study connecdenn in the various SV recycling pathways should also include studies of Rab35 to verify its role in this process and to distinguish any connecdenn- and Rab35-specific functions. If connecdenn functions exclusively through Rab35, the various Rab35-mediated trafficking routes will have to be examined for a possible connecdenn-mediated function.

C2. Specificity of the Rab35 pathway

Particular types of cargo enter cells typically through specific endocytic pathways. TfnR, for example, enters cells exclusively through CME [256], while MHCI enters through an Arf6-dependent pathway [50]. Other cargo such as β1-integrin show mixed forms of

entry, but ultimately various types of cargo are thought to merge in sorting endosomes, where they are sorted either for recycling or for degradation [179]. The cargo selected here to investigate trafficking were chosen because of similarities in trafficking patterns reported for Rab35. All three cargo, TfnR, MHCI and β1-integrin are mainly recycled back to the cell surface through an EHD1-dependent pathway [177, 215], and recycling of both TfnR and MHCI is reportedly regulated by Rab35 [172, 176]. β1-integrin has recently been reported to traffic via the Arf6-dependent endocytic pathway [217]. Although all of these cargos seemingly use an EHD1-dependent machinery for recycling, TfnR, MHCI, and β1-integrin are all trafficked differently after their exit from the sorting endosome. The differences in subsequent trafficking, like the differences in their mode of endocytosis, rely on amino acid binding motifs or domains, or post-translational modification that provide a discrete label on the cytoplasmic tail of the cargo for recruitment of specific trafficking regulators. These in turn will recruit the cargo into distinct pathways that the cell uses for specific cellular functions. This is highlighted by our results with connecdenn or Rab35 knockdown cells that show a specific inhibition of MHCI recycling. MHCI, through an unknown mechanism, selects the Rab35 pathway, which allows it to traffic back to the plasma membrane or toward the recently described Rab22-positive recycling compartment [191, 218], through which MHCI can transit back to the plasma membrane. In contrast, TfnR is first targeted to the recycling endosome through a Rab4-dependent [257] mechanism and then recycled back to the plasma membrane via a Rab11-positive trafficking pathway [53, 54], and thus can bypass the Rab35 pathway. Although β1-integrin is thought to follow a trafficking route similar to that of MHCI [218], it was not affected by Rab35 knockdown, indicating the existence of at least three independent recycling pathways, or exit routes from sorting endosomes toward recycling pathways.

The recycling of all three cargo types, TfnR, MHCI, and β1-integrin, has been shown to depend on EHD1 [177, 215, 258]. EHD1 is the first member of a four-member family also consisting of EHD2, EHD3, and EHD4. These proteins all contain an N-terminal G-domain involved in ATP binding, a central helical domain necessary for lipid binding, and a C-terminal EH domain that binds the amino acid motif, NPF. EHD proteins

generate and maintain tubular membranes, which requires an intact microtubule network, EHD protein oligomerization and ATPase activity [181]. Finally, recent structural data has provided evidence that EHD proteins may function like dynamin and allow budding off of EHD tubulated membranes [55]. EHD proteins have not been reported to directly interact with Rabs, and are thus by definition not Rab effectors. However, effectors containing NPF motifs like Rab11-FIP2 and Rabenosyn5, a Rab5 effector, bind EHD1 and may mediate recruitment for Rab-specific functions [180, 259, 260]. Thus, like dynamin at the plasma membrane, EHD proteins may be considered part of a more general complex that can be recruited to different recycling sites by distinct Rab effectors. In this respect, a Rab may be considered to function similar to adaptor proteins that recruit clathrin and accessory proteins for CCV formation at distinct locations, namely the plasma membrane, endosomes and the TGN. We currently do not know how Rab35 mediates EHD1 recruitment, and studies to address this point are complicated by the fact that EHD proteins function as homo- and hetero-oligomers [261]. Recruitment could thus rely on one of the other EHD proteins. EHD4 preferentially dimerizes with EHD1, and intriguingly, EHD4 knockdown causes swelling of sorting endosomes, increases Rab5 activity, and inhibits the formation of EHD1-positive tubules [262]. Moreover, knockdown of EHD4 caused an accumulation of a variety of cargo such as TfnR, LDLR, and MHCI in the sorting endosome, with especially strong effects on the recycling of MHCI [262]. EHD1 is firmly established to function in TfnR trafficking, yet the obvious effect on EHD1 localization in our connecdenn/Rab35 knockdown study did not show an effect on TfnR trafficking. Consequently, it could be possible that the Rab35-MHCI trafficking pathway primarily relies on EHD4; this needs to be resolved in the future. Determining the function of known and yet-to-be-discovered effectors of Rab35 may provide a direct link to the recruitment of EHD proteins to sorting endosomes and would help us to better understand the regulatory mechanisms underlying Rab35 trafficking pathways.

C3. Rationalizing connecdenn and Rab35 function based on predicted Rab effectors

To date, few Rab35 effectors have been identified, and of those identified, only p53-related protein kinase (PPRK) has been studied in any detail [263]. PPRK is a kinase that phosphorylates and regulates the activity of the transcription factor p53, an important tumor suppressor gene that functions in cell cycle check points [264]. PPRK activity in mammalian cells has been correlated with suppression of growth and division. Interestingly, recent reports have demonstrated a role for p53 in CME. p53 was shown to bind clathrin, and to regulate EGF-induced EGFR trafficking [265]. Conversely, clathrin functions as a co-factor for p53-mediated gene transcription [266, 267]. Since Rab35 has been shown to regulate cytokinesis [172], this raises the intriguing possibility that Rab35 may directly cross-link CME to gene expression and control of cell cycle progression. Although this is of great interest in and of itself, it does not immediately explain Rab35-mediated trafficking; however it may partly explain its role in cytokinesis.

Recently, yeast two-hybrid screens for Rab effectors suggested that oculocerebrorenal syndrome of Lowe (OCRL) protein and members of the Molecule interacting with CasL (MICAL) protein family are Rab35 effectors [268]. OCRL is a phospholipid 5-phosphatase that binds several Rabs, including Rab5 [269, 270]. OCRL also binds APPL1, another Rab5 effector that associates with activated RTKs and AKT, an important kinase for survival signaling [271, 272]. The OCRL-APPL1-Rab5 complex is proposed to serve as the template for signaling endosome formation [272]. Signaling endosomes are long-lived organelles in which the kinase activity of their cargo, RTKs, is maintained. Signaling endosomes are required for cellular survival and differentiation, and are well defined, at least operationally in neurons [57, 118]. The mechanisms underlying the creation and maintenance of signaling endosomes are still unknown, but recent evidence suggests that the regulation of phospholipid composition plays a role. APPL1 cannot bind PI(3)P but EEA1 another effector of Rab5 does. It was recently demonstrated that if PI(3)P levels increase, binding of EEA1 out-competes APPL1 binding for Rab5 [272]. This in turn leads to fusion of signaling endosomes to sorting endosomes, and terminates signaling endosomes. Interestingly, knockdown of Rab35

produces a dramatic up-regulation of Rab5 protein expression (>6fold, chapter 3), which then produces apparent effects on EEA1 fusion seen by increased endosome swelling and peri-nuclear re-localization. This indicates that Rab35 cooperates with Rab5 to regulate endocytic vesicle trafficking, and signaling endosome biogenesis and lifetime. Apart from regulating Rab5 expression levels, this inter-regulation may result from Rab35 competing with Rab5 for OCRL, and hence diversion of endocytic vesicles toward signaling endosomes. In the absence of Rab35, more Rab5 is expressed and activated. This leads to more Rab5-dependent PI3P synthesis, EEA1 recruitment, and displacement of APPL1-OCRL. In this context, an increase in endocytic vesicles primed for fusion would be observed, which would correlate with sorting endosome swelling due to excessive endocytic vesicle fusion, as seen in our Rab35 knockdown cells. As such, Rab35 would negatively regulate Rab5 activation/expression to control signaling endosome formation, thereby regulating cell survival or differentiation. It will thus be of interest to better understand the mechanisms of Rab35-mediated regulation of Rab5 activity and to determine the impact of this regulation on central cellular functions. In addition, it could be possible that the up-regulation of Rab5 observed upon connecdenn/Rab35 knockdown that is creating endosomal swelling also disrupts proper effector recruitment, which in turn inhibits EHD1 recruitment/tubulation. Because our phenotype on early endosome swelling is remarkably similar to over-expression of dominant active Rab5, it will also be interesting to determine if EHD1 recruitment/tabulation is affected by over-expression of dominant active Rab5. Interestingly, the Rab5 effector Rabenosyn-5 has been shown to bind EHD1, an interaction necessary for efficient recycling [180]. It is thus possible that Rab35 knockdown causes a downstream effect via Rab5 to control recycling through EHD1.

The PC12 cell line is a well-defined model to study signaling endosomes since their generation is required for cell differentiation and subsequent neurite outgrowth [116, 273]. In this system, signaling endosomes are formed upon NGF treatment, a ligand for the TrkA receptor. The TrkA receptor is subsequently endocytosed in a Rac/Arf6-dependent pathway that generates APPL1 and Rab5-positive signaling endosomes [116]. Interestingly, Rab35 has recently been reported to localize to RacA-positive structures,

and to induce changes in cell morphology as is often seen with deregulated signaling endosome biogenesis [178]. Thus, one of the functions of Rab35 may be to regulate the trafficking of specific cargo from the plasma membrane in concert with Rab5, such that signaling endosomes are formed. Knocking down Rab35 or Rab5 in PC12 cells to analyze NGF responses and neurite outgrowth would determine how these Rabs are involved in PC12 differentiation. In the event of an observed effect, studying signaling endosome-associated kinase activity and recruitment of OCRL and APPL1 will further help in understanding the interlaced function of these Rabs.

Rab35 has also been shown to interact with most members of the MICAL protein family [268]. MICAL1 was originally identified as a binding protein of Cas-L (Crk-associated substrate lymphocyte type), and is an adapter protein at focal adhesion sites that transmits various signals through cell adhesion molecule receptors such as integrins [274]. The MICAL protein family consists of 5 members in mammals (MICAL-1,-2,-3,-L1 and -L2). MICAL proteins are linked to receptor signaling by binding of the SH3 domain of Cas-L with a PPRPP amino acid motif [274]. Cas-L is predominantly expressed in lymphocytes and epithelial cells, and like MICAL1, it is required for proper integrin-mediated signaling, cell adhesion, migration, and T-cell invasion [275]. These properties are reminiscent of the neuronal requirement for dendrite development, axonal path-finding, and synapse stability, where integrins and other adhesion molecules that signal to the actin cytoskeleton for remodeling are heavily crossed-linked in function [90, 276-279]. In fact, MICAL-1,-2,-3 have all been shown to be required for semaphorin signaling to control axon growth and path finding [280]. MICAL also interacts with CRMP and together they control semaphorin signaling [281, 282]. CRMP has also been shown to interact with intersectin1 in this signaling system [283]. This raises the possibility that connecdenn may also be involved in semaphorin-dependent axon pathfinding. Furthermore, connecdenn contains a PPRPP motif that is compatible with the PPKPP motif found in MICAL1 to bind Cas-L. Connecdenn could potentially serve to bind Cas-L and coordinate Rab35, MICAL and Cas-L function to regulate actin-dependent trafficking. Altogether, both connecdenn via intersectin1/CRMP/Cas-L and Rab35 via

MICAL/Cas-L may function in semaphorin-regulated axonal pathfinding and this will be interesting to investigate.

C4. Coupling of Arf6 with Rab35 function

Although we linked connecdenn and Rab35 to CME, we have for now only observed specific effects on the trafficking of cargo entering cells through an Arf6-dependent, clathrin-independent pathway. For the moment, the most plausible explanation is that CME and Arf6-dependent endocytosis-derived vesicles merge in sorting endosomes. This allows for cargo and trafficking regulator mixing and resorting, such that trafficking regulators can then function on cargo incoming from other endocytic pathways. Arf6 has been associated with CME by increasing the plasma membrane levels of PI(4,5)P2 and thereby increasing the recruitment of AP-2 [284]. However, even if there is a role for Arf6 in CME, MHCI does not enter this pathway, as the dominant-negative dynamin mutant K44A does not inhibit its internalization while blocking TfnR endocytosis [52, 179]. However, knockdown of AP-2 does disrupt MHCI trafficking, diverting it into the degradation pathway [285]. It is thus possible that AP-2 has a clathrin-independent function with Arf6 that could explain the phenotype observed in our connecdenn/Rab35 study. The Arf6/Rab35/AP-2 association will need further clarification.

Alternatively, there exists the possibility that connecdenn/Rab35 functions directly along with Arf6 in an clathrin/AP-2-independent endocytic/recycling pathway, similar to what has been described for other CME-associated proteins such as Eps15. This could explain our observations on MHCI trafficking upon connecdenn/Rab35. Two interesting characteristics of Arf6 fit with the aforementioned projected functions of Rab35. First, many cargo that are endocytosed in an Arf6-dependent manner are involved in cell adhesion and coupled to the cytoskeleton [52, 218]. This is the case for immune synapse receptors known to traffic through Rab35-positive pathways [175, 176]. Likewise, in neurons, Arf6 has been reported to regulate neurite outgrowth, dendrite development, and synapse stability mainly via regulating the actin cytoskeleton. Thus, Arf6 and Rab35

may coordinate actin-dependent trafficking of specific cargo required for membrane remodeling. Interestingly, over-expression of dominant-active Arf6 traps Rab35 in Arf6 vesicles, where Arf6-associated cargo also accumulates [218].

Arf6 has also been suggested to function in SV recycling via enhanced recruitment of AP-2 and clathrin to the active zone since over-expression of Arf6 in PC12 cells increases SV biogenesis. Interestingly, SV recycling following bulk endocytosis in neurons shares several features that are similar to Arf6-dependent macropinocytosis. Both are rapid, activity-dependent bulk membrane endocytosis and recycling processes. Macropinocytosis creates large dynamic endosome structures from which tubules emanate that may share similarities to neuronal endosome intermediates, based on EM studies. Finally, both processes are actin-dependent [52]. Macropinocytosis requires Arf6 and Rac activity, which has not yet been studied in SV recycling but could explain the actin remodeling activity occurring during neuronal stimulation. As mentioned above, Rab35 colocalizes with Rac and actin structures and thus, could regulate fast membrane recycling from bulk endocytosis together with Arf6. It will be interesting to determine the functional relationship of connecdenn, Rab35, and Arf6 in the SV cycle.

Concluding remarks

Through the identification and characterization of connecdenn, we provided a template for the study of trafficking mechanisms in both neurons and non-neuronal cells. We identified the DENN domain of connecdenn as an additional protein module exhibiting GEF activity, with interactions with Rab35. This discovery opens new avenues in the field of Rab-mediated trafficking. Importantly, it creates new possibilities to study Rab function and their relationship to other DENN domains and DENN domain-containing proteins. Further characterization of the modules within the DENN domain will be crucial to understanding the DENN domain as a whole. My study on connecdenn also provided the opportunity to characterize Rab35-mediated trafficking, and allowed us to uncover the interdependency of separate endocytic pathways to control the fate of cargo from diverse endocytic pathways. Furthermore, the possible functional cooperation of connecdenn/Rab35 with OCRL, MICAL and Arf6 provides an interesting avenue of research to further refine our understanding of the trafficking pathways involved in neuronal and immune cell physiology.

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Annexe 1 – ethical certficates