Mechanisms for Low Density Lipoprotein Receptor Internalization

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Dedication

I dedicate this dissertation to my wonderful family; particularly to my loving father, who has been my spiritual support, and my understanding and patient husband, who put up with these years of research.

Abstract

Low density lipoprotein receptor (LDLR) internalization clears cholesterol-laden LDL particles from circulation in humans. Defective LDLR trafficking that causes elevated serum cholesterol levels have been associated with genetic diseases, such as familial hypercholesterolemia (FH) and autosomal recessive hypercholesterolemia (ARH). However, our understanding of the mechanisms underlying LDLR trafficking remains incomplete. To identify factors critical to LDLR trafficking, we pursued a genome-wide RNAi screen using *Caenorhabditis elegans* LRP-1/megalin as a model for LDLR transport. In this screen, we identified an unanticipated requirement for the clathrin-binding endocytic adaptor epsin1 in LDLR endocytosis. Epsin1 depletion reduced LDLR internalization rates in mammalian cells, similar to that observed following clathrin depletion. Genetic and biochemical analyses of epsin in *C. elegans* and mammalian cells uncovered a requirement for the ubiquitin-interaction motif (UIM) as critical for receptor transport. As the epsin UIM promotes the internalization of some ubiquitinated receptors, I predicted LDLR ubiquitination as necessary for endocytosis. However, engineered ubiquitination-impaired LDLR mutants showed modest internalization defects that were further enhanced with epsin1 depletion, demonstrating epsin1-mediated LDLR endocytosis is independent of receptor ubiquitination. Furthermore, I examined requirement of the ability of ubiquitin-binding for epsin UIM function in LDLR internalization. Genetic analyses using ubiquitin-binding-impaired EPN-1 mutants demonstrated that EPN-1 binding to ubiquitinated protein is not required for LRP-1 internalization in *C. elegans*, revealing an undefined regulatory role

of epsin UIM in LDLR internalization. Since clathrin knockdown resulted in 50% reduction in LDLR internalization, I suspected that LDLR can be internalized via an alternative mechanism. The major pathway mediated by caveolae invagination was tested and the results indicated that LDLR can undergo either caveolae- or clathrin-mediated endocytosis. Finally, I provide evidence that epsin1-mediated LDLR uptake occurs independently of either of the two documented internalization motifs (FxNPxY or HIC) encoded within the LDLR cytoplasmic tail, indicating an additional mechanism for LDLR.

Table of Contents

ACKNOWLEDGEMENTS ..... **I**

DEDICATION ..... **II**

ABSTRACT ..... **III**

TABLE OF CONTENTS ..... **V**

LIST OF TABLES ..... **IX**

LIST OF FIGURES ..... **X**

Chapter One: Introduction **1**

High blood cholesterol associated cardiovascular disease 2

Cholesterol function and synthesis 2

Lipoprotein classifications 3

LDLR superfamily members 3

Cholesterol homeostasis 4

Impaired LDLR internalization and Familial Hypercholesterolemia diseases 5

LDLR trafficking and Familial Hypercholesterolemia 6

Understanding the mechanism underlying LDLR trafficking 7

C. elegans as the model system for genetic screen 8

Overall hypothesis to investigation of LDLR trafficking 9

Figure legends 11

Figure 1-1 Schematic illustration of a generic lipoprotein particle 11

Figure 1-2 LDLR superfamily 11

Figure 1-3 Schematic diagram of LDLR trafficking 11

Figures 13

Chapter Two: *Caenorhabditis elegans* Reveals a FxNPxY-Independent LDL Internalization Mechanism Mediated by Epsin1 ..... **16**

Summary 17

Introduction 18

Results 21

Genome-wide screen identifies *dab-1* and *epn-1* in LRP-1 transport 21

Epsin1 promotes LDLR endocytosis 24

Epsin1 mediates LDLR endocytosis via an FxNPxY-independent mechanism 25
The UIM is critical for receptor internalization 26
The UIM stabilizes epsin1 complex formation with LDLR 29
Epsin-mediated LDLR endocytosis is independent of ubiquitin modification 30
Epsin1 and FxNPxY-independent LDLR internalization mechanisms.....31
Discussions 34
Epsin1 requirement in LDLR endocytosis.....34
FxNPxY- and HIC-independent LDLR uptake 34
Role of epsins in animal viability 37
Materials and Methods..... 39
Figure Legends 47
Figure 2-1: Depleting endocytic machinery phenocopies *lrp-1(lf)* molting defects.....47
Figure 2-2: Distribution of genes identified using the two-step LRP-1::GFP transport screen.....47
Figure 2-3: LRP-1::GFP accumulates on the apical surface of hyp7 in *epn-1* RNAi-fed animals. ...48
Figure 2-4: LDLR endocytosis requires epsin1.....48
Figure 2-5: Epsin1 promotes FxNPxY-independent LDLR internalization.....49
Figure 2-6: C. elegans EPN-1 UIM is essential for receptor internalization.....49
Figure 2-7: The UIM stabilizes protein complexes containing epsin1 and LDLR.....49
Figure 2-8: Epsin1 promotes HIC-independent LDLR uptake.....50
Supplemental Figure Legends 51
Sup. Figure 2-1: LRP-1::GFP accumulates at the apical surface of hyp7 in *dab-1* null animals.....51
Sup. Figure 2-2: CD8-LDLR$^{\Delta NPxY}$ internalization occurs independently of dimerization with endogenous LDLR.....51
Sup. Figure 2-3: Genomic structure of the *epn-1* gene.....51
Sup. Figure 2-4: Epsin1 recruitment to clathrin-coated pits is independent of the UIM. 52
Sup. Figure 2-5: CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization occurs via a clathrin-independent mechanism. 52
Sup. Figure 2-6: siRNA-mediated knockdown efficiency evaluated by immunoblot analysis. 53
Supplemental Tables 54
Figures 55
Author Contribution Statement..... 69
Chapter Three: Molecular dissection of EPN-1 UIMs function in LRP-1 internalization 70

Summary 71
Introduction 72
Epsin functions as an endocytic adaptor 72
Monoubiquitination and Ubiquitin-interacting-Motif (UIM) motif 72
Understanding the regulatory role of Epsin1 UIM in LDLR internalization 73
Results 75
The regulatory role of EPN-1 UIM1 in LRP-1 internalization in *C. elegans* 75
Discussions 77
Analyses of UIM structural characteristics for ubiquitin interaction 77
Diverse regulatory roles of epsin UIMs 78
The potential regulatory roles of Epsin1 UIM in LDLR internalization 79
Materials and Methods 81
Figure Legends 83
Figure 3-1 EPN-1 (*C. elegans*) and a serious of mutations in UIMs are depicted. 83
Figure 3-2 LRP-1::GFP distribution in animals expressing EPN-1 variants imaged by epifluorescence scope. 83
Figure 3-3 The amino acids in of various UIM-containing proteins required for ubiquitin interaction. 83
Figure 3-4 The potential regulatory roles of Epsin1 UIM in LDLR internalization. 84
Figures 85
Chapter Four: Caveolae-mediated LDLR internalization 89
Summary 90
Introduction 91
Clathrin-independent LDLR endocytosis 91
Results 92
LDLR employs diverse endocytic pathways 92
Discussions 93
Mechanisms underlying two parallel pathways of LDLR internalization 94
Materials and Methods 96
Figure Legends 98
Figure 4-1 Gene knockdown abrogates CD8-LDLR and CD8-LDLR$^{\Delta NPxY}$ internalization. 98
Figure 4-2 Mechanisms underlying two parallel pathways of LDLR internalization. 98

Figures 99
Chapter Five: Conclusion and Discussion 101
LDLR trafficking regulators identified by whole genome RNAi screen in *C. elegans* 102
Molecular mechanisms underlying epsin-mediated LDLR endocytosis 102
Ubiquitinated LDLR is not required for epsin function 104
Molecular dissection of LDLR internalization 105
The potential roles of epsin in hypercholesterolemia 106
Future perspectives 107
Figure legend 109
Figure 5-1 Proposed mechanisms underlying LDLR endocytosis. 109
Figure 110
References 111

List of Tables

Sup. Table 1 ..... **54**

Sup. Table 2 ..... **54**

List of Figures

Figure 1-1 Schematic illustration of a generic lipoprotein particle 13
Figure 1-2 LDLR superfamily 14
Figure 1-3 Schematic diagram of LDLR trafficking 15
Figure 2-1 Depleting endocytic machinery phenocopies *lrp-1*(*lf*) molting defects 55
Figure 2-2 Distribution of genes identified using the two-step LRP-1::GFP transport screen 56
Figure 2-3 LRP-1::GFP accumulates on the apical surface of hyp7 in *epn-1* RNAi-fed animals 57
Figure 2-4 LDLR endocytosis requires epsin1 58
Figure 2-5 Epsin1 promotes FxNPxY-independent LDLR internalization 59
Figure 2-6 *C. elegans* EPN-1 UIM is essential for receptor internalization 60
Figure 2-7 The UIM stabilizes protein complexes containing epsin1 and LDLR 61
Figure 2-8 Epsin1 promotes HIC-independent LDLR uptake 62
Figure 3-1 EPN-1 (*C. elegans*) and a serious of mutations in UIMs are depicted 85
Figure 3-2 LRP-1::GFP distribution in animals expressing EPN-1 variants imaged by epifluorescence scope 86
Figure 3-3 The amino acids in of various UIM-containing proteins required for ubiquitin interaction 87
Figure 3-4 The amino acids in of various UIM-containing proteins required for ubiquitin interaction 88
Figure 4-1 Gene knockdown abrogates CD8-LDLR and CD8-LDLR$^{\Delta NPxY}$ internalization 99
Figure 4-2 Mechanisms underlying two parallel pathways of LDLR internalization 100
Figure 5-1 Proposed mechanisms underlying LDLR endocytosis 110

Sup. Figure 2-1 LRP-1::GFP accumulates at the apical surface of hyp7 in *dab-1* null animals 63
Sup. Figure 2-2 CD8-LDLR$^{\Delta NPxY}$ internalization occurs independently of dimerization with endogenous LDLR 64
Sup. Figure 2-3 Genomic structure of the *epn-1* gene 65
Sup. Figure 2-4 Epsin1 recruitment to clathrin-coated pits is independent of the UIM 66
Sup. Figure 2-5 CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization occurs via a clathrin-independent mechanism 67
Sup. Figure 2-6 siRNA-mediated knockdown efficiency evaluated by immunoblot analysis 68

Chapter One: Introduction

High blood cholesterol associated cardiovascular disease

Coronary heart disease is the leading cause of death in the United States (Lockman *et al.*, 2005; Murphy and Xu, 2012), the risk of which increases with elevated serum cholesterol levels. One example is Familial Hypercholesterolemia (FH), a genetic disease characterized by markedly increased serum cholesterol levels, which predisposes patients to greater chances of developing premature coronary heart disease (Soutar and Naoumova, 2007). The excess circulating cholesterol in FH patients can deposit in peripheral tissues, such as eyes, hands and feet, leading to the formation of skin and tendon xanthomas (Girish and Gupta, 2005). Moreover, the deleterious pathological consequence of cholesterol buildup on the arterial wall is the development of a vascular disorder, atherosclerosis, where the atherosclerotic plaque in coronary arteries progressively narrows the blood flow which eventually gives rise to occluded blood vessel, a leading cause of coronary heart disease (Soutar and Naoumova, 2007).

Cholesterol function and synthesis

Coronary heart disease caused by high serum cholesterol levels underscores the importance of balanced cholesterol levels. Circulating cholesterol is taken up by cells and used to form cell membranes or myelin sheath of neurons; moreover, cholesterol serves as a precursor of steroid hormones, vitamin D and bile acids. Cholesterol (~75%) is largely synthesized in the liver with a rate-limiting step catalyzed by the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) while dietary cholesterol constitutes the remaining supply (Daniels *et al.*, 2009).

Lipoprotein classifications

Due to its insolubility, cholesterol is incorporated in the lipoprotein complexes to travel in the plasma circulating around the body. Lipoproteins function to transport lipids and comprise a hydrophobic center, consisting of cholesterol and triglyceride esters, and a surface monolayer of phospholipids and apolipoproteins, such as apolipoprotein A (ApoA), ApoB, ApoC and ApoE (Figure 1-1). Based on the ratio of internal cholesterol and triglyceride esters, lipoproteins classified in the order of low to high density include chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density-lipoprotein (LDL) and high-density-lipoprotein (HDL). While LDL is the predominant carrier of cholesterol, chylomicrons and VLDL serve as the major triglyceride carriers (Hegele, 2009).

LDLR superfamily members

Lipoproteins can be recognized receptors belonging to LDL receptor (LDLR) superfamily which is composed of LDLR, VLDLR, ApoER2, LRP-1, LRP-2/Megalin, LRP-4, LRP-5, and LRP-6 (Figure 1-2). The extracellular domain contains three types of repeat clusters, complement-like repeats (ligand binding domains), EGF-like and YWTD repeats (pH-dependent release of ligands). Following the single transmembrane domain is a short cytoplasmic tail carrying conserved signals critical for receptor trafficking, such as NPxY motif (asparagine-proline-x-tyrosine, where x is any amino acid) (Figure 1-2) (Willnow *et al.*, 1999; Morris and Cooper, 2001; He *et al.*, 2002; Mishra *et al.*, 2002a; Strickland *et al.*, 2002; Willnow *et al.*, 2007).

LDLR superfamily members recognize different ligands on the basis of the compositions of their extracellular domains, which render them to function in various cellular processes. For example, VLDLR primarily regulates the uptake of VLDL enriched with triglycerides utilized as the main energy source in tissues, such as skeletal muscle, heart and brain (Go and Mani, 2012). LRP-2/megalin is abundantly expressed in different epithelial tissues, such as kidney, and internalizes HDL as well as vitamin binding protein, steroid, retinoid and hormones. LRP-2 deficiency has been linked to vitamin D deficiency in patients with kidney disease (Marzolo and Farfan, 2011). LRP-5 and LRP-6 are highly homologous and both function as co-receptors for Wnt/β-catenin signaling (Go and Mani, 2012).

Cholesterol homeostasis

The LDLR superfamily also functions to regulate cholesterol levels. Excess circulating cholesterol is predominantly removed in the liver where LDLR is abundantly expressed. LDLR recognizes and internalizes the serum cholesterol-laden LDL particles to clear excess cholesterol in the plasma (Innerarity and Mahley, 1978; Weisgraber *et al.*, 1978). Cholesterol levels are not only maintained by LDLR-mediated LDL clearance but also biosynthesis of cholesterol and LDLR. In response to the rising cellular cholesterol levels as a result of LDL internalization, cholesterol synthesis is reduced by inhibiting the activity of HMG-CoA reductase; meanwhile, LDLR expression level is decreased by deactivating its transcription factor, sterol regulatory element-binding protein, SREBP-2 (Horton *et al.*, 2002). Taken together, the biosynthesis of cholesterol and

LDLR are controlled in response to the cellular cholesterol levels to maintain cholesterol homeostasis.

Impaired LDLR internalization and Familial Hypercholesterolemia diseases

The requirement for LDLR in maintaining serum cholesterol levels was revealed by the fundamental work led by Goldstein and Brown who first described FH, a genetic disease caused by a loss of LDLR activity contributing to high serum LDL levels (Goldstein and Brown, 1973; Brown and Goldstein, 1974; Anderson *et al.*, 1977).

Defective LDLR internalization was the first uncovered pathological mechanism of FH. Microscopic analysis on a FH patient, J.D., showed that LDLR failed to cluster in the clathrin-coated pit and appeared scattered on the cell surface indicative of impaired LDLR internalization. The associated mutation was then determined as a mutated endocytic motif, NPV**Y** to NPV**C**, on the LDLR cytoplasmic tail (Brown and Goldstein, 1976; Chen *et al.*, 1990). Additional studies revealed that LDLR undergoes clathrin-mediated endocytosis, starting with sequential recruitment of numerous adaptors, such as AP-2, and clathrin molecules to form clathrin-coated pits. These coated pits then pinch off the plasma membrane, assisted by dynamin, to form clathrin-coated vesicles (Figure 1-3) (Anderson *et al.*, 1977; Goldstein *et al.*, 1979).

The importance of LDLR internalization to maintain cholesterol levels was underscored by the identification of autosomal recessive hypercholesterolemia (ARH). ARH (also

referred to as LDLRAP1) functions as an endocytic adaptor for LDLR and regulates LDLR internalization by binding to the NPxY motif via its phosphotyrosine binding (PTB) domain. It is the mutations in the PTB domain of ARH that disrupt ARH-LDLR interaction, resulting in impaired LDLR internalization and consequently, elevated cholesterol levels (Norman *et al.*, 1999; Garcia *et al.*, 2001; Mishra *et al.*, 2002b).

LDLR trafficking and Familial Hypercholesterolemia

It is not surprising that efficient LDL clearance also requires proper LDL binding to LDLR. Indeed, mutations in LDLR that impair LDL binding have been shown to cause FH. In addition to LDL binding and LDLR internalization, efficient LDL clearance also requires sufficient levels of surface LDLR that are maintained by both LDLR biosynthesis and LDLR recycling from endosomes. LDLR is synthesized in the endoplasmic reticulum (ER) followed by post-translational modification in Golgi apparatus. The mature LDLR is transported to the cell surface where they recognize and internalize circulating LDL particles. After being internalized, the LDLR-LDL complex is directed to the early endosome, the acidity of which dissociates LDL from LDLR. At this point, LDLR can be recycled back to the cell surface to internalize LDL particles or the separated LDL and LDLR may undergo lysosomal protein degradation (Figure 1-3). The importance of sufficient surface LDLR is highlighted by the identification of FH-causing mutations in LDLR that impair its recycling and transport to the plasma membrane. In fact, mutations in LDLR responsible for FH have been compiled and categorized in association with LDLR activities, including 1) biosynthesis; 2) transport

to plasma membrane; 3) LDL binding; 4) receptor endocytosis; and 5) receptor recycling (Brown and Goldstein, 1986; Hobbs *et al.*, 1990) (Figure 1-3).

Dominantly inherited FH caused by LDLR mutations results in a prevalence of 1 in 500 (heterozygous FH) and 1 in 1,000, 000 (homozygous FH). Besides mutations in LDLR, severe hypercholesterolemia has also been linked to mutations in other genes that regulate LDLR activities, such as apolipoprotein B (reduced LDL-LDLR binding) and protein convertase subtilisin/kexin type 9 (enhanced LDLR degradation) with prevalence of 1in 1,000 and 1 in 2,500, respectively (Rader *et al.*, 2003). Moreover, the cause of elevated cholesterol levels can be polygenic whereas the disease causing genes have yet to be discovered.

Understanding the mechanism underlying LDLR trafficking

These FH-causing mutations reveal the significance of understanding how this sequence of tightly regulated trafficking processes is orchestrated. However, LDLR trafficking regulators, particularly those that direct recycling and transport LDLR to the plasma membrane, remain largely unknown. Moreover, despite our advances in the understanding of LDLR endocytosis, the observation that ARH deficient fibroblasts are still capable of internalizing LDLR reveals deficiencies in our comprehension in LDLR uptake (Eden *et al.*, 2002). My thesis aims to broaden our knowledge with regards to LDLR trafficking by first addressing 1) the identification of regulatory proteins mediating LDLR trafficking and 2) the regulatory mechanisms underlying LDLR trafficking.

C. elegans as the model system for genetic screen

To identify LDLR trafficking regulators, we employed a genetic approach, taking advantage of multiple factors that make *C. elegans* a premiere genetic model organism. These include ease of performing genome-wide RNAi screens and the ability to directly observe GFP-tagged proteins in live animals that is essential for identifying potential trafficking defects. Importantly, the requirement for cholesterol and LDLR in multiple cellular processes is also conserved in *C. elegans*.

Cholesterol is critical for *C. elegans* development

Like humans, cholesterol is essential for multiple processes including viability in *C. elegans*. Moreover, these processes require LDLR to mediate uptake of cholesterol. For example, a LDLR family member, RME-2 functions to take up yolk, a type of lipoprotein in *C. elegans*, a process critical for oocyte development (Figure 1-2) (Sharrock, 1983; Grant and Hirsh, 1999).

Another LDLR member, LRP-1, is required for *C. elegans* growth and development (Figure 1-2), without which, animals arrest during larval development, exhibiting molting defects that are reminiscent of phenotypes displayed by wild type animals suffering from cholesterol starvation. The similarity in these phenotypes and established functions for LDLR superfamily in regulating cholesterol biology implicates a role for LRP-1 in regulating cholesterol uptake that is critical for viability and molting during larval development (Chitwood, 1992; Yochem and Greenwald, 1993; Willnow *et al.*,

1999). Moreover, like mammalian LDLR, the NPxY motif is conserved in the *C. elegans* LRP-1 cytoplasmic tail and is known to bind *C. elegans* DAB-1 (ceDAB-1), the sole homolog of mammalian ARH and Dab2 (Kamikura and Cooper, 2003). Additionally, loss of ceDAB-1 function in animals results in molting defects similar to *lrp-1* animals, in support of a role for ceDAB-1 in regulating LRP-1 endocytosis.

In summary, given that *C. elegans* LRP-1 has been implicated for a role in cholesterol uptake and cytoplasmic tail shares common trafficking signal, NPxY, with mammalian LDLR, we determined to focus on *C. elegans* LRP-1 to identify mammalian LDLR trafficking regulators by performing whole genome RNAi screen. In the screen, gene knockdowns that resulted in molting defect/larval death as well as disrupted LRP-1 distribution were selected and investigated for their roles in mammalian LDLR trafficking.

Overall hypothesis to investigation of LDLR trafficking

With an overall goal to identify additional trafficking regulators of LDLR and the comprehensive information obtained from the whole genome RNAi screen in *C. elegans*, I would expect to advance the mechanistic insights into a broad spectrum of LDLR activities. For example, gene knockdowns that result in receptor accumulation in intracellular compartments indicate a role in endosomal sorting. The investigation as to the regulatory mechanism underlying these identified genes would not only extend the knowledge concerning the functional roles of LDLR but also its pathological relevance associated with FH. In addition to understanding the biological activity of LDLR, I

would also expect to isolate genes that could be potential candidates responsible for hypercholesterolemia patients who carry no known mutations.

My thesis is comprised of four chapters starting with this introduction chapter. In chapter two, I describe the genome-wide RNAi screen in *C. elegans* and my analysis on epsin, which was identified in the screen, as an endocytic adaptor protein for LDLR. In chapter three, I describe my analysis of the regulatory role(s) for epsin UIMs in LDLR internalization. Chapter four includes the revelation of caveolae-mediated LDLR endocytosis. Chapter five provides a summary of my thesis with a discussion on the biological significance of the work.

Figure legends

Figure 1-1 Schematic illustration of a generic lipoprotein particle

The hydrophobic cholesterol (C) and triglyceride (T) are surrounded by phospholipid monolayer (blue). Apolipoproteins are inserted in the phospholipid monolayer. Different types of lipoproteins possess different apolipoproteins, including ApoA, ApoB, ApoC or ApoE.

Figure 1-2 LDLR superfamily

LDLR superfamily members are type I transmembrane protein and share similar protein structure. Three types of repeats constitute the extracellular domain. Complement-like repeats serve as the ligand binding domains. EGF-like and YWTD repeats are required for pH-dependent ligand release. LDLR members can be modified with a sugar molecule attached to the O-link sugar domain. Single transmembrane domain is followed by the cytoplasmic tail possessing intracellular trafficking signals, such as NPxY motif.

Figure 1-3 Schematic diagram of LDLR trafficking

Biosynthesis and post-translational modification of LDLR occur in ER and Golgi apparatus; then, LDLR is transported to the cell surface. LDLR recognizes LDL and

undergoes clathrin-mediated endocytosis, which involves the recruitment of AP-2 and clathrin molecules. The clathrin-coated pit detaches from the plasma membrane facilitated by dynamin activity. After endocytosis, LDLR is directed to endosomal sorting process. LDL is released from LDLR in the early endosome and degraded in the lysosome. LDLR recycles back the plasma membrane or undergoes lysosomal protein degradation.

Figures

Figure 1-1 Schematic illustration of a generic lipoprotein particle

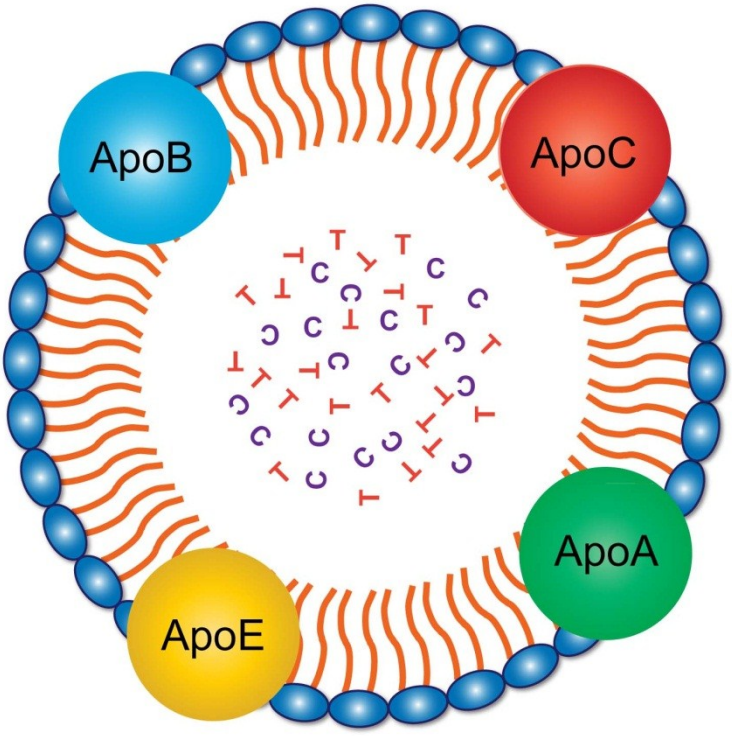


Figure 1-2 LDLR superfamily

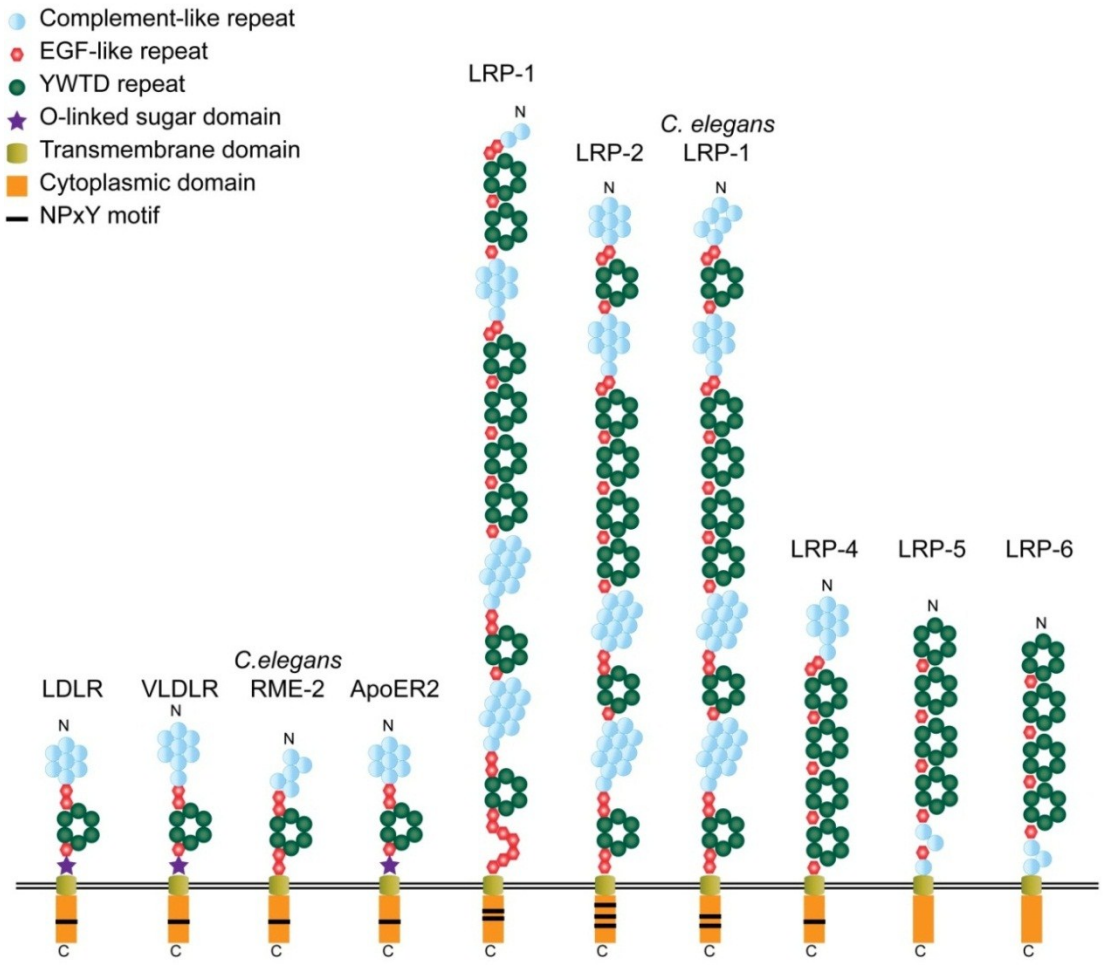
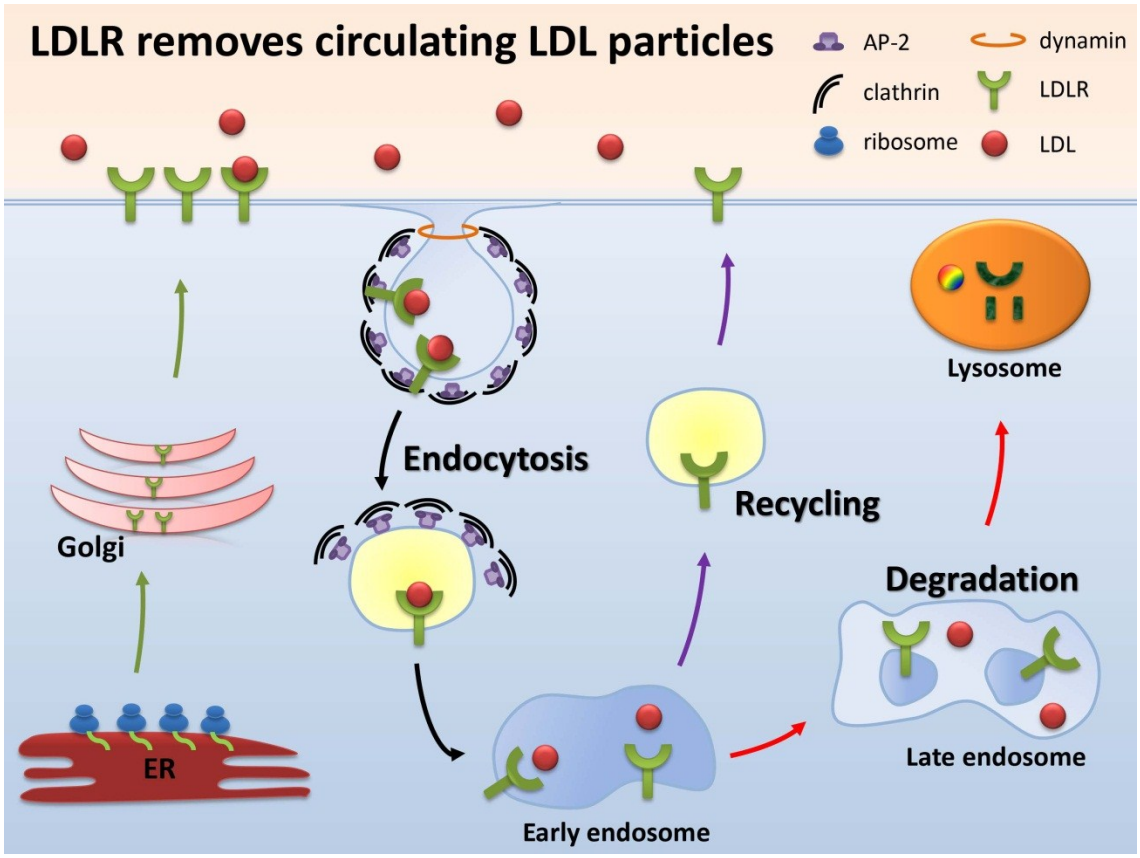


Figure 1-3 Schematic diagram of LDLR trafficking



Chapter Two: *Caenorhabditis elegans* Reveals a FxNPxY-Independent LDL Internalization Mechanism Mediated by Epsin1

The content of chapter two is published in a peer-reviewed journal: Molecular Biology of the Cell

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Summary

Low density lipoprotein receptor (LDLR) internalization clears cholesterol-laden LDL particles from circulation in humans. Defects in clathrin-dependent LDLR endocytosis promote elevated serum cholesterol levels and can lead to atherosclerosis. However, our understanding of the mechanisms that control LDLR uptake remains incomplete. To identify factors critical to LDLR uptake, we pursued a genome-wide RNAi screen using *Caenorhabditis elegans* LRP-1/megalin as a model for LDLR transport. In doing so, we discovered an unanticipated requirement for the clathrin-binding endocytic adaptor epsin1 in LDLR endocytosis. Epsin1 depletion reduced LDLR internalization rates in mammalian cells, similar to that observed following clathrin depletion. Genetic and biochemical analyses of epsin in *C. elegans* and mammalian cells uncovered a requirement for the ubiquitin-interaction motif (UIM) as critical for receptor transport. As the epsin UIM promotes the internalization of some ubiquitinated receptors, we predicted LDLR ubiquitination as necessary for endocytosis. However, engineered ubiquitination-impaired LDLR mutants showed modest internalization defects that were further enhanced with epsin1 depletion, demonstrating epsin1-mediated LDLR endocytosis is independent of receptor ubiquitination. Finally, we provide evidence that epsin1-mediated LDLR uptake occurs independently of either of the two documented internalization motifs (FxNPxY or HIC) encoded within the LDLR cytoplasmic tail, indication and additional mechanism for LDLR.

Introduction

Clearance of cholesterol-laden low density lipoprotein (LDL) from circulation occurs via endocytosis of the LDL receptor (LDLR) (Anderson *et al.*, 1977). Following receptor-ligand internalization, the complex is targeted to the endosomal pathway, the acidity of which dissociates LDL from the receptor. LDL is then targeted to the degradative pathway, while the receptor is recycled back to the plasma membrane to promote additional rounds of LDL endocytosis. Maintaining robust LDLR internalization rates are critical since failure to do so can result in hypercholesterolemia and lead to atherosclerosis (Soutar and Naoumova, 2007).

LDLR uptake is a highly coordinated process that is dependent on a clathrin-mediated internalization mechanism. Clathrin coat assembly drives plasma membrane invagination and formation of cargo-containing endocytic vesicles, but it is not sufficient for LDLR uptake (Maldonado-Baez and Wendland, 2006). Also required are endocytic adaptor proteins that act as intermediates that link clathrin to membranes and cargo destined for internalization. In particular, LDLR endocytosis relies on AP-2 and ARH (autosomal recessive hypercholesterolemia) or Dab2 (disabled homolog 2). AP-2 is a heterotetrameric adaptor protein complex consisting of two large subunits (α, β 2) that mediate interaction with clathrin, phospholipids, and a host of accessory factors; a medium subunit (μ2) that directly engages endocytic cargo; and a small subunit (σ 2) thatappears to stabilize the complex (Robinson, 2004; Edeling *et al.*, 2006). AP-2 serves a general requirement in the formation of endocytic clathrin coats and is essential to internalization of myriad receptors including LDLR, EGFR (epidermal growth factor

receptor), and the transferrin receptor (Motley *et al.*, 2003; Boucrot *et al.*, 2010). By contrast, ARH and Dab2 are related monomeric adaptor proteins that perform more selective, tissue-specific roles in receptor endocytosis (Morris and Cooper, 2001; Eden *et al.*, 2002; Mishra *et al.*, 2002a; Sirinian *et al.*, 2005; Mettlen *et al.*, 2010).

ARH and Dab2 are modular scaffolding proteins containing an amino-terminal phosphotyrosine-binding (PTB) domain that engages both phospholipids and the FxNPxY internalization motif present within the cytoplasmic tail of target receptors like LDLR (Yun *et al.*, 2003). The carboxy terminal region encompasses several small protein interaction modules that facilitate recruitment of core endocytic machinery like AP2 and clathrin, as well as additional accessory factors. For example, Dab2 interacts with EH (Eps15 homology) domain proteins like Eps15 and intersectin to coordinate β 1 integrin endocytosis (Teckchandani *et al.*, 2012) and FCHO-2 (FCH only domain-2) (Henne *et al.*, 2010) to promote AP-2-independent LDLR uptake (Mulkearns and Cooper, 2012). These extended interactions, beyond the core endocytic machinery, implicate a dynamic protein-interaction network, the activity of which is likely a key regulatory step in governing receptor internalization efficiency.

To gain additional insight into the regulatory steps that control LDLR endocytosis and identify additional factors critical for receptor internalization, we employed an unbiased genome-wide RNAi screen using a C. elegans LDLR superfamily member and megalin homologue, LRP-1, as a model for LDLR transport. In doing so, we discovered a requirement for epsin1 in LDLR internalization.

Epsins family members are clathrin-associated proteins conserved from yeast to mammals (Legendre-Guillemin *et al.*, 2004), where they coordinate internalization of signaling receptors and their ligands (Wang and Struhl, 2004; Kazazic *et al.*, 2009; Dores *et al.*, 2010; Sorensen and Conner, 2010; Xie *et al.*, 2012), control synaptic vesicle recycling (Jakobsson *et al.*, 2008), and perform a key role in influenza virus uptake (Chen and Zhuang, 2008). Here, we provide evidence that epsin1 promotes LDLR internalization via a FxNPxY-independent pathway. We complement C. elegans in vivo approaches with loss-of-function and biochemical analyses using mammalian cell culturesystems to evaluate epsin1 mode of action in LDLR endocytosis.

Results

Genome-wide screen identifies *dab-1* and *epn-1* in LRP-1 transport

To identify factors that promote LDL receptor internalization, we pursued a genome-wide RNAi feeding strategy in *C. elegans* to screen for genes involved in endocytosis of LRP-1, an LDLR superfamily member that possesses two conserved [F/V]xNPxY internalization motifs (FTNPVY 4658 , VDNPLY 4744) (Yochem and Greenwald, 1993). Given that animals lacking LRP-1 arrest during larval development and have trouble molting (Figure 2-1) (Yochem *et al.*, 1999), we reasoned that defects in LRP-1 trafficking should mimic *lrp-1* loss-of-function (lf) phenotypes. Indeed, RNAi-induced clathrin-heavy chain (CHC-1) depletion leads to molting defects and larval arrest (Figure 2-1). This indicated that *lrp-1*(lf) phenotypes could serve as an initial visual screen to identify LRP-1 transport genes. As with animal viability, molting is orchestrated by myriad processes, many of which are unrelated to LRP-1 transport defects (Frand *et al.*, 2005). Therefore, to focus on genes involved in LRP-1 trafficking, we examined RNAi-fed animals that phenocopy *lrp-1*(lf) for alterations in LRP-1 localization. To facilitate the assessment of LRP-1 localization, we used LH191, *an lrp-1(ku156); rrf-3(pk1426)* strain with a genome-integrated *lrp-1::gfp* transgenic array (*eqIs1*) that fully rescues the *lrp-1*(lf) phenotypes (Figure 2-1). LRP-1::GFP is enriched in hyp7, a large hypodermal syncytial cell that encompasses the animal body and spans the anterior/posterior axis, and localizes to the apical region of the cell (Figure 2-1), similar to endogenous LRP-1 (Yochem *et al.*, 1999).

Using the described two-step strategy, we screened a bacterial RNAi library that encompasses over 80% of the 19,000 *C. elegans* open reading frames (Fraser *et al.*, 2000; Kamath and Ahringer, 2003). We identified 235 genes that alter LPR-1::GFP distribution within hyp7 when expression of these genes is reduced by RNAi feeding (Sup. Table 1). Consistent with published studies, 31% of these identified genes are critical for molting (Frand *et al.*, 2005); while 26% are involved in oocyte yolk uptake, a process dependent on receptor-mediated endocytosis (Figure 2-2A) (Balklava *et al.*, 2007). Given the nature of our screening strategy, the largest group of genes identified (~22%) were those implicated in intracellular transport (Figure 2-2B, Sup. Table 2).

As LDLR internalization occurs via clathrin-mediated endocytosis, it is not surprising that the screen identified genes encoding DYN-1/dynamin 1 and the major coat constituents of the clathrin-mediated endocytic pathway, including CHC-1/clathrin heavy chain and subunits of the adaptor protein complex AP-2. Similar to CHC-1 depletion, LRP-1::GFP redistributed to the plasma membrane following RNAi-induced depletion of DYN-1/dynamin, DPY-23/μ2 adaptin or APS-2/σ2 adaptin (Figure 2-3), although the extent of localization defect was somewhat variable between animals, likely reflecting knockdown efficiency. Suprisingly, RNAi-induced depletion of the single beta adaptin subunit, APB-1, resulted in a distinct, albeit abnormal, LRP-1::GFP distribution that is reminiscent of endosomal accumulation. This putative endosomal distribution of LRP-1::GFP is consistent with AP1 functioning in receptor sorting decisions within the endosome (Reusch *et al.*, 2002; Gravotta *et al.*, 2012; Shafaq-Zadah *et al.*, 2012; Xu *et al.*, 2012; Zhang *et al.*, 2012). Given that *C. elegans* encodes a

single beta adaptin subunit, it is possible that the spatial and functional distinctions observed for mammalian AP-1 and AP-2 (Robinson, 2004) are not as well resolved in nematodes. Alternatively, this distinct LRP-1::GFP distribution may reflect cross-reactivity of the RNAi clone, resulting in the knock-down of other gene(s).

As anticipated, we also identified *dab-1*, the single *C. elegans* homolog of autosomal recessive hypercholesterolemia (ARH) and Disabled 2 (Dab2). Both ARH and Dab2 are essential for efficient LDLR endocytosis in mammals and promote LDLR uptake by coupling the receptor to other components of the endocytic machinery (Garcia *et al.*, 2001). *dab-1* RNAi frequently resulted in molting defects (data not shown) (Kamikura and Cooper, 2006), as well as significant plasma membrane accumulation of LRP-1::GFP (Figure 2-3), consistent with impaired LRP-1 endocytosis. These observations were also confirmed in *dab-1(gk291)* null animals (Sup. Figure 2-1). Given the critical role of mammalian ARH and Dab2 in LDLR endocytosis (Garcia *et al.*, 2001; Maurer and Cooper, 2006), the identification of *dab-1* validates our strategy of using *C. elegans* LRP-1 trafficking defects to elucidate genes involved in LDLR transport. In addition to the aforementioned genes, the screen also uncovered *epn-1* as a candidate gene required for LRP-1 endocytosis. Indeed, RNAi-depletion of EPN-1 resulted in a redistribution of LRP-1 to the plasma surface, like that seen following DAB-1 depletion (Figure 2-3).

Epsin1 promotes LDLR endocytosis

epn-1 is the sole gene in *C. elegans* to encode epsin (Chen *et al.*, 1998), a clathrin-binding protein that is thought to serve as an endocytic adaptor to coordinate internalization of some receptors (Maldonado-Baez and Wendland, 2006). To determine whether the role for epsin in LRP-1 internalization is also conserved with mammalian LDLR, we first examined uptake of DiI-labeled LDL (DiI-LDL) in HeLa cells following epsin1 siRNA-mediated depletion. Relative to control cells, a marked reduction in internalized DiI-LDL was observed following a reduction in epsin1 expression, similar to that observed following clathrin heavy chain depletion (Figure 2-4A). By comparison, epsin1 knockdown did not alter FITC-labeled transferrin internalization in the same cells, consistent with published reports that epsin1 is not necessary for transferrin uptake (Huang *et al.*, 2004). We interpret these observations to indicate that epsin1 is essential for robust internalization of endogenous LDLR.

To measure changes in LDLR internalization following epsin1 knockdown, we employed a CD8-LDLR chimera, which has been successfully used to quantitate LDLR internalization kinetics (Motley *et al.*, 2003) by tracking uptake of an antibody directed at the extracellular CD8 epitope (51.1) (Martin *et al.*, 1984). Consistent with the DiI-LDL uptake data, epsin1 depletion impaired CD8-LDLR uptake relative to controls when evaluated qualitatively by immunofluorescence after incubating CD8-LDLR-expressing cells for 5 min with 51.1 antibody (Figure 2-4B,C). Likewise, quantitative analysis revealed a significant reduction in CD8-LDLR internalization rate following

epsin1 depletion, similar to that observed following depletion of clathrin heavy chain (Figure 2-4D) or Dab2 (Figure 2-4E). By comparison, ARH depletion showed an intermediate internalization defect in HeLa cells, in line with published reports (Maurer and Cooper, 2006). Collectively, these observations strongly support the conclusion that epsin1 plays a role in promoting LDLR endocytosis.

Epsin1 mediates LDLR endocytosis via an FxNPxY-independent mechanism

To resolve the mechanism by which epsin1 mediates LDLR endocytosis, we evaluated the possibility that it serves in concert with ARH and Dab-2 via the FxNPxY mechanism. To test this, we generated a CD8-LDLR mutant where the NPxY region of the FxNPxY internalization motif was deleted (CD8-LDLR$^{\Delta NPxY}$) to eliminate the contribution of Dab2 and ARH in CD8-LDLR uptake. As expected, the CD8-LDLR$^{\Delta NPxY}$ internalization rate was markedly reduced relative to that of the CD8-LDLR control (Figure 2-5). Moreover, CD8-LDLR$^{\Delta NPxY}$ internalization rate was not significantly altered following Dab2 depletion, consistent with the critical role of Dab2 binding to the FxNPxY motif in LDLR endocytosis.

Interestingly, an appreciable degree of CD8-LDLR$^{\Delta NPxY}$ uptake was observed (Figure 2-5), raising the possibility that FxNPxY-independent LDLR internalization mechanisms exist, like that previously reported (Michaely *et al.*, 2007). Alternatively, LDLR can dimerize (van Driel *et al.*, 1987). Thus, the observed CD8-LDLR$^{\Delta NPxY}$ uptake might result from dimerization and co-internalization with endogenous LDLR, similar to

published reports (Yoshida *et al.*, 1999; Zou and Ting, 2011). We ruled out this latter possibility by testing CD8-LDLR$^{\Delta NPxY}$ internalization kinetics in CHO *ldlA* cells that lack functional LDLR (Kingsley and Krieger, 1984). As in HeLa cells, a similar degree of CD8-LDLR$^{\Delta NPxY}$ uptake was also observed in CHO *ldlA* cells, as well as the parental cell line (Sup. Figure 2-2). This indicated that the observed uptake for CD8-LDLR$^{\Delta NPxY}$ did not arise from dimerization with endogenous receptor, the expression of which is significantly less than that of recombinant CD8-LDLR. We thus conclude that internalization observed for CD8-LDLR$^{\Delta NPxY}$ occurred independent of the mechanism requiring the FxNPxY motif.

We next evaluated how epsin mediates LDLR internalization. If like Dab2, epsin1 also relies on the FxNPxY motif, epsin1 depletion should not further impair CD8-LDLR$^{\Delta NPxY}$ uptake. Surprisingly and in contrast to Dab2 depletion, epsin1 siRNA decreased CD8-LDLR$^{\Delta NPxY}$ internalization even further, thus revealing epsin1 promotes LDLR endocytosis via an FxNPxY-independent mechanism.

The UIM is critical for receptor internalization

Epsin is a modular protein (Figures 2-6A and 2-7A) consisting of an epsin N-terminal homology domain (ENTH) that directly binds phosphatidylinositol-4,5-bisphosphate (Itoh *et al.*, 2001) and Cdc42 GAPs (Aguilar *et al.*, 2006), a middle region encoding multiple ubiquitin-interacting motifs (UIM) that engage ubiquitinated endocytic cargo (Hawryluk *et al.*, 2006; Kazazic *et al.*, 2009), and a series of small motifs that support

interaction with clathrin, AP-2, and EH domain-containing proteins like Eps15 (Chen *et al.*, 1998; Drake *et al.*, 2000). To further resolve how epsin1 controls LDLR transport, we pursued a functional dissection of EPN-1 in *C. elegans*, given that epsin overexpression in mammalian cells is dominant-negative for endocytosis of multiple receptors (Sugiyama *et al.*, 2005; Kazazic *et al.*, 2009; Sorensen and Conner, 2010). Animals homozygous for a strong loss-of-function *epn-1* allele *tm3357* (Sup. Figure 2-3) arrest as larvae at a stage before molting defects are apparent, but can be fully rescued by an *epn-1::mChr* extra-chromosomal array (Figure 2-6A).

To establish EPN-1 regions that are essential for LRP-1 internalization, a series of deletion mutants was generated and tested for their ability to rescue larval lethality. In combination, we also imaged LRP-1::GFP distribution in hyp7 *in epn-1(tm3357)* animals with an *lrp-1(ku156) eqIs1(lrp-1::gfp)* background by total internal reflective fluorescence microscopy (TIRF) to selectively visualize LRP-1::GFP localization differences at the plasma membrane. Full-length *epn-1::mChr* fully rescues the *epn-1* larval lethality, resulting in healthy adult animals that exhibit LRP-1::GFP distribution in hyp7 that is indistinguishable from that of control LH191 *lrp-1(ku156) eqIs1(lrp-1::gfp)* animals (Figure 2-6). On the other hand, an epn-1 construct lacking the ENTH (EPN-1$^{\Delta ENTH}$) fails to rescue *epn-1(tm3357)* larval lethality (Figure 2-6A). By contrast, engineered versions of EPN-1 lacking specific motifs that support interaction with AP-2 or eps15 fully rescue *epn-1* larval lethality. In each case, LRP-1::GFP localization is indistinguishable from that in control animals (Figure 2-6D and data not shown),

indicating the AP-2- and EH-binding motifs in EPN-1 are not required for viability or LRP-1 trafficking.

Remarkably, an EPN-1 mutant lacking the UIM (EPN-1$^{\Delta UIM}$) fully rescues larval lethality, yet LRP-1::GFP localization is diffuse at the apical cell surface of hyp7 (Figure 2-6E). In addition to the diffused LRP-1::GFP localization, epn-1 ΔUIM animals also resemble dab-1 mutant animals in that they are viable, fertile, Dpy (short and fat), and exhibiting partial molting defects (Kamikura and Cooper, 2003). Interestingly, rescue of *epn-1* lethality by EPN-1$^{\Delta UIM}$ is lost in the *dab-1* null background. This synthetic lethality of *dab-1;epn-1*$^{\Delta UIM}$ animals indicates *epn-1* and *dab-1* function in parallel pathways and is reminiscent of our mammalian analysis that epsin1 and Dab2 act by distinct mechanisms to internalize LDLR. To determine which of the two EPN-1 UIMs is required for LRP-1 internalization, we tested the ability of engineered EPN-1 forms lacking only one UIM to rescue LRP-1 distribution in *epn-1* mutant animals. As expected, both constructs rescue *epn-1* larval lethality. Animals expressing EPN-1$^{\Delta UIM2}$ (lacking the second UIM) displayed apparently wild-type LRP-1::GFP distribution. However, animals expressing EPN-1$^{\Delta UIM1}$ exhibited diffuse cell surface accumulation of LRP-1::GFP (not shown) that was similarly observed in LH191 animals following *epn-1* RNAi feeding or in *dab-1(gk291)* animals in a *lrp-1(ku156) eqIs1(lrp-1::gfp)* background (Figure 2-6F,G). Taken together, these observations reveal a striking requirement for the first EPN-1 UIM in promoting internalization of LDLR superfamily members, similar to that recently reported for Notch transport in

Drosophila (Xie *et al.*, 2012). Remarkably, this requirement is not coupled to the as-yet-uncharacterized role of epsin that is essential for animal viability.

The UIM stabilizes epsin1 complex formation with LDLR

Robust receptor uptake necessitates tight spatio-temporal assembly of protein complexes that couple cargo to the endocytic machinery. Like epsin1, LDLR colocalizes with clathrin in coated pits at the cell surface (Anderson *et al.*, 1977; Chen *et al.*, 1998). In support of epsin1 mediating LDLR endocytosis, TIRF imaging revealed that epsin1 and LDLR colocalize at the cell surface in mammalian cells (Sup. Figure 2-4). Given the critical role of the UIM in receptor internalization, we postulated that the UIM might influence epsin1 targeting to clathrin-coated pits. However, no significant alteration in epsin1 recruitment to clathrin coated structures was observed when comparing epsin1-mChr and epsin1$^{\Delta UIM}$-mChr by live cell imaging (Sup. Figure 2-4), a result that is in agreement with published reports (Chen and Zhuang, 2008).

Given that epsin1 recruitment to clathrin-coated pits occurs independently of the UIM domain, we postulated that the UIM domain might function as a protein-protein interaction platform to promote assembly of an endocytic network that drives receptor internalization, like that recently suggested for yeast epsins (Dores *et al.*, 2010). To test this idea, complex formation was evaluated via co-immunoprecipitation assays in cells that co-expressed CD8-LDLR together with either WT or epsin1 deletion mutants (Figure 2-7A). WT epsin1 readily co-immunoprecipitated with CD8-LDLR (Figure 2-

7B), indicating that epsin1 and CD8-LDLR are present in a complex. Similarly, engineered versions of epsin1 lacking the ENTH domain, the region spanning the clathrin and AP-2 binding region, or the NPF motifs also co-immunoprecipitated with CD8-LDLR. By contrast, epsin1$^{\Delta UIM}$ did not co-immunoprecipitate with CD8-LDLR (Figure 2-7C), indicating that the UIM is critical for epsin1 incorporation into CD8-LDLR complexes.

Epsin-mediated LDLR endocytosis is independent of ubiquitin modification

Drosophila and mammalian epsins can bind and promote internalization of ubiquitinated cargo (Chen *et al.*, 2002; Sigismund *et al.*, 2005). Given the important role of the epsin1 UIM in receptor uptake, we postulated that LDLR ubiquitination might be essential for epsin1-mediated LDLR endocytosis. To test this notion, we first evaluated the internalization kinetics of a ubiquitination-impaired form of CD8-LDLR (CD8-LDLR$^{Ub\text{-}mut}$, Figure 2-8A), using established mutations that block LDLR ubiquitination (K790R,K795R,K809R and C818A, Zelcer et al., 2009). We reasoned that if ubiquitination was critical for epsin1 activity, internalization of the CD8-LDLR$^{ub\text{-}mut}$ should be impaired. Moreover, CD8-LDLR$^{Ub\text{-}mut}$ internalization defects should not be further impacted following epsin1 depletion. Relative to the CD8-LDLR control, the internalization rate of CD8-LDLR$^{Ub\text{-}mut}$ was appreciably reduced, although this defect was not as severe as that observed for CD8-LDLR following epsin1 depletion (Figure 2-8B). Moreover, contrary to our prediction, CD8-LDLR$^{Ub\text{-}mut}$ uptake rate was further

decreased following epsin1 knockdown. Taken together, these results indicate that epsin1-mediated LDLR uptake can occur independent of LDLR ubiquitination status.

Epsin1 and FxNPxY-independent LDLR internalization mechanisms

Our observations clearly indicate that epsin1 can promote LDLR uptake via a mechanism independent of the FxNPxY motif (Figure 2-5). Consistently, published observations reveal that LDLR internalization can occur via the HIC motif (Michaely *et al.*, 2007), which is positioned C-terminal of the FxNPxY motif within the LDLR cytoplasmic tail (Figure 8A). Indeed, mutating the HIC motif (CD8-LDLR$^{HIC\text{-}mut}$, HIC mutated to AAA) reduced receptor internalization rates relative to control CD8-LDLR (Fig 2-8C). Interestingly, the cysteine residue within the HIC motif (C818, Figure 2-8A) is also deemed essential for the ubiquitin-modification of LDLR (Zelcer *et al.*, 2009). Hence it was mutated in CD8-LDLR$^{Ub\text{-}mut}$. This raised the possibility that the internalization defects observed for CD8-LDLR$^{Ub\text{-}mut}$ might not result from changes in LDLR ubiquitination status, but instead result from a defective HIC motif. To test this, we compared the internalization kinetics of CD8-LDLR$^{Ub\text{-}mut}$ with that of CD8-LDLR$^{HIC\text{-}mut}$ and a CD8-LDLR form where only the lysines were mutated (CD8-LDLR$^{3K\text{-}mut}$, K790R,K795R,K809R). CD8-LDLR$^{3K\text{-}mut}$ internalization was similar to that of control CD8-LDLR (Figure 2-8C). By comparison, indistinguishable defects in receptor uptake rate were observed for CD8-LDLR$^{HIC\text{-}mut}$ and CD8-LDLR$^{Ub\text{-}mut}$ (Figure 2-8C).

The striking similarity in the internalization kinetics for both CD8-LDLR$^{HIC\text{-}mut}$ and CD8-LDLR$^{Ub\text{-}mut}$ raised the possibility that the mechanisms that drive their endocytosis might be linked. To address this possibility, we tested the uptake of a CD8-LDLR form that was mutant for HIC and ubiquitination (CD8-LDLR$^{Ub\text{-}HIC\text{-}mut}$). We reasoned that if both mechanisms were linked, CD8-LDLR$^{Ub\text{-}HIC\text{-}mut}$ internalization rate should be similar to CD8-LDLR forms that cannot be ubiquitinated or those lacking the HIC motif. Indeed, CD8-LDLR$^{Ub\text{-}HIC\text{-}mut}$ uptake was indistinguishable from either CD8-LDLR$^{Ub\text{-}mut}$ or CD8-LDLR$^{HIC\text{-}mut}$ (Figure 2-8C). Combined with the observation that CD8-LDLR$^{3K\text{-}mut}$ endocytosis is normal, we interpret these latter findings to indicate that ubiquitination is not critical for LDLR uptake. Instead, the impaired internalization observed for CD8-LDLR$^{Ub\text{-}mut}$ likely arises from mutating the cysteine residue, which is critical for HIC-mediated LDLR uptake.

Consistent with published observations, our findings reinforce a model where at least two independent mechanisms drive LDLR endocytosis — one reliant on the FxNPxY motif, and another on the HIC motif. However, our findings also suggest that epsin1-mediated LDLR uptake can occur independent of either internalization motif. To better test this latter notion, we measured the internalization kinetics of a ubiquitination-defective form of CD8-LDLR that is incapable of FxNPxY- or HIC-mediated uptake (CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$) following epsin1 depletion. Consistent with two distinct internalization mechanisms, CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization rate was reduced relative to CD8-LDLR$^{\Delta NPXY}$. Moreover, further reductions in CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization rate were observed following epsin1 depletion (Figure 2-8D), similar to

that observed for a CD8-LDLR form lacking a functional FxNPxY that cannot be ubiquitinated (CD8-LDLR$^{\Delta NPXY;Ub\text{-}mut}$, Figure 2-8E) and in agreement with an additional epsin-dependent LDLR uptake mechanism. To determine whether this epsin-mediated internalization is reliant on clathrin, we next measured CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ uptake following clathrin-depletion. Following clathrin heavy chain knockdown, CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ was not impaired, like that observed for CD8-LDLR uptake (Figure 2-8). Instead, CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization was markedly increased (Sup. Figure 2-5), indicating that when clathrin-mediated uptake is impaired, alternative internalization pathways can be stimulated, similar to that previously reported (Damke *et al.*, 1995). Taken together, these findings reveal a distinct epsin1-mediated pathway that promotes LDLR uptake via a clathrin-independent pathway.

Discussions

Epsin1 requirement in LDLR endocytosis

In this study, we describe a genome-wide RNAi screen identifying EPN-1/epsin as an essential factor in promoting internalization of the *C. elegans* LDLR superfamily member, LRP-1. We provide genetic, cell-based, and biochemical evidence that demonstrate a conserved activity for mammalian epsin1 in LDLR endocytosis. The requirement for epsin family members in promoting internalization of the LDLR family members is further underscored by the identification of EPN-1/epsin in a previous *C. elegans* screen for genes involved in yolk uptake into oocytes, a process also mediated by an LDLR superfamily member, RME-2 (Balklava *et al.*, 2007).

Interestingly, these findings contrast two studies where siRNA-mediated epsin1 depletion in human HeLa SS6 cells or monkey BSC-1 cells was not found to disrupt LDLR internalization (Keyel *et al.*, 2006; Chen and Zhuang, 2008). This apparent discrepancy may reflect differences in siRNA-induced epsin1 depletion or in experimental conditions and approach used to measure LDLR internalization, similar to that recently demonstrated for AP-2 (Boucrot *et al.*, 2010).

FxNPxY- and HIC-independent LDLR uptake

The cytoplasmic LDLR tail encodes multiple internalization signals that are critical for maintaining robust receptor internalization. The canonical FxNPxY motif (Chen *et al.*,

1990) is recognized by the PTB domain-containing endocytic adaptor proteins ARH and Dab-2 (Garcia *et al.*, 2001; Morris and Cooper, 2001; Mishra *et al.*, 2002a; Mishra *et al.*, 2002b) that couple the receptor to core endocytic machinery to drive LDLR endocytosis. In the absence of a functional FxNPxY motif, LDLR internalization can occur via an HIC-dependent mechanism (Michaely *et al.*, 2007), although the identity of endocytic adaptors essential for HIC-mediated endocytosis are currently unknown. By comparison, our loss-of-function analyses indicate that epsin1 can promote LDLR internalization via a mechanism that is independent of clathrin or either internalization motif. An FxNPxY-independent mechanism for epsin is also supported by our observations in *C. elegans*. Despite LRP-1 internalization defects, *dab-1* as well as *epn-1*$^{\Delta UIM}$-expressing *epn-1* mutant animals are viable and fertile. However, EPN-1$^{\Delta UIM}$-expressing *dab-1;epn-1* double mutant animals exhibit synthetic larval lethality, consistent with both genes acting in parallel pathways. While our data demonstrates epsin1 promotes LDLR endocytosis independently of the FxNPxY and HIC mechanisms, our observations do not enable us to resolve the potential role for epsin1 in also facilitating LDLR uptake via an FxNPxY- or HIC-dependent route.

Our *in vivo* dissection of EPN-1 to rescue *epn-1(tm3357)* lethality provided us the ability to distinguish protein motifs that are critical for EPN-1 function from protein domains that have regulatory roles. The inability for EPN-1$^{\Delta ENTH}$ to rescue *epn-1(tm3357)* lethality highlights the necessity of the ENTH domain to epsin function, a result that was also uncovered by functional assays in other systems (Wendland *et al.*, 1999; Overstreet *et al.*, 2003; Brady *et al.*, 2008; Xie *et al.*, 2012). In contrast to the

ENTH domain, EPN-1 transgenes that lack the UIM or protein modules important for interaction with AP-2 or EH domain-containing factors rescue *epn-1(tm3357)* lethality, revealing these motifs as having regulatory or redundant roles. However, only EPN-1 forms lacking the UIM show abnormal LRP-1::GFP accumulation at the plasma membrane, pinpointing the UIM as essential for LRP-1 internalization. Reinforcing the importance of the UIM, our co-immunoprecipitation analyses in mammalian cells also defined the UIM as necessary for epsin1 to stabilize complex formation with the LDL receptor.

The requirement for the UIM in epsin1-mediated receptor transport is echoed in a recent *in vivo* dissection of *Drosophila* epsin that also revealed the importance of the UIM in Notch signaling (Xie *et al.*, 2012), in addition to previous studies that implicate a critical role for the UIM in receptor transport (Shih *et al.*, 2002; Overstreet *et al.*, 2003; Sugiyama *et al.*, 2005; Kazazic *et al.*, 2009; Dores *et al.*, 2010). However, the mechanistic role for the UIM remains unclear. Our observations, combined with that of others (Chen and Zhuang, 2008), indicate that epsin1 recruitment to clathrin-coated pits is UIM-independent. By contrast, epsin1 interaction with ubiquitinated EGF receptor relies on the UIM, where UIM-mediated binding to ubiquitinated EGF receptor is thought to promote receptor recognition and packaging for endocytosis (Sigismund *et al.*, 2005; Hawryluk *et al.*, 2006; Kazazic *et al.*, 2009).

By comparison, ubiquitination is known to direct LDLR for degradation within the lysosome (Zelcer *et al.*, 2009). However, our observations indicate that LDLR

ubiquitination is not essential for epsin-mediated receptor endocytosis — epsin1 depletion further reduced the modestly impaired CD8-LDLR$^{Ub\text{-}mut}$ internalization rate. These findings, which are consistent with those in yeast where ubiquitinated cargo can be endocytosed in the absence of epsin binding to ubiquitin (Dores *et al.*, 2010), raise the possibility that the epsin1 UIM serves as a protein-protein interaction platform, as previously suggested (Dores *et al.*, 2010). We do not favor a direct interaction between epsin1 and LDLR given that *in vitro* binding and yeast two hybrid assays failed to detect a robust interaction (data not shown). Instead, it is possible that epsin1 interacts with other factors that may be ubiquitin-modified to coordinate LDLR uptake. Alternatively, the UIM might be important for regulating epsin1 endocytic activity. For example, the UIM is essential for epsin1 ubiquitination within the ENTH domain (Oldham *et al.*, 2002). Additional analysis is required to resolve these possibilities.

Role of epsins in animal viability

Our studies reveal that epsin function is required for viability in *C. elegans*, similar to *Drosophila* and mice. Indeed, epsin loss in *Drosophila* and mice result in embryonic lethality, although the precise defect that leads to lethality remains to be determined (Tian *et al.*, 2004; Chen *et al.*, 2009; Xie *et al.*, 2012). Likewise, the cause for *epn-1* larval lethality in *C. elegans* is not known, although one possibility may be LRP-1 trafficking defects. Indeed, *lrp-1* null animals exhibit early larval lethality (Yochem *et al.*, 1999). *epn-1* larval lethality is rescued by EPN-1 ΔUIM as a result of a partial

suppression of *epn-1* LRP-1 trafficking defects. The synthetic lethality of *dab-1;epn-1*$^{\Delta UIM}$ animals is suggestive of enhanced LRP-1 trafficking defects, which is reminiscent of our data indicating that epsin1 promotes LDLR internalization in parallel to the FxNPxY mechanism that relies on Dab2. Alternatively, *epn-1* lethality may be LRP-1-independent and a result of more pleiotropic defects in epn-1-mediated transport of unidentified factors or the disruption of critical signaling pathways that rely on receptor transport. While the cause for *epn-1* lethality is unknown, it is clear from the *dab-1;epn-1*$^{\Delta UIM}$ synthetic lethality that *epn-1* acts in parallel with *dab-1* in *C. elegans* as do epsin1 and Dab2 in mammalian LDLR internalization.

Materials and Methods

Animal strains: C. elegans strains are grown on nematode growth medium plates at 20°C as described by (Brenner, 1974). The following alleles were used: *lrp-1(ku156)*, *dab-1(gk291)* and *epn-1(tm3357)*. Bristol N2 serves as the wild-type *C. elegans* strain. The following alleles were used:

LGI: *lrp-1(ku156)*.

LGII: *dab-1(gk291), rrf-3(pk1426)*

LGX: *epn-1(tm3357), unc-7(35)*.

The *epn-1(tm3357)* strain was kindly provided by Dr. Shohei Mitani (National Bioresource Project at the Tokyo Women's Medical University School of Medicine). We obtained a twice-outcrossed *epn-1(tm3357)* strain from Dr. Erik Jorgensen (University of Utah) that we outcrossed seven additional times.

C. elegans expression vectors: epn-1 genomic DNA was kindly provided by Dr. Erik Jorgensen (University of Utah). All epn-1 expression constructs were cloned in the pGEM-3Zf(+)vector. *epn-1* wild type and deletion DNA was cloned using engineered BglII and NotI restriction sites. mCherry was inserted in frame at the 3' end of epn-1 just prior to the stop codon using engineered NotI and BamHI restriction sites.

Pepn-1::epn-1::mCherry (*epn-1* genomic DNA, including 1551bp of sequences upstream of the start codon and 1659 bp of sequences downstream of the *epn-1* coding region)

Pepn-1::epn-1ΔENTH::mCherry (*epn-1* genomic DNA, deletion from a.a. 19-140)

Pepn-1::epn-1ΔUIM::mCherry (*epn-1* genomic DNA, deletion from a.a. 193-232)

Pepn-1::epn-1ΔUIM1::mCherry (*epn-1* genomic DNA, deletion from a.a. 193-207)

Pepn-1::epn-1ΔUIM2::mCherry (*epn-1* genomic DNA, deletion from a.a. 218-232)

Pepn-1::epn-1ΔAP2::mCherry (*epn-1* genomic DNA, deletion from a.a. 285-332)

Pepn-1::epn-1ΔNPF::mCherry (*epn-1* genomic DNA, deletion from a.a. 425-469)

Generated C. elegans transgenic strains:

LH191: *lrp-1(ku156) eqIs1; rrf-3(pk1426)*

LH801: *lrp-1(ku156) eqIs1; dab-1(gk291)*

LH757: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1::mCherry*

LH764: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1ΔUIM::mCherry*

LH819: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1ΔUIM1::mCherry*

LH824: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1ΔUIM2::mCherry*

LH770: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1ΔAP2::mCherry*

LH761: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1ΔNPF::mCherry*

eqIs1 is a spontaneously-integrated transgene containing *lrp-1::gfp* that rescues *lrp-1* mutations. It was generated in the following fashion. Two plasmids, MH#jy209b and MH#jy207b, contain overlapping DNA from the *lrp-1* locus that can result in an intact gene to rescue *lrp-1* mutant animals (Yochem *et al.*, 1999). GFP was inserted in frame with the cytoplasmic tail of LRP-1 in MH#jy207b at the PacI/SalI restriction sites. The resulting LRP-1::GFP-pBluescript(SK+) plasmid was injected into *unc-13 lrp-1(ku156); nDp4/+* animals, together with MH#jy209b and a co-injection marker, pRF4, which confers dominant rolling. Rescue of *ku156* is indicated by the production of healthy, viable *unc-13* progeny that were picked for further propagation. One such animal and its progeny appeared to have a spontaneous insertion at or near the *lrp-1(ku156)* locus and showed GFP expression in hyp7 with a pattern identical to that observed in wild-type animals stained with anti-LRP-1 antibodies (Yochem *et al.*, 1999). The proximity to the *lrp-1* locus and an absence of rolling are consistent with gene conversion, but this has not been examined at the DNA level. *eqIs1* was then separated from the *unc-13* marker by standard genetic recombination and then isolating an offspring homozygous for the *eqIs1* were isolated. *eqIs1* was crossed into *rrf-3(pk1426)* and *dab-1(gk291)* animals to generate the LH191 and LH801 strains, respectively.

All *epn-1* expression constructs were injected at 1 ng/µL with 70 ng/µL *str-1::gfp* as the coinjection marker into *lrp-1(ku156) eqIs1; epn-1(tm3357)/unc-7(e5)* animals.

C. elegans RNAi screen: We used an established bacterial RNAi library that covers an estimated 86% of the *C. elegans* genome and adapted an established RNAi by feeding protocol (Kamath and Ahringer, 2003). Briefly, we placed three gravid LH191 animals on each plate seeded with a single bacterial RNAi clone. Gravid animals were used in the screen to bypass any embryonic requirement of the gene targeted by RNAi. After three days, the progeny of the RNAi-treated adults were examined for *lrp-1* null phenotypes, which include larval arrest and/or defective molting. These animals were then further examined under light microscopy for alterations in LRP-1::GFP distribution, as compared to control LH191 animals.

Reagents: The monoclonal antibodies TD.1, E7, and 4A4 were used to recognize clathrin heavy chain, β-tubulin and LDLR. The mouse hybridomas 4A4 (CRL-1898), 51.1(HB-230) and TD.1 (CRL-2232) were obtained from the American Type Culture Collection (ATCC). Rabbit antibody against CD8-α (H-160) and mouse antibody against Dab2 (610464) were from Santa Cruz Biotechnology and BD biosciences, respecitvely. Rabbit polyclonal antiserum against Epsin1 was a generous gift from Dr. Sandra Schmid (University of Texas, Southwestern Medical Center). BSC-1 cells stably expressing CLC-GFP were a generous gift from Dr. Tom Kirchhausen (Harvard

University). Lipofectamine LTX and Plus Reagent and Lipofectamine RNAi^{MAX} were used for DNA and siRNA transfection following the manufacturers' instructions (Invitrogen). CHO ldlA cells lacking functional LDLR and the parental CHO cell line were a gift from Monty Krieger (MIT).

Plasmid used to generate adenovirus coding for mouse CD8-LDLR chimera was a gift from Dr. Margaret Robinson (The University of Cambridge) and used in experiments for Figs. 4 and 7. Human cDNA for LDLR was a gift from Dr. Peter Tontonoz (UCLA) and was used to generate adenovirus encoding the extracellular domain of human CD8 fused to LDLR starting with the LDLR transmembrane domain (WT, ΔNPxY, Ub-mut, HIC-mut, 3K-mut, Ub-HIC-mut, ΔNPxY-HIC-mut, ΔNPxY-Ub-mut) and was used in experiments for Figs. 5, 8, and Sup. Fig. 2. Rat epsin1 sequence is based on NM_057136. myc-tagged Rat epsin1 cDNA is kindly provided by Dr. Pietro De Camilli (Yale University). Epsin1 truncations were engineered using PCR-based mutagenesis and subcloned to the EcoRI/XhoI site of pcDNA3-myc plasmid. Epsin1 domain deletions spanned the following amino acids: ENTH (19-132), UIM (184-248), CL/AP-2 (257-484), and NPF (501-575). For carboxy-terminal fusions, mCherry was subcloned into the XhoI/XbaI site of pcDNA containing myc-epsin1 or myc-epsin1ΔUIM.

Co-Immunoprecipitation: tTA HeLa cells were infected with CD8-LDLR adenovirus and transfected with myc-tagged epsin1 wild type or deletion constructs 4-6 hours after

seeding in 60mm dish. Cells were harvested 18 hours after transfection and lysed in IP buffer (50mM Tris-Cl pH7.5, 150mM NaCl, 2mM EDTA and 1% NP-40) with protease inhibitor complex (Sigma, P8340). CD8-LDLR containing protein complexes were immunoprecipitated using either 51.1 or 4A4 antibody followed by incubation with Protein G argarose beads (EMD Biosciences). Resulting IP products were subjected to immunoblot analysis.

Immunolocalization: tTA HeLa cells grown on coverslips were fixed with ice-cold acetone for 10 minutes followed by ice-cold methanol for 2 minutes. Cells were then washed with 1X PBS and incubated with primary antibody at room temperature for 1 hour. After three washes with 1X PBST, cells were incubated with secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen) at room temperature for 1 hour. Samples were imaged with a Zeiss TIRF microscope. For live cell imaging, $8X10^4$ BSC-1 cells stably expressing clathrin-light-chain-eGFP were seeded in 35 mm dish and transfected with either myc-epsin1-mCherry or myc-epsin1 ΔUIM-mCherry. Samples were visualized with the Zeiss TIRF scope.

siRNA-mediated knockdown: $3.1X10^5$ tTA HeLa cells were plated in 6-well plate and transfected with siRNA on day 1 and day 2. For internalization assay, cells were then infected with CD8-LDLR expressing adenovirus on day 3 for additional 18 hours. Knockdown siRNAs were for each target include: ARH,

AACAGCATGATTCTGACAGGGTTGG; CHC, TAATCCAATTCGAAGACCAAT; Epsin1, GGAAGACGCCGGAGTCATT; Dab2, AGGTTGGAACCAGCCTTCACCCTTT. Each siRNA was obtained from Invitrogen. Silencer negative control siRNA (#1) was from Ambion. The extent of expression knockdown was evaluated by immunoblot (Sup. Figure 2-6).

Internalization assay: For quantitative uptake assays, tTA HeLa cells were resuspended in DMEM/10%FBS with CD8 mAb (51.1) followed by incubation of ^{125}I-Protein A (Perkin Elmer) in DMEM/0.5% BSA at 4°C. Cells were transferred to a 37°C water bath to allow for internalization for each indicated time points. Internalization was stopped by moving cells to 4°C. Surface-bound ligands were stripped by acid wash (0.2 M acetic acid, 0.5 M NaCl). The amount of internalized CD8-LDLR was determined by γ counting and expressed as a percentage of total surface-bound γ count.

For epifluorescence analysis, tTA HeLa cells grown on coverslips were transfected with either control or indicated siRNA. After 70 hours of knockdown, cells were incubated with 10 µg/mL Dil-labeled LDL (Biomedical Technology Inc.) and 5µg/mL Alexa Fluor-488-labeled transferrin (Invitrogen) at 37°C for 20 minutes. Coverslips were then transferred to ice, washed with 1X PBS once and fixed with ice-cold 3.7% formaldehyde. Cells were mounted and imaged by epifluorescence using a Zeiss Axio

Imager M1 and captured with a monochrome Jenoptik CCD camera. Images were imported, cropped and illustrated using the Adobe Creative Suite CS3.

Figure Legends

Figure 2-1: **Depleting endocytic machinery phenocopies *lrp-1(lf)* molting defects.**

Wild-type *C. elegans* animals (A) fail to molt when fed *chc-1* RNAi (B), similar to *lrp-1(ku156)* animals (C). *lrp-1(ku156)* molting defects and animal viability is rescued with an *lrp-1::gfp* transgene (*eqIs1*, D). LRP-1::GFP can be detected along the anterior-posterior axis within hyp7 (E). Arrowheads indicate unshed cuticle of animals exhibiting molting defects. scale bar = 20 μm.

Figure 2-2: **Distribution of genes identified using the two-step LRP-1::GFP transport screen.**

A) Venn diagram indicating the number of unique and overlapping genes identified from *C. elegans* screens for genes involved in molting (Frand *et al.*, 2005) and yolk uptake in oocytes (Balklava *et al.*, 2007). Numbers in parentheses indicate the total number of genes identified. B) Functional grouping of genes identified in the screen (see Sup. Table 2).

Figure 2-3: **LRP-1::GFP accumulates on the apical surface of hyp7 in *epn-1* RNAi-fed animals.**

LRP-1::GFP was analyzed by epifluorescence in LH191 (control) animals fed with RNAi directed against each indicated gene. scale bar = 10 μm

Figure 2-4: **LDLR endocytosis requires epsin1.**

tTA HeLa cells were transfected with control siRNA or siRNAs targeting the indicated gene. LDL uptake was then qualitatively evaluated by epifluorescence (A) or measured by tracking internalization of a CD8-LDLR chimera (B,C). A) Control cells or those depleted of CHC or epsin1 were incubated with FITC-labeled transferrin (5μg/mL) and Dil-labeled LDL (10μg/mL) for 20 minutes at 37°C to analyze ligand uptake. Panels include cells (arrowheads) we conclude were not depleted of either CHC or epsin1, thus serving as internal controls for ligand uptake. LDLR chimera uptake was qualitatively measured by 51.1 mAb uptake using epifluorescence in tTA HeLa cells treated with control siRNA (B) or siRNA targeting epsin1 (C). D,E) tTA HeLa cells were treated with siRNA targeting the indicated factor and infected with CD8-LDLR adenovirus to quantitatively measure internalization kinetics. Control and epsin1 data were split into two graphs to facilitate visualization. Uptake experiments for each factor were performed in tandem. scale bar = 20 μm, error bars indicate ±SD of at least three independent experiments.

Figure 2-5: **Epsin1 promotes FxNPxY-independent LDLR internalization.**

tTA HeLa cells were infected with adenovirus encoding the indicated CD8-LDLR chimera and internalization was quantitatively measured and compared to Dab2 or epsin1 depleted cells. Error bars indicate ±SD of at least three independent experiments.

Figure 2-6: C. elegans **EPN-1 UIM is essential for receptor internalization.**

A) Diagram indicating the domain structure of EPN-1 and the constructs used for the functional dissection in *eqIs1; epn-1(tm3357)* animals. The associated table indicates the capacity of each EPN-1 mutant form to rescue *epn-1(tm3357)* lethality. The number of independent animal lines and LRP-1::GFP localization data for each line is also indicated. B-G) LRP-1::GFP localization analysis in hyp7 by TIRF microscopy for each indicated line or RNAi-fed LH191 animal. Animals expressing EPN-1$^{\Delta UIM}$ rescue viability defects, but LRP-1::GFP accumulates on the surface of hyp7, like that for *dab-1(gk291)* animals (F) and *epn-1* RNAi-fed LH191 (G). scale bar = 20 μm

Figure 2-7: **The UIM stabilizes protein complexes containing epsin1 and LDLR.**

A) Diagram illustrating the mammalian epsin1 domain structure and constructs used for immunoprecipitation analysis. B) tTA HeLa cells expressing the indicated recombinant protein were lysed and subjected to co-immunoprecipitation with the 51.1 mAb, which recognizes the extracellular CD8 epitope of CD8-LDLR. Bottom two panels indicate

CD8-LDLR and epsin1 expression levels. (C) CD8-LDLR-expressing tTA HeLa cells were transfected with plasmid encoding each epsin1 constructs shown in (A). CD8-LDLR was then immunoprecipitated with the mAb 4A4 antibody that recognizes the LDLR cytoplasmic tail. Protein samples were then evaluated for epsin1 binding by immunoblot analysis.

Figure 2-8: **Epsin1 promotes HIC-independent LDLR uptake.**

A) Schematic amino acid sequence of the human cytoplasmic domain (K790 to A839) for the CD8-LDLR chimera (WT) and the various mutant forms tested. Numbers indicate amino acid positions relative to full-length human LDLR. B-E) tTA HeLa cells were infected with adenovirus encoding the indicated CD8-LDLR chimera and internalization was quantitatively measured and compared to cells treated with control siRNA or siRNA targeting epsin1. Error bars indicate ± SD of 3 or more independent experiments.

Supplemental Figure Legends

Sup. Figure 2-1: **LRP-1::GFP accumulates at the apical surface of hyp7 in *dab-1* null animals.**

In vivo epifluorescence analysis of LRP-1::GFP in LH191 and *dab-1(gk291)* transgenic animals. For images in the left and middle panels, samples were focused at the apical surface of hyp7, while an equatorial focal plane was used in the right panel. Equatorial images reveal autofluorescent gut granules (indicated by arrowheads) that do not reflect LRP-1::GFP fluorescence. scale bar = 20 μm

Sup. Figure 2-2: **CD8-LDLR$^{\Delta NPxY}$ internalization occurs independently of dimerization with endogenous LDLR.**

Control CHO cells or CHO *ldlA* cells were infected with adenovirus encoding CD8-LDLR or CD8-LDLR$^{\Delta NPxY}$ and internalization kinetics were measured (see Materials and Methods). Error bars indicate ± SD of three independent experiments.

Sup. Figure 2-3: **Genomic structure of the *epn-1* gene.**

tm3357 is a 329 base pair deletion that removes sequences between nucleotides 606-934 and introduces seven base pairs of *non-epn-1* sequence (GATATAT). The deletion starts in the second intron, removing most of the second intron through the beginning of the

fourth exon. The deletion and insertion mutation is predicted to cause a frame shift, resulting in a premature stop to produce a truncated product, if stable, to contain only the ENTH domain. Open boxes represent exons while inverted 'V's represent introns.

Sup. Figure 2-4: **Epsin1 recruitment to clathrin-coated pits is independent of the UIM.**

A-C) Co-immunolocalization analysis of endogenous LDLR (A, red) and epsin1 (B, Green) in tTA HeLa cells. D-I) BSC-1 cells stably expressing clathrin light chain (CLC) fused to eGFP (CLC-GFP, green) were transfected with plasmid encoding WT epsin1 or epsin1ΔUIM fused to mCherry (mChr, red). Fixed cell samples (A-C) or live cells (D-I) were imaged by TIRF microscopy. Yellow indicates colocalization in overlay panels C, F, and I. scale bar = 10 μm

Sup. Figure 2-5: **CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization occurs via a clathrin-independent mechanism.**

tTA HeLa cells were treated with control siRNA or siRNAs targeting clathrin heavy chain (CHC KD). Cells were then infected with adenovirus encoding CD8-LDLR or a mutant CD8-LDLR form lacking functional NPXY or HIC motifs (ΔNPXY;HIC-mut). Internalization kinetics were then evaluated (see Materials and Methods). Error bars reflect ± SD of 5 independent experiments.

Sup. Figure 2-6: **siRNA-mediated knockdown efficiency evaluated by immunoblot analysis.**

tTA HeLa cells were treated with the indicated siRNA (see Materials and Methods). Cell lysates were then analyzed by immunoblot to determine the extent of expression knockdown. E7 was used to detect β-tubulin, which served as a protein loading control.

Supplemental Tables

Sup. Table 1

Identified genes that altered LRP-1::GFP localization when depleted by RNAi, organized by chromosome number.

Sup. Table 2

Identified genes that altered LRP-1::GFP localization when depleted by RNAi, organized by functional group.

Supplemental tables are available for download at Molecular Biology of the Cell website: http://www.molbiolcell.org/content/24/3/308/suppl/DC1

Figures

Figure 2-1 Depleting endocytic machinery phenocopies *lrp-1*(*lf*) molting defects

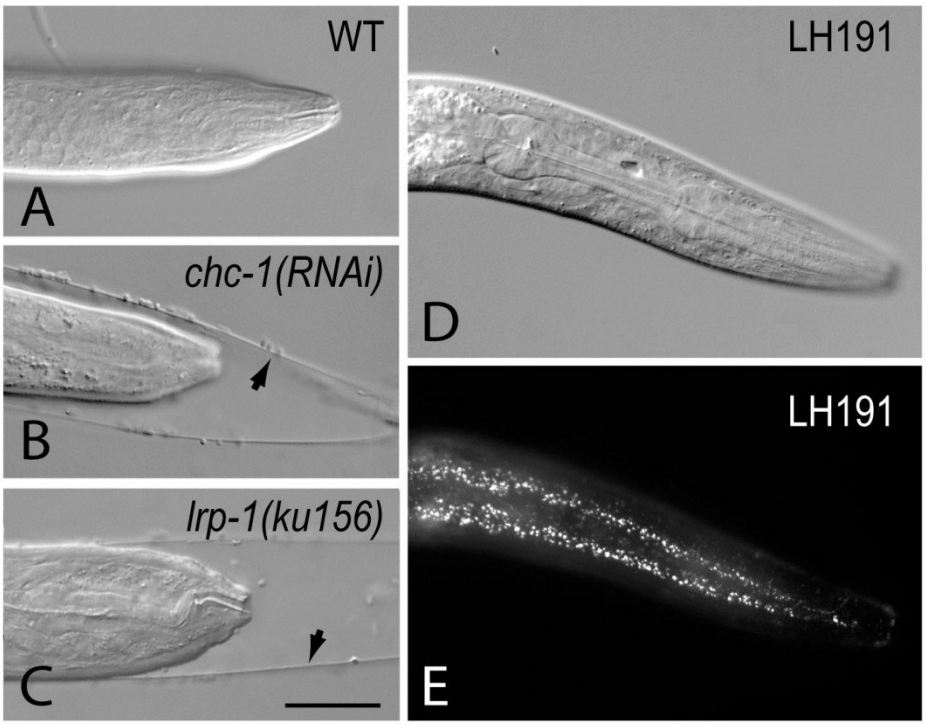


Figure 2-2 Distribution of genes identified using the two-step LRP-1::GFP transport screen

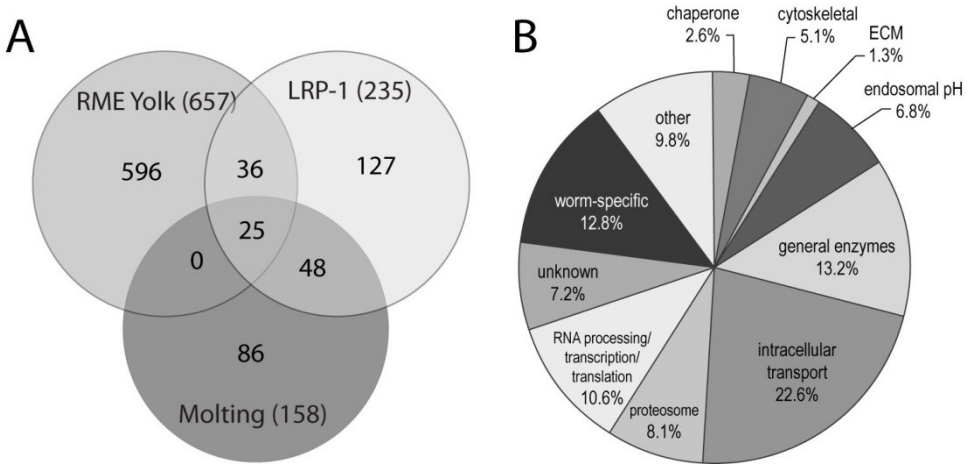


Figure 2-3 LRP-1::GFP accumulates on the apical surface of hyp7 in *epn-1* RNAi-fed animals

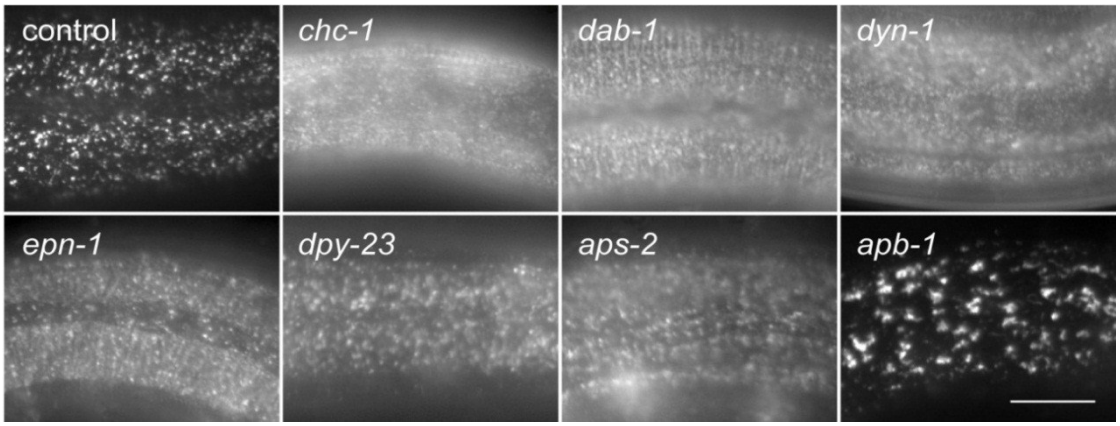


Figure 2-4 LDLR endocytosis requires epsin1

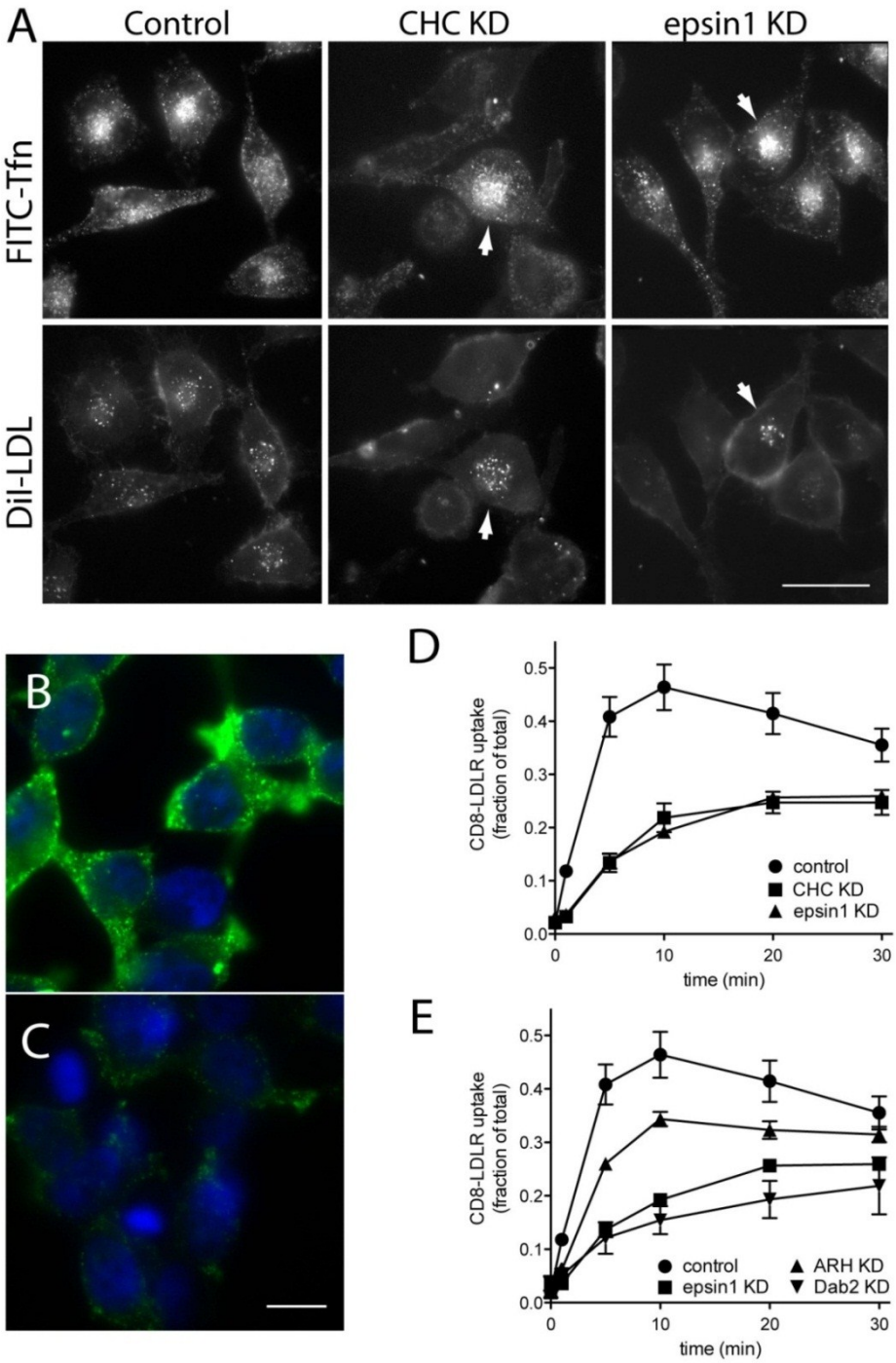


Figure 2-5 Epsin1 promotes FxNPxY-independent LDLR internalization

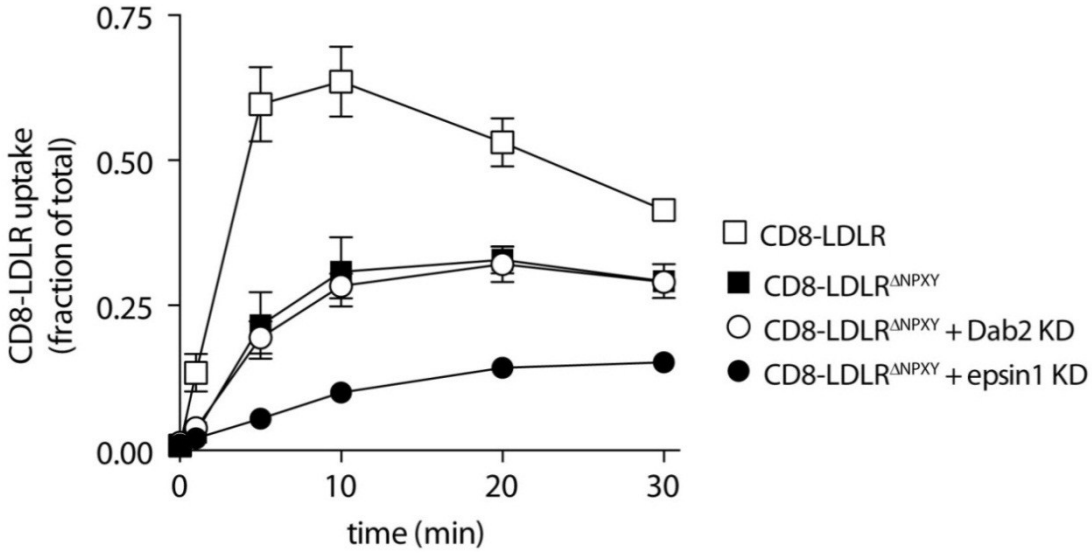


Figure 2-6 *C. elegans* EPN-1 UIM is essential for receptor internalization

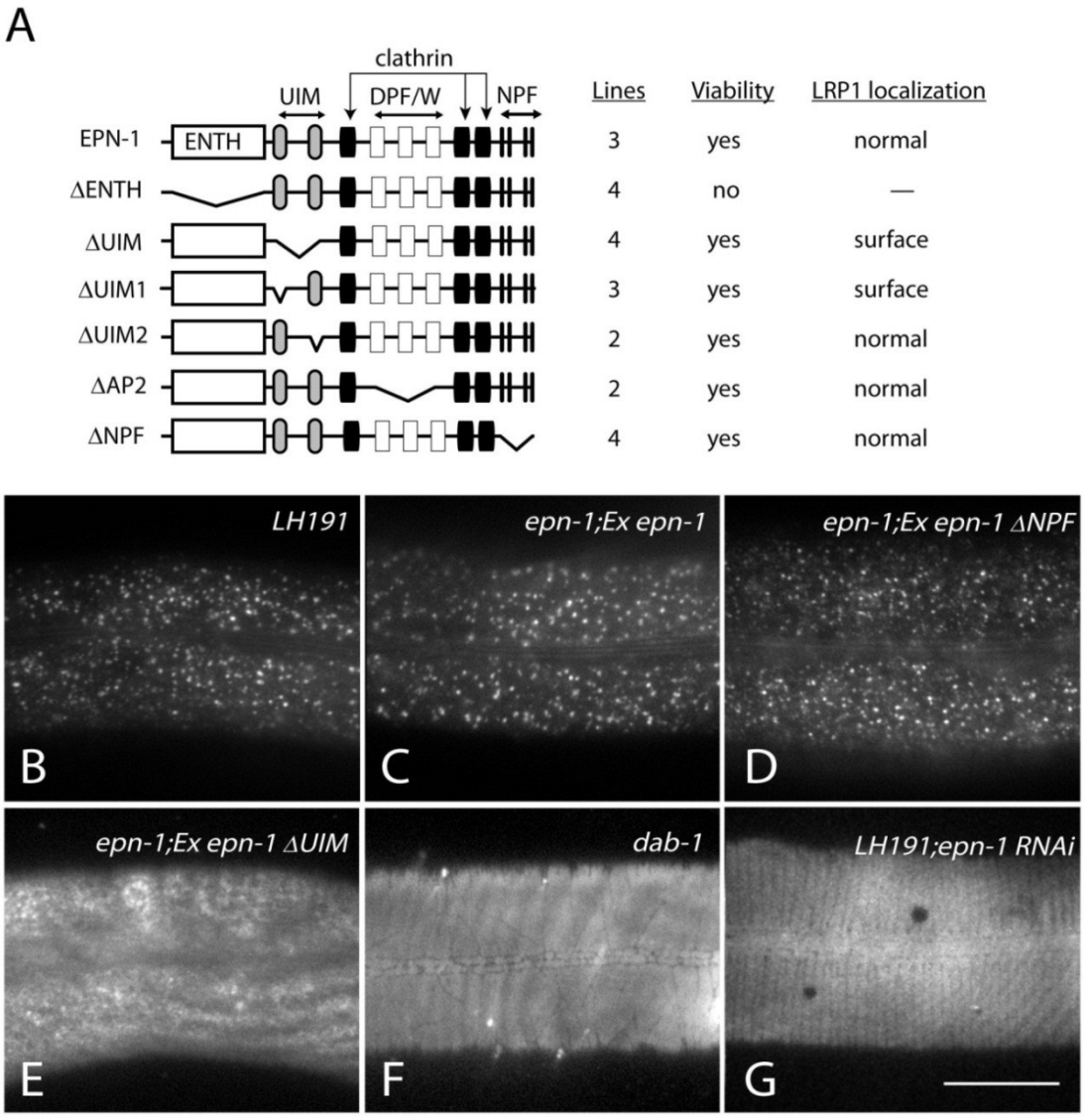


Figure 2-7 The UIM stabilizes protein complexes containing epsin1 and LDLR

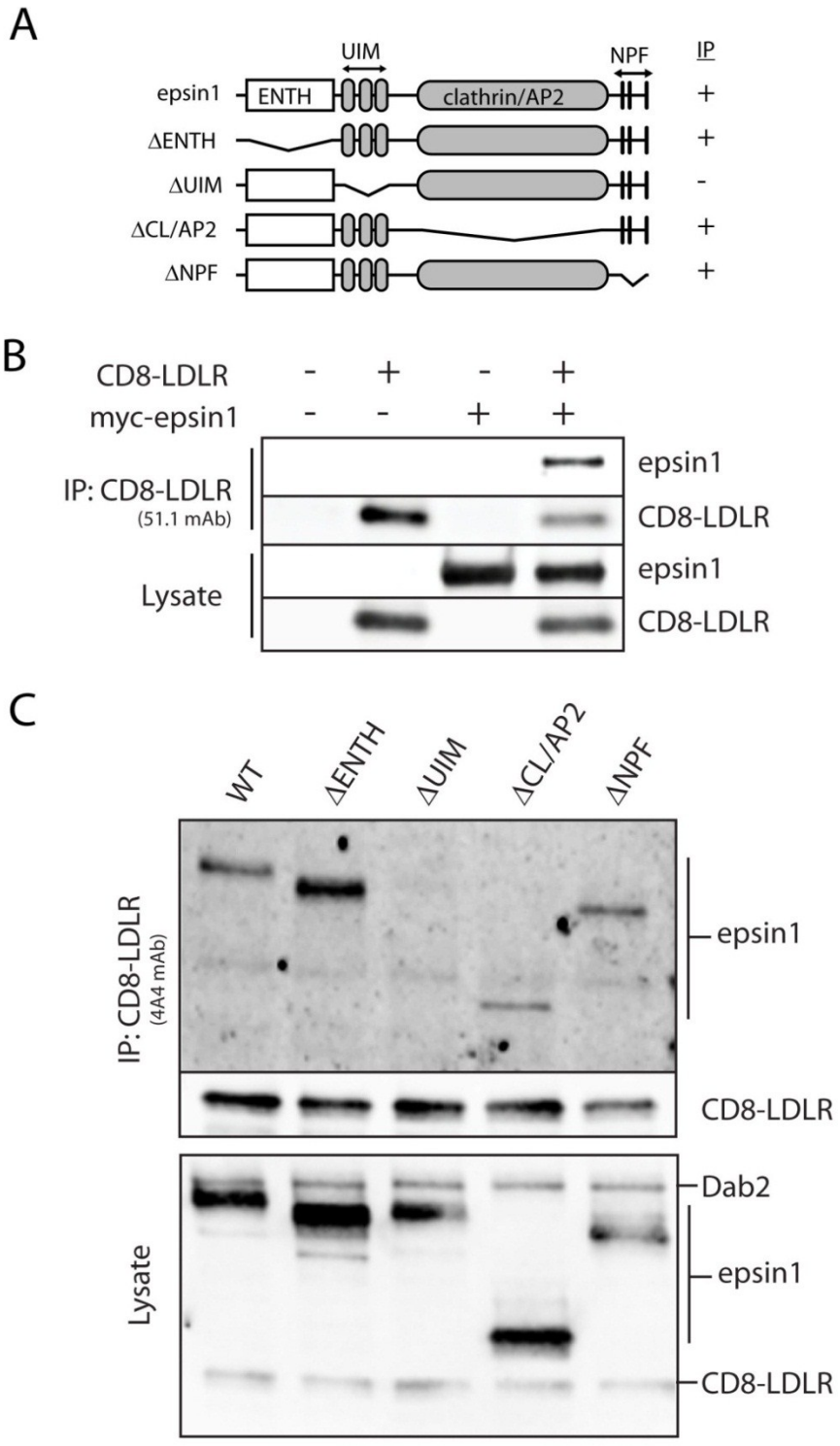
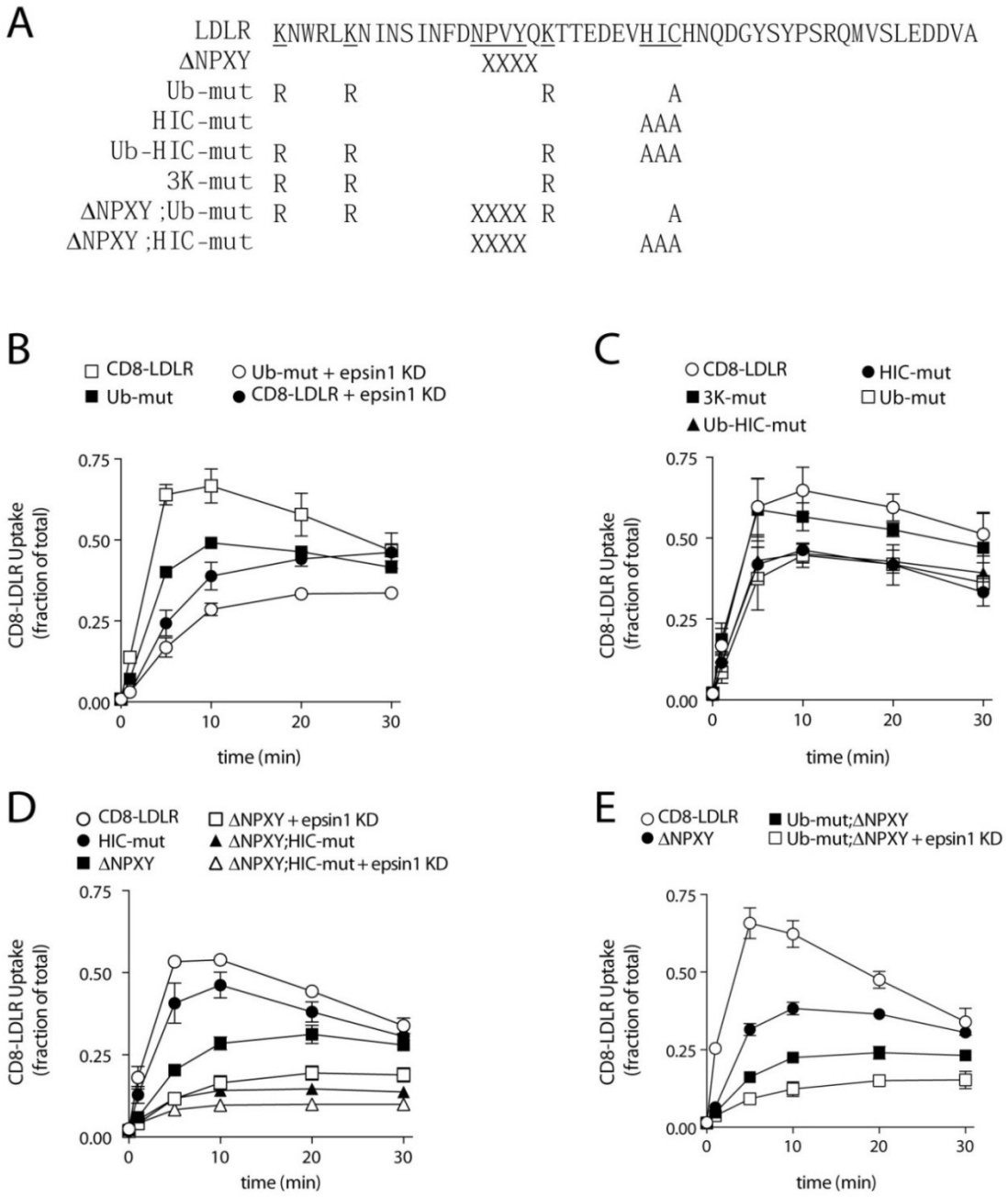
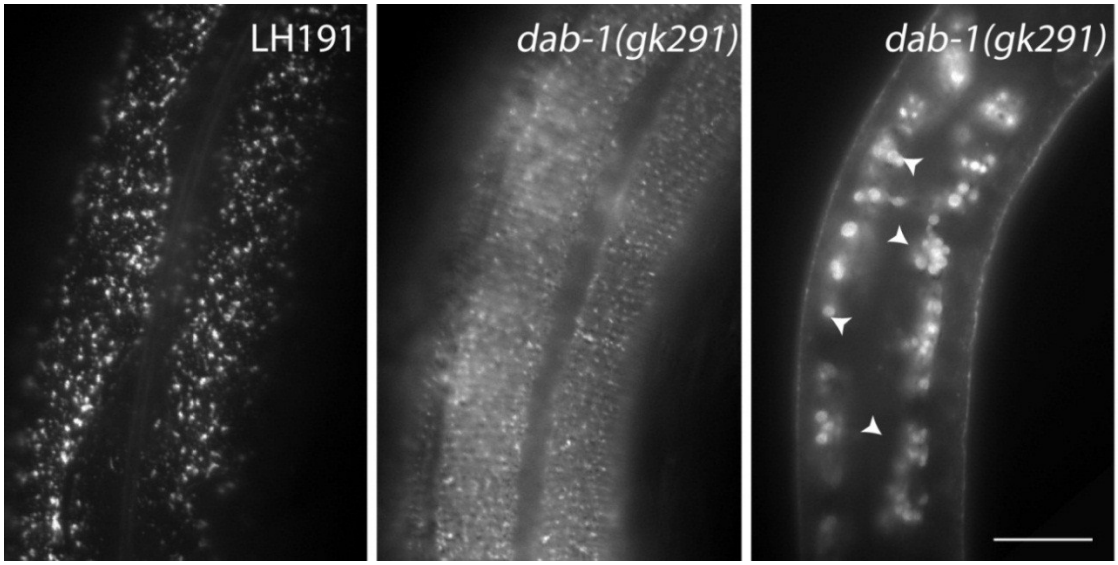


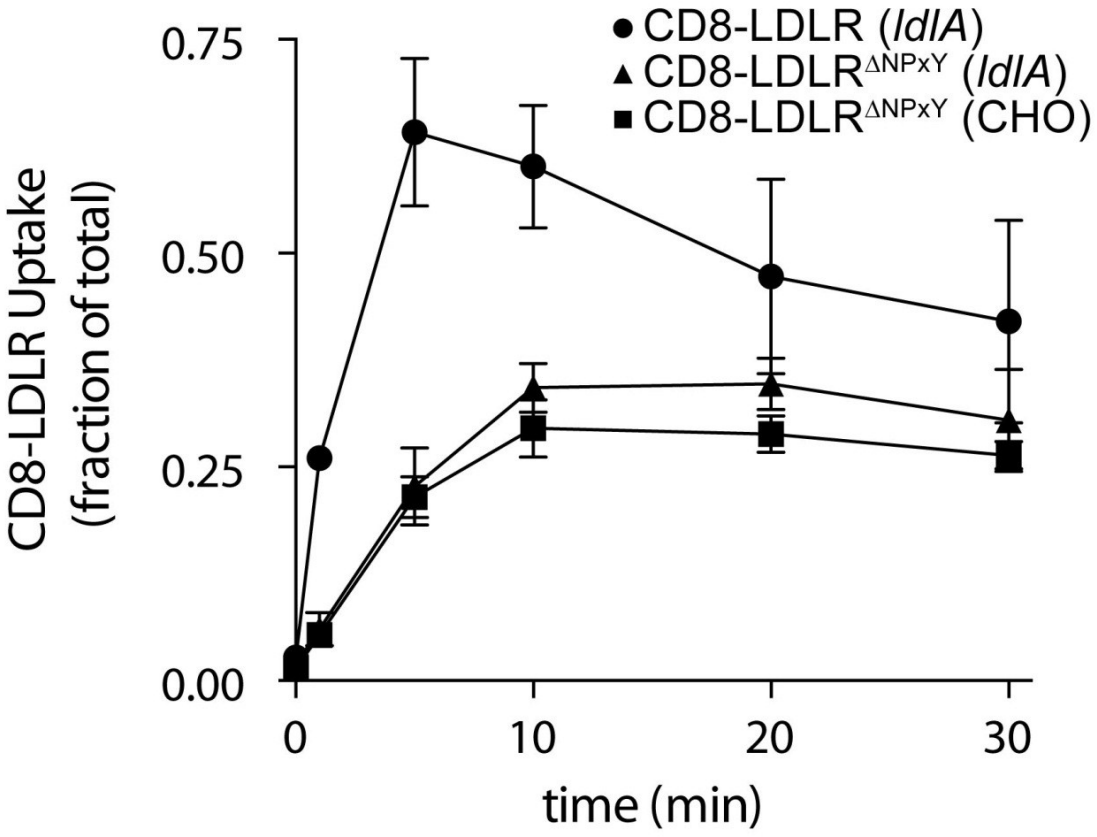
Figure 2-8 Epsin1 promotes HIC-independent LDLR uptake



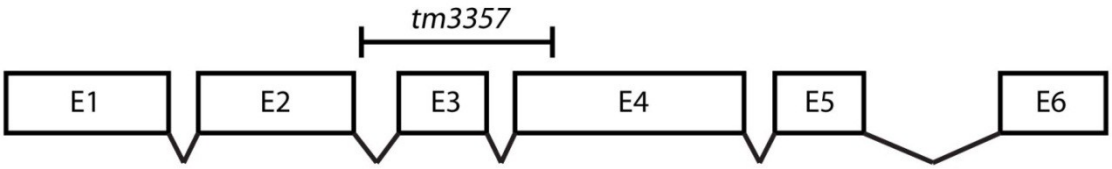
Sup. Figure 2-1 LRP-1::GFP accumulates at the apical surface of hyp7 in *dab-1* null animals



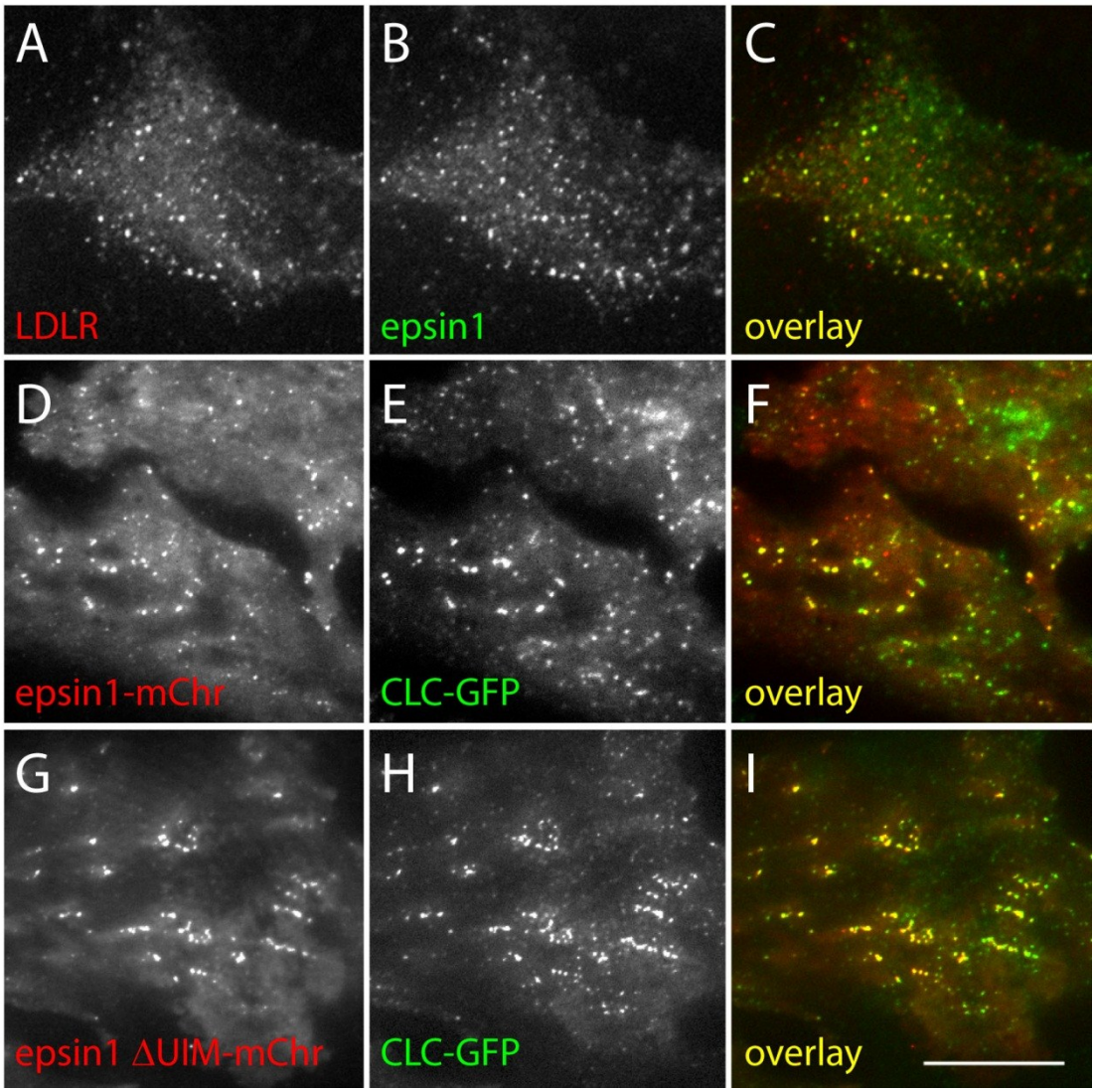
Sup. Figure 2-2 CD8-LDLR$^{\Delta NPxY}$ internalization occurs independently of dimerization with endogenous LDLR



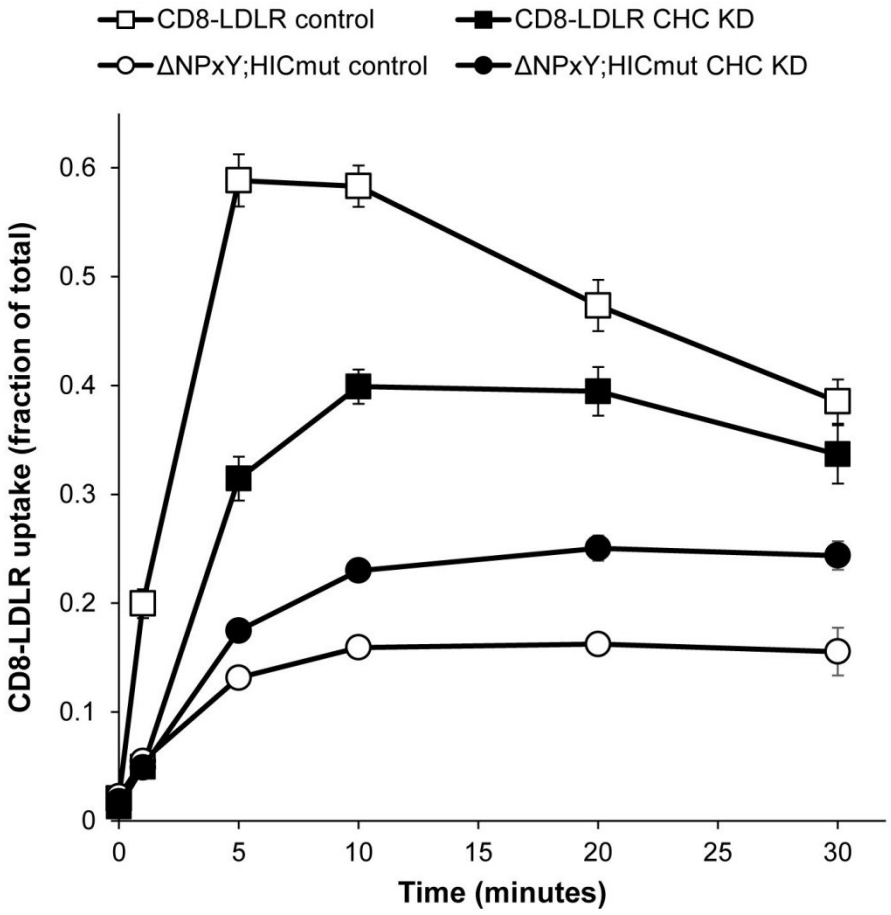
Sup. Figure 2-3 Genomic structure of the *epn-1* gene



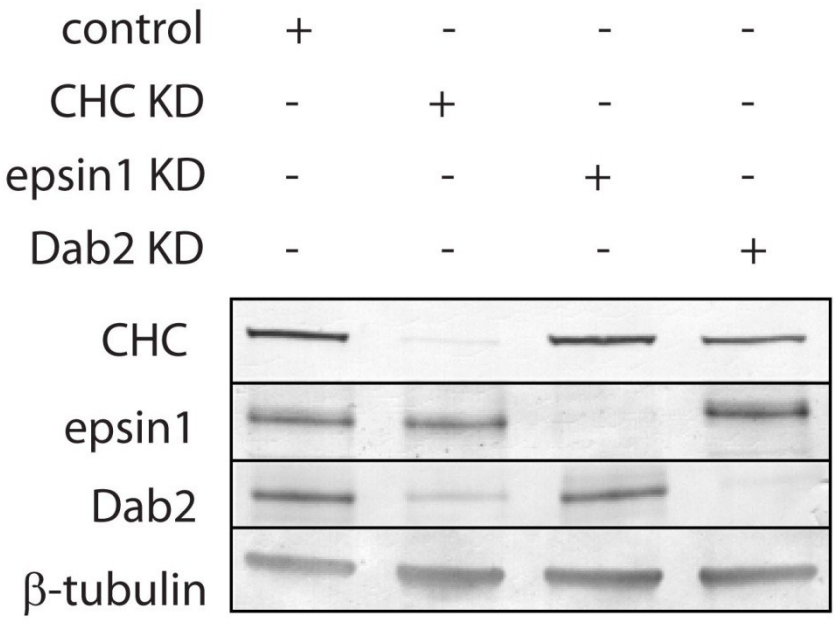
Sup. Figure 2-4 Epsin1 recruitment to clathrin-coated pits is independent of the UIM



Sup. Figure 2-5 CD8-LDLR$^{\Delta NPXY;HIC\text{-}mut}$ internalization occurs via a clathrin-independent mechanism



Sup. Figure 2-6 siRNA-mediated knockdown efficiency evaluated by immunoblot analysis



Author Contribution Statement

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Authors: Yuan-Lin Kang, John Yochem, Leslie Bell, Erika B. Sorensen, Lihsia Chen, Sean D. Conner

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Author contributions:

Y.L.K. designed research, conducted experiments, analyzed data and wrote the paper.

J.Y. generated transgenic *C. elegans* line: LH191. J.Y. and L.B. performed whole genome RNAi screen in *C. elegans*.

E.B.S. performed experiments on CHC and epsin1 knockdown on Dil-LDL and FITC-transferrin uptake assay in mammalian cells.

L.C. and S.D.C. designed research, analyzed data, supervised the project and wrote the paper.

Chapter Three: Molecular dissection of EPN-1 UIMs function in LRP-1 internalization

Summary

Epsin was first revealed as an endocytic adaptor for LDLR and UIMs of epsin are critical for LDLR uptake (chapter two). However, the hypothesis that epsin directly binds to ubiquitinated LDLR during endocytosis seems unlikely because epsin knockdown reduces the internalization of ubiquitination-impaired LDLR. The alternative mechanisms include that epsin UIMs bind to other ubiquitinated protein or acquire undefined functions to mediate LDLR uptake. I have shown that the first of two UIMs in EPN-1 is critical for LRP-1 uptake while UIM2 is dispensable in *C. elegans*. Next, I assessed whether mutated EPN-1 UIM1that cannot bind to ubiquitin affects LRP-1 endocytosis. To do so, ubiquitin-binding impaired UIM1 was generated and used to examine how LRP-1 endocytosis is affected. Our preliminary results reveal that the ability of UIM1 to engage ubiquitin is not essential for EPN-1 function in LRP-1 uptake, suggesting alternative roles for epsin UIMs.

Introduction

Epsin functions as an endocytic adaptor

Epsin1 acts as an endocytic adaptor characterized by the modular organization and is evolutionarily conserved from yeast, *C. elegans*, *Drosophila* and mammals. The amino-terminal encodes the epsin NH_2-terminal homology (ENTH) domain, which has high affinity for phosphatidylinositol-4, 5-bisphosphate (PIP2), a component of plasma membrane. Next are three UIMs, which can bind to ubiquitinated proteins. The central part of Epsin1 is comprised of two clathrin-binding motifs and eight DPF/W motifs that engage with clathrin and AP2, respectively (Rosenthal *et al.*, 1999; Drake *et al.*, 2000). The C-terminus of Epsin1 contains three NPF motifs that can interact with EH-domain containing proteins, such as Eps15 (Chen *et al.*, 1998).

Monoubiquitination and Ubiquitin-interacting-Motif (UIM) motif

Ubiquitination has long been described as a post-translational modification to target proteins with a chain of covalently bound ubiquitin units to the proteasome for degradation. For the past decade, several lines of evidence uncovered that monoubiquitination serves as a regulatory signal in multiple cellular processes. One of the examples is that monoubiquitination serves as an endocytic signal for the endocytosis of various transmembrane proteins, such as G-protein-coupled receptors (Hicke, 1999; Shih *et al.*, 2000).

Monoubiquitinated proteins can be recognized by a wide range of adaptor proteins, such as Epsin, Eps15 , HRS and STAM, all of which contain a conserved protein-protein interaction motif, ubiquitin-interacting-motif (UIM) (Hofmann and Falquet, 2001). A variety of cellular processes are facilitated by interaction between UIM-containing adaptors and monoubiquitinated proteins. Endocytosis of epidermal growth factor receptor (EGFR) is a well-characterized example to demonstrate the requirement of UIM-ubiquitin interaction in receptor internalization. Ubiquitinated EGFR recruits Epsin and Eps15 which directly interact with the ubiquitin moiety attached on the EGFR (Wendland, 2002). The assembly of the clathrin-coated pit is then completed by Epsin and Eps15 recruiting AP2 and clathrin (Polo *et al.*, 2003; Hawryluk *et al.*, 2006; Hirano *et al.*, 2006; Kazazic *et al.*, 2009).

From an evolutionary perspective, the yeast Epsin, Ent1, UIM of which is required for receptor endocytosis (Shih *et al.*, 2002; Aguilar *et al.*, 2003). Also, analyses of *Drosophila* Epsin, Liquid facets (*lqf*), showed a requirement of its UIM for regulating Notch signaling pathway (Tian *et al.*, 2004; Wang and Struhl, 2004; Xie *et al.*, 2012). Together, receptor endocytosis mediated by the association between ubiquitinated receptor and UIM-containing endocytic adaptors is an evolutionarily conserved mechanism employed by a wide range of organisms.

Understanding the regulatory role of Epsin1 UIM in LDLR internalization

Our observations in both worms and mammalian cells converge at the idea that epsin1 mediates LDLR endocytosis in a UIM-dependent manner. However, my results showed

the LDLR ubiquitination is not required for epsin1 function in LDLR internalization. Possible interpretations as to epsin1 UIM function in LDLR endocytosis include: 1) there is an intermediate ubiquitinated adaptor recognized by epsin1 to link to LDLR; 2) epsin1 UIM acquires a role other than simply engaging ubiquitinated LDLR to be a part of LDLR endocytic assembly.

I showed the requirement of EPN-1 UIM1 for LRP-1 internalization in chapter two. In this chapter, I tested whether the ability to bind ubiquitin of EPN-1 UIM1 is necessary for LRP-1 uptake by a series of mutational analyses in *C. elegans*.

Results

The regulatory role of EPN-1 UIM1 in LRP-1 internalization in *C. elegans*

In chapter two, I showed that deletion of EPN-1 UIM1 causes impaired LRP-1 internalization. To analyze the function of EPN-1 UIM1 in LRP-1 uptake, I aimed to test whether the ability of worm EPN-1 UIM1 to bind ubiquitin plays a regulatory role in this process (Figure 3-1). Based on the study reported by Xie *et al.*, the first three Glu (E) residues of *Drosophila* epsin UIM1 were shown as critical for ubiquitin binding and epsin function in eye development. Corresponding E residues in EPN-1 UIM1 were mutated to Ala (A) residues, Glu (E) 201, 202, 202 to Ala (A) (Figure 3-1). The mutated EPN-1 was used to examine the ability to rescue *epn-1(tm3357)* caused LRP-1 trafficking defects. My results showed that *epn-1* animals expressing these mutations are healthy and indistinguishable from WT animals. Importantly, animals expressing EPN-1 UIM1$^{EEE\rightarrow AAA}$ showed apparent WT LRP-1 distribution (Figure 3-2A, B), in contrast to surface LRP-1 accumulation in animals expressing EPN-1ΔUIM1. These results raised the possibilities that 1) ubiquitin binding is not required or 2) the presence of UIM2 may compensate for EPN-1 function in LRP-1 internalization. To distinguish between these possibilities, I tested the ability of EPN-1 UIM1$^{EEE\rightarrow AAA}$; ΔUIM2 to rescue *epn-1(tm3357)* caused LRP-1 trafficking defects (Figure 3-1). Animals expressing EPN-1 UIM1$^{EEE\rightarrow AAA}$; ΔUIM2 did not show surface accumulation of LRP-1, indicating apparent WT LRP-1 internalization. But interestingly, adult animals expressing EPN-1 UIM1$^{EEE\rightarrow AAA}$; ΔUIM2 exhibited dumpy phenotype and abnormal

intracellular LRP-1 accumulation (Figure 3-2C), similar to that in *epn-1(tm3357)* animals (Figure 3-2D), indicating that while UIM2 does not play a role in LRP-1 endocytosis, UIM2 is likely to regulate another LRP-1 intracellular trafficking step.

Discussions

I previously showed that LDLR ubiquitination is not required for epsin1 UIM function in LDLR internalization, raising the possibility that epsin1 recognizes an intermediate ubiquitinated endocytic adaptor. In support with the hypothesis, my *in vitro* GST pull-down results indicated that there is no strong direct interaction between epsin1 and LDLR. One compelling candidate is Eps15, an endocytic adaptor known to be ubiquitinated in the endocytic assembly (Klapisz *et al.*, 2002; Polo *et al.*, 2002). To assess whether epsin1 functions via binding to ubiquitinated Eps15, I measured LDLR internalization rate following Eps15 knockdown. My preliminary result indicated that Eps15 takes no part in LDLR internalization (data not shown). To explore other potential candidates, systematic proteomic analysis can be performed by analyzing the Co-IP product of LDLR. The addition of ubiquitin to the protein will then be assessed to evaluate the possibility that epsin1 targets to the ubiquitinated adaptor in the process of LDLR endocytosis.

Analyses of UIM structural characteristics for ubiquitin interaction

EPN-1 UIM1$^{EEE\rightarrow AAA}$ used as ubiquitin-binding impaired mutant did not alter LRP-1 internalization. The conserved UIM protein sequences may correlate with conserved function in engaging ubiquitin; however, the amino acids in UIM crucial for ubiquitin-binding remain debatable, raising the possibility that EPN-1 UIM1$^{EEE\rightarrow AAA}$ may not entirely lose the ability to bind ubiquitin.

Several groups have made efforts to resolve the conformational determinant(s) in UIM essential for ubiquitin interaction. First, the structures of yeast Vps27 UIMs were resolved and ten residues were suggested as important for engaging ubiquitin (labeled with # in Figure 3-3A) (Swanson *et al.*, 2003). Second, protein structure analysis of Hrs UIM showed that leucine (Leu) residues (in bold) are critical to ubiquitin binding, in particular Leu263 and Leu267 (in red) relative to Leu265 and Leu269 (in black, Figure 3-3B). Moreover, they also demonstrated that Hrs UIM is capable of binding to two ubiquitins via two groups of residues (Figure 3-3B) (Shekhtman and Cowburn, 2002; Hirano *et al.*, 2006). To resolve whether EPN-1 UIM1$^{EEE\rightarrow AAA}$ mutant indeed fails to interact with ubiquitin, *in vitro* ubiquitin binding and more mutational analyses can be performed (Figure 3-3C).

Diverse regulatory roles of epsin UIMs

Multiple studies show that epsin1 promotes various receptor endocytosis via direct UIMs binding to ubiquitinated proteins, such as EGFR, Insulin receptor, Protease-activated Receptor-1 (PAR-1) and μ-opioid neuropeptide receptors (Sugiyama *et al.*, 2005; Kazazic *et al.*, 2009; Chen *et al.*, 2011; Henry *et al.*, 2012). However, our results suggest that ubiquitin binding is not likely to play a role in uptake of LDLR superfamily members. This suggests that UIM may have additional roles besides ubiquitin binding. Indeed, emerging evidence implicates that the UIM function is not limited to binding to the ubiquitin moiety of ubiquitinated proteins. Diverse biological activities ranging from receptor trafficking to protein degradation are regulated by a broad spectrum of

UIM-containing proteins. Alternative functions of UIM have been proposed and may provide explanations for epsin function in LDLR uptake. First, a yeast study is that the structural conformation of UIM could serve as a protein-protein interaction platform (Dores *et al.*, 2010). Second, one proposed model for the function of UIM-containing proteins is described as coupled monoubiquitination mechanism. UIM-containing adaptor proteins are often ubiquitinated which requires the presence of its own UIM. One of the most studied examples for coupled monoubiquitination mechanism is Eps15, with two UIMs harboring distinct functions. The Eps15 UIM2 interacts with ubiquitinated protein whereas the integrity of Eps15 UIM1 is critical for recruiting Nedd4 family of E3 ubiquitin ligase to catalyze the ubiquitination of Eps15 (Polo *et al.*, 2002; Woelk *et al.*, 2006).

The coupled monoubiquitination mechanism is possibly applicable to EPN-1 mediated LRP-1 internalization. Because epsin1 has been described to be ubiquitinated (Oldham *et al.*, 2002), the possible interpretation can be that EPN-1 UIM1 functions to recruit ubiquitin ligase for self-ubiquitination in order to enter the LRP-1 endocytic assembly, instead of binding to ubiquitinated cargo in the process of LRP-1 endocytosis.

The potential regulatory roles of Epsin1 UIM in LDLR internalization

My genetic analysis in worms and biochemical evidence in mammalian cells implicated a requirement of epsin UIM for regulating LDLR internalization. To evaluate the regulatory roles of epsin UIM, three potential mechanisms were proposed and investigated as illustrated in Figure 3-4. First, epsin recognizes the ubiquitin moiety of

ubiquitinated LDLR via the UIM (Figure 3-4A) to promote LDLR endocytosis. However, epsin1 knockdown further decreased the internalization of ubiquitination-impaired LDLR, suggesting that receptor ubiquitination is not required. Second, the possible indirect interaction between epsin and LDLR was implicated by *in vitro* GST binding and yeast two hybridization analyses. One possible mechanism is that epsin directly binds to the ubiquitinated intermediate protein (Figure 3-4B). On the other hand, epsin interacts with an intermediate protein that is not ubiquitinated (Figure 3-4C). Genetic analysis in worms suggested that the ability of ubiquitin-binding is not critical for EPN-1 function in LRP-1 internalization. The results I have accumulated support the last possibility (Figure 3-4C). While the nature of the interaction between epsin and LDLR has not been entirely understood, it will be critical to understand the endocytic signal(s) on the LDLR tail required for epsin interaction and identify the possible intermediate protein linking LDLR and epsin.

In summary, the efforts devoted to understanding the regulatory roles of worm EPN-1 UIMs will provide functional implications as to the mechanism underlying mammalian epsin-mediated LDLR internalization. In light of previous studies, the possible regulatory roles might be applicable for EPN-1 UIM1, including 1) ubiquitin binding; 2) ubiquitin ligase recruitment. By understanding the function of worm EPN-1 UIM1, it will be beneficial for determining the multi-functionalities of epsin UIMs on LDLR endocytosis.

Materials and Methods

Animal strains: C. elegans strains are grown on nematode growth medium plates at 20°C as described by (Brenner, 1974). The following alleles were used: *lrp-1(ku156)*, *dab-1(gk291)* and *epn-1(tm3357)*. Bristol N2 serves as the wild-type *C. elegans* strain. The following alleles were used:

LGI: *lrp-1(ku156)*.

LGX: *epn-1(tm3357), unc-7(35)*.

The *epn-1(tm3357)* strain was kindly provided by Dr. Shohei Mitani (National Bioresource Project at the Tokyo Women's Medical University School of Medicine). We obtained a twice-outcrossed *epn-1(tm3357)* strain from Dr. Erik Jorgensen (University of Utah) that we outcrossed seven additional times.

C. elegans expression vectors: epn-1 genomic DNA was kindly provided by Dr. Erik Jorgensen (University of Utah). All epn-1 expression constructs were cloned in the pGEM-3Zf(+)vector. *epn-1* wild type and deletion DNA was cloned using engineered BglII and NotI restriction sites. mCherry was inserted in frame at the 3' end of epn-1 just prior to the stop codon using engineered NotI and BamHI restriction sites.

Pepn-1::epn-1 UIM 1$^{EEE\rightarrow AAA}$*::mCherry* (*epn-1* genomic DNA, Glu 201, 202, 202 mutated to Ala)

Pepn-1::epn-1 UIM 1$^{EEE \rightarrow AAA}$*;* Δ*UIM2::mCherry* (*epn-1* genomic DNA, Glu 201, 202, 202 mutated to Ala, deletion from a.a. 218-232)

Generated C. elegans transgenic strains:

LH843: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1 UIM 1*$^{EEE \rightarrow AAA}$*::mCherry*

LH859: *lrp-1(ku156) eqIs1; epn-1(tm3357)*; *eqEx Pepn-1::epn-1 UIM 1*$^{EEE \rightarrow AAA}$*;* Δ*UIM2::mCherry*

For epifluorescence analysis, animals were mounted in 2% agarose pad and LRP-1::GFP distribution was imaged by epifluorescence using a Zeiss Axio Imager M1 and captured with a monochrome Jenoptik CCD camera. Images were imported, cropped and illustrated using the Adobe Creative Suite CS3.

Figure Legends

Figure 3-1 EPN-1 (*C. elegans*) and a serious of mutations in UIMs are depicted.

The number of transgenic lines expressing each EPN-1 variants, their abilities to rescue *epn-1(tm3357)* lethality and LRP-1 localization are described in the columns on the right. UIMs in blue possess wild type sequences while UIM1 in purple contains mutations of E→A as illustrated.

Figure 3-2 LRP-1::GFP distribution in animals expressing EPN-1 variants imaged by epifluorescence scope.

(A, B) LRP-1::GFP in wild type and EPN-1 UIM $1^{EEE\rightarrow AAA}$ expressing animals localizes to distinct puncta. (C) LRP-1::GFP in EPN-1 UIM $1^{EEE\rightarrow AAA}$; ΔUIM2 expressing animals exhibits apparent intracellular accumulation. (D) LRP-1::GFP in *epn-1(tm3357)* animals appears to accumulate in intracellular compartments. The scale bar indicates 20μm.

Figure 3-3 The amino acids in of various UIM-containing proteins required for ubiquitin interaction.

(A) Ten residues in yeast Vps27 are important for ubiquitin binding (labeled with "#" on top) (Swanson *et al.*, 2003). (B) Hirano *et al.* proposed double-sided ubiquitin binding

attributable to two sets of residues in Human Hrs UIM (depicted as sequence in red and green under the UIM full sequence). (C) The protein sequences of EPN-1 UIM1 and UIM2.

Figure 3-4 The potential regulatory roles of Epsin1 UIM in LDLR internalization.

Three functionalities of epsin UIM are illustrated as potential mechanisms underlying LDLR internalization. (A) Direct interaction between UIM of epsin and ubiquitinated LDL. (B) Indirect interaction between UIM of epsin and LDLR via an ubiquitinated intermediated LDLR. (C) Indirect interaction between UIM of epsin and LDLR via an intermediated protein that is not ubiquitinated.

Figures

Figure 3-1 EPN-1 (*C. elegans*) and a serious of mutations in UIMs are depicted

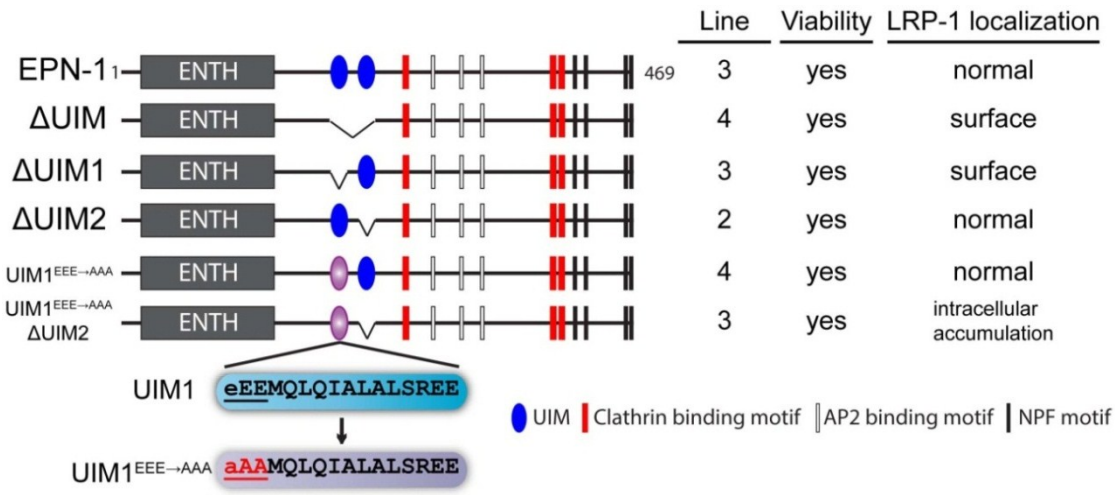


Figure 3-2 LRP-1::GFP distribution in animals expressing EPN-1 variants imaged by epifluorescence scope

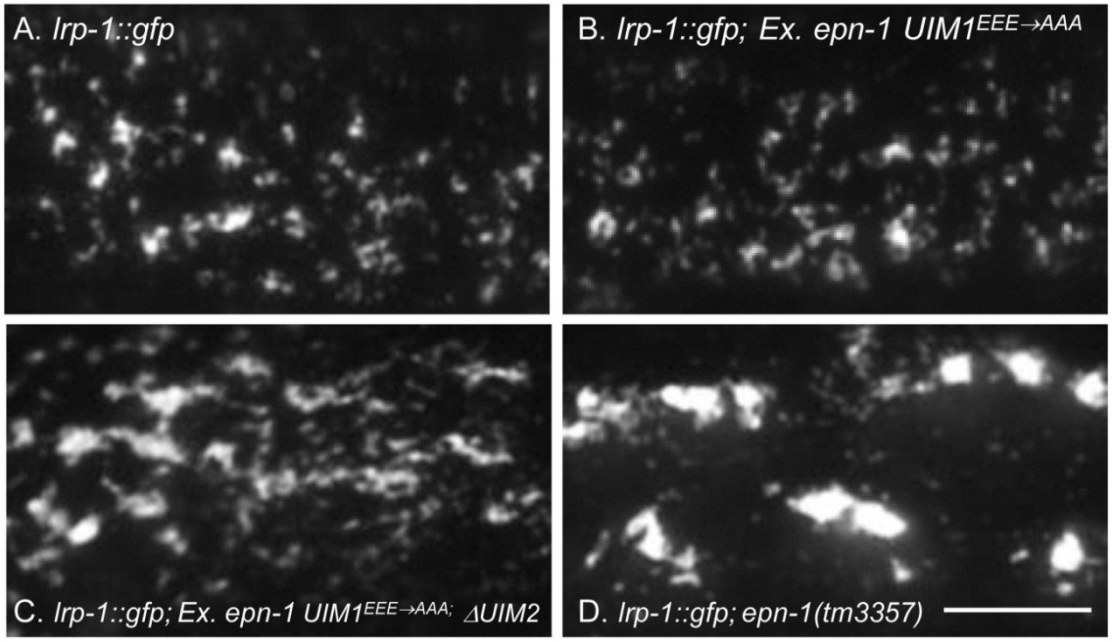


Figure 3-3 The amino acids in of various UIM-containing proteins required for ubiquitin interaction

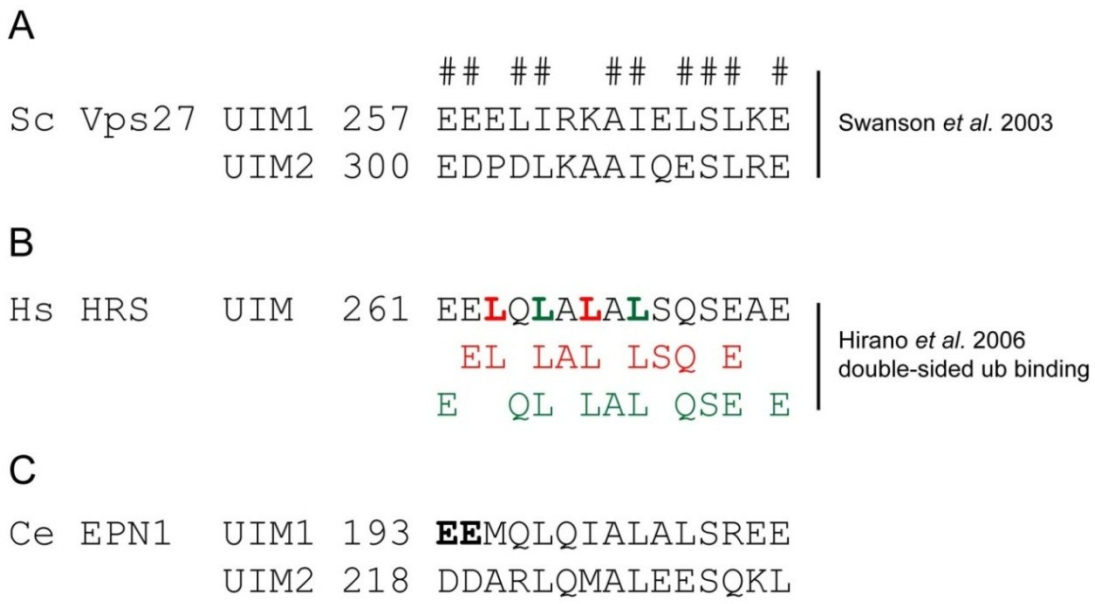
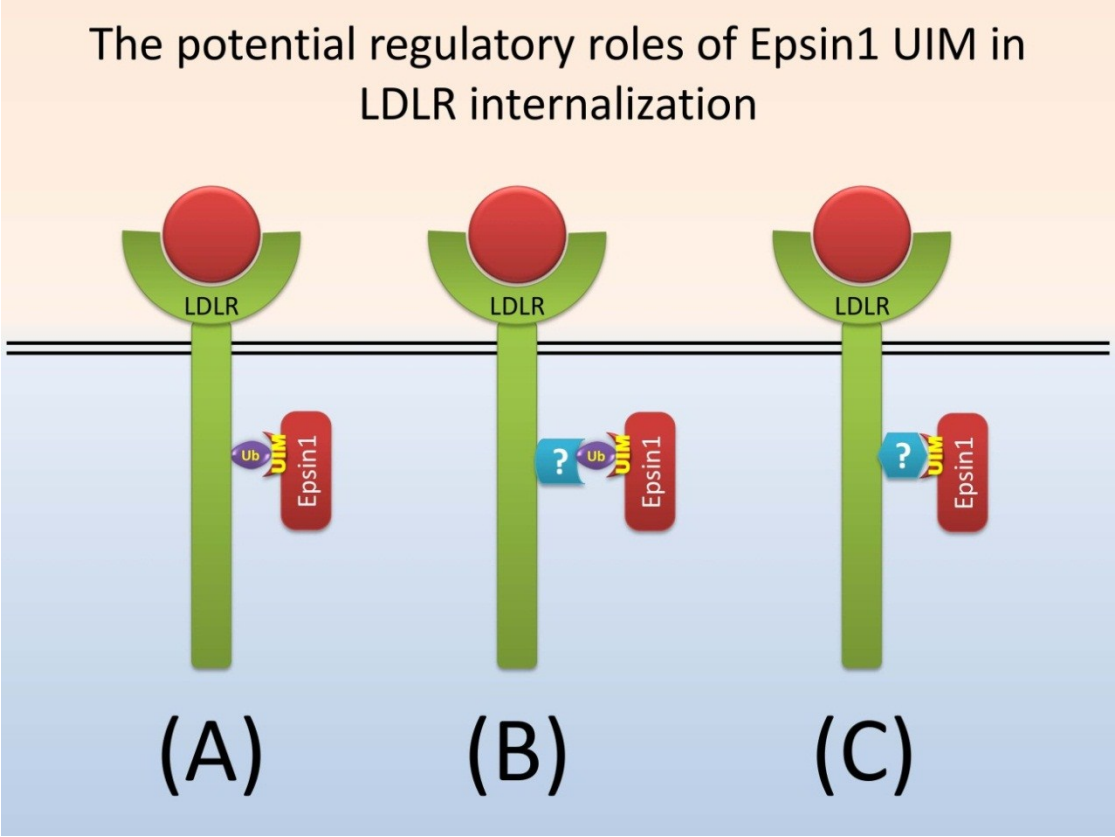


Figure 3-4 The amino acids in of various UIM-containing proteins required for ubiquitin interaction



Chapter Four: Caveolae-mediated LDLR internalization

Summary

LDLR mutations identified in FH patients highlight the physiological importance of LDLR trafficking in maintaining serum cholesterol levels. The mutation in the NPxY motif on LDLR tail results in defective LDLR internalization, a critical process to remove serum cholesterol-laden LDL particles. In chapter 2, I showed that reduced LDLR internalization following clathrin knockdown supports the well-accepted model that LDLR internalization undergoes clathrin-mediated pathway. However, clathrin knockdown did not completely diminish LDLR internalization. Additionally, biochemical analyses demonstrated the colocalization of LDLR and caveolin1 in the rodent liver (Ness *et al.*, 2003; Truong *et al.*, 2006). These observations led me to postulate that LDLR undergoes caveolae-mediated endocytosis as well. I provide preliminary results in this appendix showing that LDLR can undergo caveolae-mediated endocytosis. Further investigation remains required to uncover the regulatory mechanism with regards to caveolae-mediated LDLR internalization.

Introduction

Clathrin-independent LDLR endocytosis

Although it is widely accepted that LDLR endocytosis relies on the formation of clathrin-coated vesicle (Anderson *et al.*, 1977; Goldstein *et al.*, 1979), I demonstrated in the chapter two that clathrin knockdown did not completely abolish LDLR internalization, suggesting that clathrin-mediated endocytosis is not the sole mechanism for LDLR internalization. This finding raised the possibility that LDLR undergoes endocytosis via an alternative clathrin-independent pathway. Caveolae-dependent endocytosis serves as another major endocytic pathway that is characterized by caveolin-1 (Cav1)-enriched invagination in a plasma membrane microdomain with abundant cholesterol and sphingolipids (Lajoie and Nabi, 2010). In a previous study, one group analyzed liver homogenates from both rats and hamsters by means of discontinuous sucrose fractionation and co-immunoprecipitation, and demonstrated that LDLR coincides with Cav1-enriched fractions (Ness *et al.*, 2003). Similarly, another study showed that in Cav1 overexpressing hepatoma cells, HepG2, LDLR was found to be colocalized with Cav1 by immunofluorescent staining and protein fractionation analyses (Truong *et al.*, 2006). Based on my observation and these previous reports, I hypothesized that LDLR can be internalized via caveolae-mediated pathway. To test this hypothesis, I performed internalization assays to assess whether Cav1 knockdown alters LDLR internalization.

Results

LDLR employs diverse endocytic pathways

To test the hypothesis that LDLR undergoes caveolae-mediated endocytosis, I examined whether Cav1 knockdown impairs LDLR internalization. Indeed, Cav1 knockdown via siRNA in HeLa cells resulted in a striking decrease in LDLR internalization. This decrease was to a similar degree as compared to that following CHC, epsin1 and Dab2 depletions, respectively (Figure 4-1A). This observation suggests that caveolae contributes to LDLR internalization to a comparable level as clathrin, epsin and Dab2.

Considering the important role of NPxY motif in LDLR uptake (Morris and Cooper, 2001; Mishra *et al.*, 2002a), I speculated that caveolae-mediated LDLR uptake requires NPxY. If this hypothesis is true, Cav1 knockdown should not alter the internalization rate of $LDLR^{\Delta NPxY}$. Instead, Cav1 knockdown gave rise to a marked reduction in the internalization rate of $LDLR^{\Delta NPxY}$ (Figure 4-1B). This result reveals that caveolae-mediated LDLR uptake can occur independently of the NPxY motif. Interestingly, I previously demonstrated that epsin1 also functions independently of NPxY motif, raising the possibility that epsin1 functions in the caveolae pathway. To test this hypothesis, I compared the internalization rate of $LDLR^{\Delta NPxY}$ following epsin1 and Cav1 depletions, respectively (Figure 4-1B). Strikingly, epsin1 and Cav1 knockdowns led to comparable reductions in $LDLR^{\Delta NPxY}$ uptake, consistent with the possibility that epsin1 can mediate LDLR internalization in caveolae-mediated endocytosis.

Discussions

Multiple studies have established calthrin-mediated pathway as the prevalent manner for LDLR uptake (Anderson *et al.*, 1977; Goldstein *et al.*, 1979). Here, I present data indicating caveolae as an alternative way for LDLR to enter the cell. Moreover, my work demonstrates that caveolae-mediated LDLR endocytosis occurs independently of the NPxY motif, revealing a distinct LDLR internalization mechanism.

It is not clear as to the significance of LDLR utilizing two internalization pathways. One possibility is that different internalization pathways are employed based on ligand specificity. For example, LDLR relies on the NPxY motif via clathrin-mediated pathway to take up serum LDL (Chen *et al.*, 1990). However, LDLR utilizes HIC motif to internalize β-VLDL (Michaely *et al.*, 2007). While it has not been determined whether HIC-dependent β-VLDL uptake by LDLR occurs in a clathrin-independent pathway, my work in chapter two and this appendix showed that HIC motif- and caveolae-mediated LDLR internalization do not rely on NPxY motif. These observations raise the possibility that LDLR internalizes LDL and β-VLDL via clathrin and caveolae pathways, respectively.

As my results reveal that both epsin1- and Cav1-mediated LDLR uptake occur independently of NPxY motif, epsin1 may act as an endocytic factor in the caveolae-mediated LDLR uptake process (Figure 4-1B). One question of interest to ask is whether epsin1 acts as an endocytic adaptor for LDLR to internalize β-VLDL via caveolae-mediated pathway.

Another possible explanation for the presence of two distinct LDLR internalization pathways is functional differences based on tissue specificity. For example, Cav1 deficient mice show impaired LDL transcytosis across endothelial cells, which apparently slows down the development of atherosclerosis (Frank *et al.*, 2003; Frank *et al.*, 2008). While the role of LDLR in internalizing LDL in endothelial cells has not been investigated, it will be of interest to test the hypothesis that LDL uptake in vascular endothelial cells occurs via caveolae-mediated LDLR endocytosis.

Mechanisms underlying two parallel pathways of LDLR internalization

The revelation that LDLR internalization undergoes either clathrin- or caveolae-mediated pathways implicates a new scheme that LDLR may employ different pathways in response to physiological contexts. First, while LDLR reaches early endosome, the trafficking regulators incorporated with one of the pathways may preferably direct LDLR to lysosomal protein degradation to decrease the amount of surface LDLR. On the other hand, the other pathway may preferably direct LDLR to recycling pathway to increase the number of LDLR on the cell surface (Figure 4-2A).

Second, the selection of entry method may be ligand specific. LDLR has been shown to internalize both LDL and β-VLDL depending on different endocytic signals, NPxY and HIC, respectively (Innerarity and Mahley, 1978; Chen *et al.*, 1990; Michaely *et al.*, 2007). Based on the observations that LDL uptake relies on NPxY motif in a clathrin-dependent manner and that β-VLDL uptake relies on HIC motif independent of NPxY

motif, it is likely that LDLR internalizes β-VLDL via caveolae-mediated pathway (Figure 4-2B).

Third, LDLR employs different mechanism in a tissue-specific manner. LDLR is highly expressed in the liver where most circulating LDL is removed via clathrin-mediated pathway. On the other hand, Cav1 expression has been associated with atherosclerosis in the blood vessel, a pathological consequence of LDL transcytosis across vascular endothelial cells and LDL accumulation-induced inflammatory responses (Frank *et al.*, 2008). However, LDLR-mediated LDL transcytosis in the endothelial cells has not been studied and it will be critical address the questions: first, whether LDLR promotes LDL uptake via caveolae-mediated pathway which reflects the pro-atherogenic role of LDLR in the blood vessel; second, elucidation of the endocytic machinery involved in LDLR will also be crucial to understand the potential pathological role of LDLR that would offer therapeutic applications for atherosclerosis (Figure 4-2C).

Materials and Methods

Reagents: The mouse hybridomas 51.1(HB-230) was obtained from the American Type Culture Collection (ATCC). Lipofectamine RNAi^{MAX} was used for siRNA transfection following the manufacturers' instructions (Invitrogen).

Plasmid used to generate adenovirus coding for mouse CD8-LDLR chimera was a gift from Dr. Margaret Robinson (The University of Cambridge). Human cDNA for LDLR was a gift from Dr. Peter Tontonoz (UCLA) and was used to generate adenovirus encoding the extracellular domain of human CD8 fused to LDLR starting with the LDLR transmembrane domain (WT, ΔNPxY).

siRNA-mediated knockdown: 3.1X10^5 tTA HeLa cells were plated in 6-well plate and transfected with siRNA on day 1 and day 2. For internalization assay, cells were then infected with CD8-LDLR expressing adenovirus on day 3 for additional 18 hours. Knockdown siRNAs were for each target include: CHC, TAATCCAATTCGAAGACCAAT; Epsin1, GGAAGACGCCGGAGTCATT; Dab2, AGGTTGGAACCAGCCTTCACCCTTT; Cav1, CCCACTCTTTGAAGCTGTTGGGAAA. Each siRNA was obtained from Invitrogen. Silencer negative control siRNA (#1) was from Ambion.

Internalization assay: For quantitative uptake assays, tTA HeLa cells were resuspended in DMEM/10%FBS with CD8 mAb (51.1) followed by incubation of ^{125}I-Protein A (Perkin Elmer) in DMEM/0.5% BSA at 4°C. Cells were transferred to a 37°C water bath to allow for internalization for each indicated time points. Internalization was

stopped by moving cells to 4°C. Surface-bound ligands were stripped by acid wash (0.2 M acetic acid, 0.5 M NaCl). The amount of internalized CD8-LDLR was determined by γ counting and expressed as a percentage of total surface-bound γ count.

Figure Legends

Figure 4-1 Gene knockdown abrogates CD8-LDLR and CD8-LDLR$^{\Delta NPxY}$ internalization.

(A, B) siRNA-mediated knockdown was utilized to deplete the expression of epsin1, Dab2, CHC and Cav1, respectively while scrambled siRNA was used as control. The impact of gene depletion on CD8-LDLR (A) and CD8-LDLR$^{\Delta NPxY}$(B) uptake was measured by internalization assay over a 30-minute time period.

Figure 4-2 Mechanisms underlying two parallel pathways of LDLR internalization.

Illustration of three possible mechanisms explains LDLR internalization employs two parallel pathways, clathrin-mediated and caveolae-mediated endocytosis. (A) The endocytic machinery in one route preferably directs LDLR to lysosomal protein degradation while the other route targets LDLR to recycling pathway. (B) The selection of endocytic pathways is determined by the type of ligands, such as LDL and β-VLDL. (C) LDLR internalization is regulated in a tissue-specific manner. In the liver, LDLR is internalized via clathrin-mediated pathway. In the vascular endothelial cells, LDLR internalization possibly undergoes caveolae-mediated pathway.

Figures

Figure 4-1 Gene knockdown abrogates CD8-LDLR and CD8-LDLR$^{\Delta NPxY}$ internalization

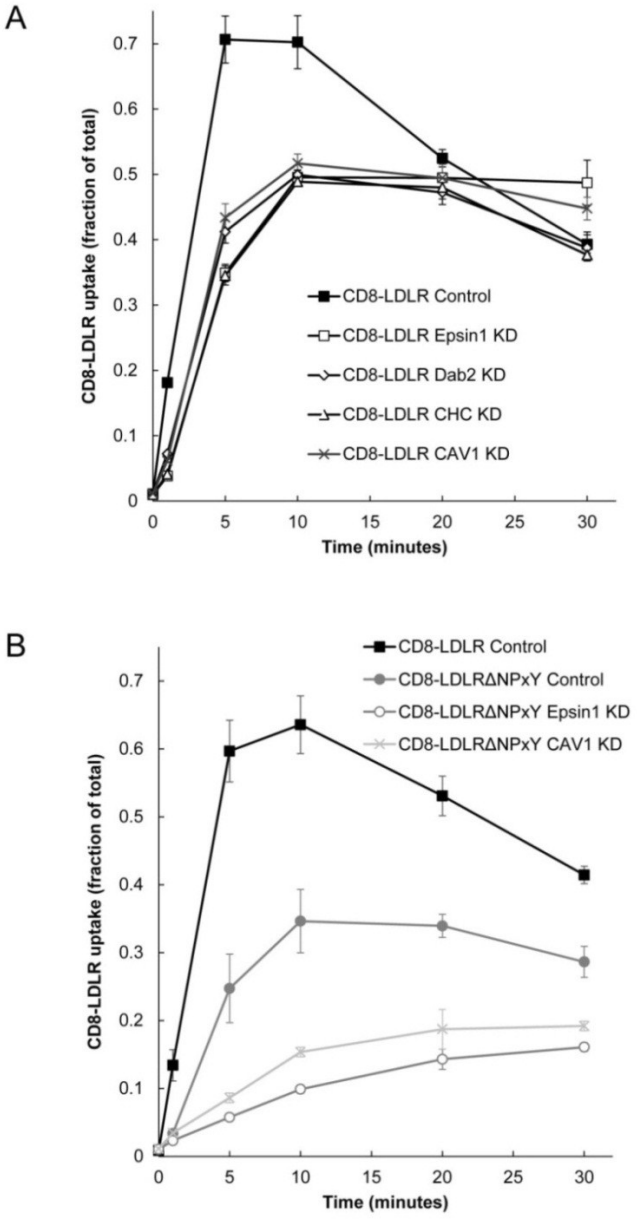
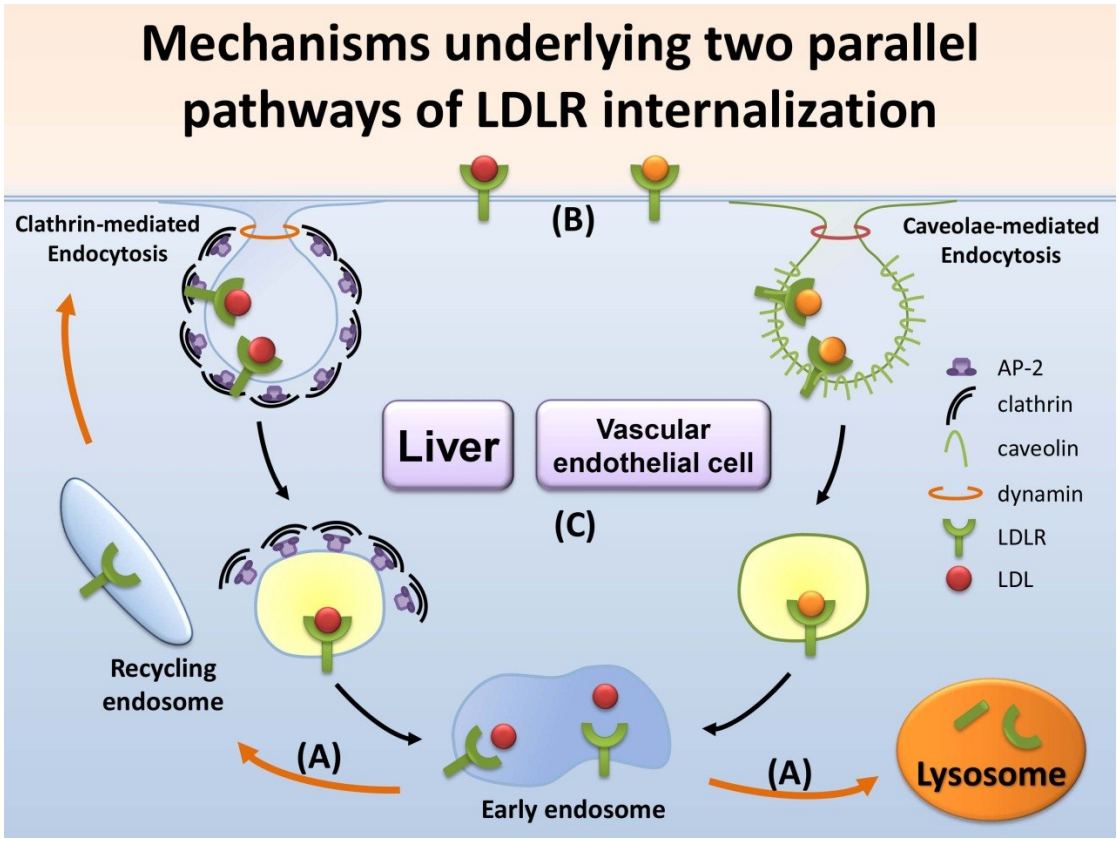


Figure 4-2 Mechanisms underlying two parallel pathways of LDLR internalization



Chapter Five: Conclusion and Discussion

LDLR trafficking regulators identified by whole genome RNAi screen in *C. elegans*

Familial hypercholesterolemia is characterized by elevated serum cholesterol. The identification of mutations in LDLR emphasizes the importance of LDLR trafficking, the mechanism of which remains largely unknown outside the scope of endocytosis. The long-term goal project is to better resolve the mechanism underlying transport of LDLR as its critical role in clearing serum LDL. The whole genome RNAi screen results suggest that *C. elegans* is an advantageous genetic model to identify additional regulators for LDLR trafficking. Meanwhile, it also indicates that the rationale underlying the screen is valid. LDLR superfamily members share common trafficking regulatory mechanisms. Also, receptor trafficking is critical for LDLR superfamily function. In *C. elegans*, defective LRP-1 trafficking phenocopies the molting defects caused by loss of *lrp-1*.

Molecular mechanisms underlying epsin-mediated LDLR endocytosis

Epsin identified as an endocytic factor for LDLR

In addition to the known component of clathrin coat, *chc-1*, and endocytic adaptors such as dynamin, subunits of AP-2, and Dab2, we identified epsin encoded by *epn*-1 as a major factor in mediating LRP-1 endocytosis. LDLR internalization assays in mammalian cells revealed a conserved function for mammalian epsin in mediating LDLR endocytosis.

The functionalities of epsin UIMs in LDLR trafficking

As a first approach to ascertain how epsin UIMs function to mediate internalization of LDLR superfamily members, I assessed 1) whether all epsin UIMs are required and 2) whether UIM-ubiquitin interaction is required using genetic assays in *C. elegans*. My study revealed that worm EPN-1 selectively uses UIM1 to regulate LRP-1 uptake and the ability of EPN-1 UIM1 to bind ubiquitin is not required to regulate LRP-1 uptake. The potential alternative function for EPN-1 UIM1 could be a regulatory role in ubiquitination of EPN-1 based on two previous observations. First, epsin has been implicated to be predominantly monoubiquitinated that might be required for epsin function. Second, the occurrence of self-monoubiquitination of epsin depends on the presence of its own UIM (Oldham *et al.*, 2002; Timsit *et al.*, 2005). If EPN-1 indeed selectively uses its UIM1 for self-monoubiquitination as a requirement for regulating LRP-1 internalization, I postulate that mammalian epsin1 selectively uses some of its three UIMs, which may function to regulate self-ubiquitination instead of interacting with an ubiquitinated protein, to regulate LDLR internalization.

I demonstrated that worm EPN-1 UIM1 is required for LDR-1 uptake; however, the question whether EPN-1 UIM2 has a role in LRP-1 trafficking remains unclear. One surprising observation that worms expressing EPN-1 UIM1$^{EEE\rightarrow AAA}$; ΔUIM2 exhibited intracellular accumulation of LRP-1 suggests that EPN-1 UIM2 may have a role in intracellular trafficking. Indeed, LRP-1 in *epn-1(tm3357)* larvi appears to accumulate in a type of intracellular compartment (Figure 3-2C, D in chapter three). Moreover, LDLR

internalization following epsin1 knockdown in mammalian cells not only indicates a defect in internalization but also recycling (Figure 4-1A). Together, epsin may selectively use different UIMs to regulate both LDLR internalization and recycling.

Ubiquitinated LDLR is not required for epsin function

Function-structure analyses demonstrated EPN-1 UIM1 as critical for LRP-1 endocytosis in *C. elegans*. Also, the importance of the UIMs in receptor internalization is underscored by the requirement of the UIMs in epsin1 to stabilize LDLR-epsin1 protein complex. Epsin has long been associated with governing receptor internalization by directly engaging ubiquitin-modified receptor (Sugiyama *et al.*, 2005; Kazazic *et al.*, 2009; Chen *et al.*, 2011; Henry *et al.*, 2012; Xie *et al.*, 2012). However, our studies reveal that ubiquitin modification of LDLR is not required for epsin-mediated LDLR internalization. This result raises the possibility that instead of directly engaging LDLR, epsin is associated with LDLR via an ubiquitinated intermediate protein. Indeed, GST pull-down and yeast two hybrid assays did not indicate epsin interaction with LDLR, consistent with the idea of a linker protein.

Considering my observations in epsin UIMs and LDLR ubiquitination, the possible mechanism by which epsin1 regulates LDLR trafficking is that epsin1 needs to be monoubiquitinated, a process that requires its own UIM(s), before being recruited to the LDLR endocytic assembly. Moreover, in the endosomal sorting process, epsin1 plays a role in directing LDLR to recycling pathway where epsin1 UIM(s) is required, likely via indirect interaction with LDLR.

Molecular dissection of LDLR internalization

To delineate the critical endocytic signal(s) on the LDLR cytoplasmic tail responsible for epsin1-mediated LDLR endocytosis, two well-defined endocytic signals, NPxY and HIC, with mutually exclusive function with respective to LDLR endocytosis, were examined extensively (Chen *et al.*, 1990; Michaely *et al.*, 2007). Mutation and internalization assays of LDLR led to the conclusion that epsin1 can function independently of NPxY and HIC motifs, revealing a third unidentified mechanism. To uncover the not-yet-defined endocytic motif, a series of mutations could be created and used for internalization assay and its physiological significance could be investigated.

LDLR internalizes LDL via clathrin-mediated endocytosis, which requires the interaction between the endocytic signal, NPxY, and PTB domain containing protein adaptors, ARH or Dab2 (Figure 4-1). Interestingly, my work showed that LDLR also undergoes caveolae-mediated endocytosis independent of NPxY motif. Considering that epsin can function independent of NPxY motif, it raises the possibility that epsin can regulate LDLR internalization via caveolae-mediated pathway (Figure 4-1B and Figure 5-1).

Taken together, LDLR internalization employs multiple mechanisms in response to physical contexts (Figure 5-1). 1) The types of ligands: LDL uptake depends on NPxY motif while β-VLDL uptake depends on HIC motif. 2) LDLR internalized via caveolae-mediated pathway is directed to lysosome to reduce LDL uptake (Truong *et al.*, 2006) while LDLR internalized via clathrin-mediated pathway is targeted to recycling

endosome to promote active LDL uptake. 3) The types of tissues: LDL uptake in the liver undergoes clathrin-mediated pathway while LDL uptake in vascular endothelial cells undergoes caveolae-mediated pathway (discussed in the chapter 4).

The potential roles of epsin in hypercholesterolemia

To reconcile the fact that there have been no reported mutations in epsin linked to hypercholesterolemia, it is possibly due to the redundancy between epsin1 and epsin2 which is supported by the embryonic death in mouse resulting from epsin1 and epsin2 double knockout in contrast to respective viable single knockout mouse (Chen *et al.*, 2009). Alternatively, epsin may not participate in the NPxY-dependent pathway; instead, epsin may coordinate LDLR to take up β-VLDL as a way to regulate the LDL levels based on the observations that epsin-mediated and HIC-dependent LDLR internalization are independent of NPxY-motif, respectively.

On the other hand, epsin may regulate LDLR internalization via caveolae-mediated endocytosis based on our results demonstrating independence of NPxY motif for both epsin and caveolae-mediated LDLR uptake. While it is critical to first elucidate the role of LDLR in vascular endothelial cells, interestingly, a recent report demonstrated that loss of epsin1 and epsin2 in vascular endothelial cells leads to disorganized vasculature as a consequence of impaired VEGFR2 endocytosis (Pasula *et al.*, 2012). It would be of great interest to examine whether epsin-mediated LDLR endocytosis occurs in the vascular endothelial cells and functionally correlates to the development of atherosclerosis, a characteristic feature of FH.

Future perspectives

LDLR maintains serum cholesterol levels by recognizing and internalizing circulating LDL. Defective LDLR trafficking has been associated with familial hypercholesterolemia; however, besides the established knowledge regarding LDLR internalization, the mechanisms underlying LDLR trafficking, such as transport to the plasma membrane and endosomal sorting, remain largely unknown.

Hypercholesterolemia is a rising health problem because of its association with atherosclerosis and coronary heart disease. The cause for hypercholesterolemia is multifactorial, including a diet high in saturated fat as well as genetic mutations. While genetic cause is more likely polygenic mutations that predispose patients to hypercholesterolemia, the severe cases can be monogenic such as familial hypercholesterolemia caused by mutated LDLR, protein convertase subtilisin/kexin type 9 (PCSK9) or ApoB (Bhatnagar *et al.*, 2008). PCSK9 mutations promote LDLR degradation, resulting in excessive LDL particles in the circulation (Naoumova *et al.*, 2005; Costet *et al.*, 2008). Mutations in APOB gene diminish the affinity between LDL and LDLR, leading to elevated serum LDL levels (Soria *et al.*, 1989).

Considering that both impaired LDLR recycling and degradation can cause hypercholesterolemia, the regulatory mechanism underlying LDLR intracellular sorting for recycling or degradation has not been studied. Our whole genomic RNAi screen successfully enabled us to discover additional LDLR trafficking regulators. These identified factors that when knocked down, cause abnormal intracellular accumulation

of LRP-1, are likely to regulate intracellular sorting, directing LDLR to recycling pathway or lysosomal protein degradation. Understanding the mechanisms underlying LDLR recycling and degradation will provide insights into the pathological mechanism as to FH caused by mutations that impair LDLR recycling or degradation (Miyake *et al.*, 1989; Naoumova *et al.*, 2005).

My work on identifying proteins critical for LDLR trafficking and understanding the regulatory mechanism not only sheds light on the LDLR transport but also provides potential candidates that may account for hypercholesterolemia and further serve a target for therapeutic application.

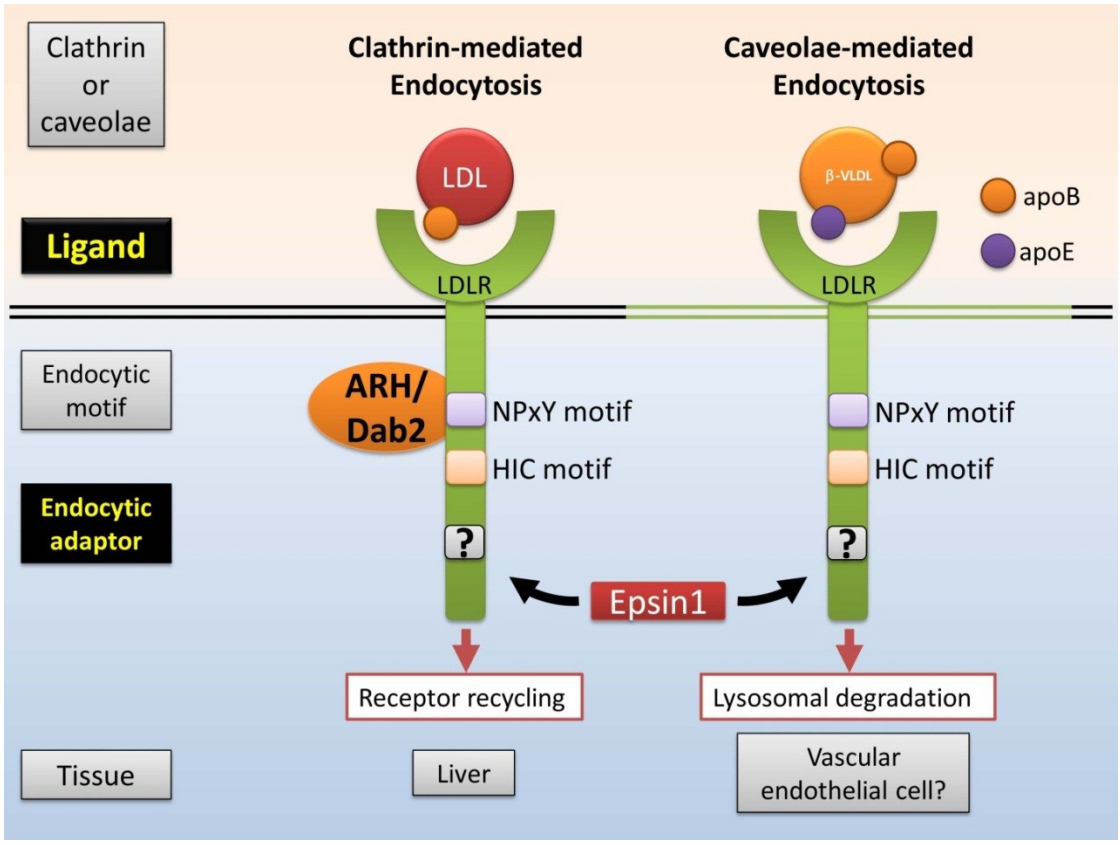
Figure legend

Figure 5-1 Proposed mechanisms underlying LDLR endocytosis.

LDLR endocytosis undergoes either clathrin- or caveolae-dependent pathways. First, internalization of LDL particles requires interaction between ARH/Dab2 and NPxY motif (He *et al.*, 2002; Mishra *et al.*, 2002b; Maurer and Cooper, 2006) while internalization of β-VLDL particles requires the HIC motif (Michaely *et al.*, 2007). Second, Epsin1 is likely to regulate LDLR endocytosis in both clathrin- and caveolae-mediated endocytosis. However, the endocytic signal(s) critical for epsin1 function in LDLR internalization remain to be resolved. Third, LDLR internalized via clathrin-mediated pathway is recycled back to the plasma membrane to promote active LDL uptake; on the contrary, LDLR internalized via caveolae-mediated pathway is degraded in the lysosome. Fourth, LDLR removes serum LDL particles via clathrin-mediated pathway in the liver to maintain serum cholesterol levels while LDLR internalizes across vascular endothelial cells via caveolae-mediated pathway, likely a pro-atherogenic process.

Figure

Figure 5-1 Proposed mechanisms underlying LDLR endocytosis



References

Aguilar, R.C., Longhi, S.A., Shaw, J.D., Yeh, L.Y., Kim, S., Schon, A., Freire, E., Hsu, A., McCormick, W.K., Watson, H.A., and Wendland, B. (2006). Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. Proc Natl Acad Sci U S A *103*, 4116-4121.

Aguilar, R.C., Watson, H.A., and Wendland, B. (2003). The yeast Epsin Ent1 is recruited to membranes through multiple independent interactions. The Journal of biological chemistry *278*, 10737-10743.

Anderson, R.G., Goldstein, J.L., and Brown, M.S. (1977). A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts. Nature *270*, 695-699.

Balklava, Z., Pant, S., Fares, H., and Grant, B.D. (2007). Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. Nat Cell Biol *9*, 1066-1073.

Bhatnagar, D., Soran, H., and Durrington, P.N. (2008). Hypercholesterolaemia and its management. Bmj *337*, a993.

Boucrot, E., Saffarian, S., Zhang, R., and Kirchhausen, T. (2010). Roles of AP-2 in clathrin-mediated endocytosis. PLoS One *5*, e10597.

Brady, R.J., Wen, Y., and O'Halloran, T.J. (2008). The ENTH and C-terminal domains of Dictyostelium epsin cooperate to regulate the dynamic interaction with clathrin-coated pits. Journal of cell science *121*, 3433-3444.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Brown, M.S., and Goldstein, J.L. (1974). Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. Proc Natl Acad Sci U S A *71*, 788-792.

Brown, M.S., and Goldstein, J.L. (1976). Analysis of a mutant strain of human fibroblasts with a defect in the internalization of receptor-bound low density lipoprotein. Cell *9*, 663-674.

Brown, M.S., and Goldstein, J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science *232*, 34-47.

Chen, B., Dores, M.R., Grimsey, N., Canto, I., Barker, B.L., and Trejo, J. (2011). Adaptor protein complex-2 (AP-2) and epsin-1 mediate protease-activated receptor-1 internalization via

phosphorylation- and ubiquitination-dependent sorting signals. The Journal of biological chemistry *286*, 40760-40770.

Chen, C., and Zhuang, X. (2008). Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. Proc Natl Acad Sci U S A *105*, 11790-11795.

Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P., and De Camilli, P. (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature *394*, 793-797.

Chen, H., Ko, G., Zatti, A., Di Giacomo, G., Liu, L., Raiteri, E., Perucco, E., Collesi, C., Min, W., Zeiss, C., De Camilli, P., and Cremona, O. (2009). Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. Proc Natl Acad Sci U S A *106*, 13838-13843.

Chen, W.J., Goldstein, J.L., and Brown, M.S. (1990). NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. The Journal of biological chemistry *265*, 3116-3123.

Chen, X., Zhang, B., and Fischer, J.A. (2002). A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev *16*, 289-294.

Chitwood, D.J. (1992). Nematode sterol biochemistry. American Oil Chemists' Society: Champaign, IL.

Costet, P., Krempf, M., and Cariou, B. (2008). PCSK9 and LDL cholesterol: unravelling the target to design the bullet. Trends Biochem Sci *33*, 426-434.

Damke, H., Baba, T., van der Bliek, A.M., and Schmid, S.L. (1995). Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. The Journal of cell biology *131*, 69-80.

Daniels, T.F., Killinger, K.M., Michal, J.J., Wright, R.W., Jr., and Jiang, Z. (2009). Lipoproteins, cholesterol homeostasis and cardiac health. Int J Biol Sci *5*, 474-488.

Dores, M.R., Schnell, J.D., Maldonado-Baez, L., Wendland, B., and Hicke, L. (2010). The function of yeast epsin and Ede1 ubiquitin-binding domains during receptor internalization. Traffic *11*, 151-160.

Drake, M.T., Downs, M.A., and Traub, L.M. (2000). Epsin binds to clathrin by associating directly with the clathrin-terminal domain. Evidence for cooperative binding through two discrete sites. The Journal of biological chemistry *275*, 6479-6489.

Edeling, M.A., Mishra, S.K., Keyel, P.A., Steinhauser, A.L., Collins, B.M., Roth, R., Heuser, J.E., Owen, D.J., and Traub, L.M. (2006). Molecular switches involving the AP-2 beta2 appendage regulate endocytic cargo selection and clathrin coat assembly. Dev Cell *10*, 329-342.

Eden, E.R., Patel, D.D., Sun, X.M., Burden, J.J., Themis, M., Edwards, M., Lee, P., Neuwirth, C., Naoumova, R.P., and Soutar, A.K. (2002). Restoration of LDL receptor function in cells from patients with autosomal recessive hypercholesterolemia by retroviral expression of ARH1. J Clin Invest *110*, 1695-1702.

Frand, A.R., Russel, S., and Ruvkun, G. (2005). Functional genomic analysis of C. elegans molting. PLoS Biol *3*, e312.

Frank, P.G., Pavlides, S., Cheung, M.W., Daumer, K., and Lisanti, M.P. (2008). Role of caveolin-1 in the regulation of lipoprotein metabolism. American journal of physiology. Cell physiology *295*, C242-248.

Frank, P.G., Woodman, S.E., Park, D.S., and Lisanti, M.P. (2003). Caveolin, caveolae, and endothelial cell function. Arterioscler Thromb Vasc Biol *23*, 1161-1168.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature *408*, 325-330.

Garcia, C.K., Wilund, K., Arca, M., Zuliani, G., Fellin, R., Maioli, M., Calandra, S., Bertolini, S., Cossu, F., Grishin, N., Barnes, R., Cohen, J.C., and Hobbs, H.H. (2001). Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. Science *292*, 1394-1398.

Girish, M.P., and Gupta, M.D. (2005). Images in clinical medicine. Xanthomatous pseudospectacles in familial hypercholesterolemia. N Engl J Med *352*, 2424.

Go, G.W., and Mani, A. (2012). Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. Yale J Biol Med *85*, 19-28.

Goldstein, J.L., Anderson, R.G., and Brown, M.S. (1979). Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature *279*, 679-685.

Goldstein, J.L., and Brown, M.S. (1973). Familial hypercholesterolemia: identification of a defect in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol. Proc Natl Acad Sci U S A *70*, 2804-2808.

Grant, B., and Hirsh, D. (1999). Receptor-mediated endocytosis in the Caenorhabditis elegans oocyte. Molecular biology of the cell *10*, 4311-4326.

Gravotta, D., Carvajal-Gonzalez, J.M., Mattera, R., Deborde, S., Banfelder, J.R., Bonifacino, J.S., and Rodriguez-Boulan, E. (2012). The clathrin adaptor AP-1A mediates basolateral polarity. Dev Cell *22*, 811-823.

Hawryluk, M.J., Keyel, P.A., Mishra, S.K., Watkins, S.C., Heuser, J.E., and Traub, L.M. (2006). Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. Traffic *7*, 262-281.

He, G., Gupta, S., Yi, M., Michaely, P., Hobbs, H.H., and Cohen, J.C. (2002). ARH is a modular adaptor protein that interacts with the LDL receptor, clathrin, and AP-2. The Journal of biological chemistry *277*, 44044-44049.

Hegele, R.A. (2009). Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet *10*, 109-121.

Henne, W.M., Boucrot, E., Meinecke, M., Evergren, E., Vallis, Y., Mittal, R., and McMahon, H.T. (2010). FCHo proteins are nucleators of clathrin-mediated endocytosis. Science *328*, 1281-1284.

Henry, A.G., Hislop, J.N., Grove, J., Thorn, K., Marsh, M., and von Zastrow, M. (2012). Regulation of endocytic clathrin dynamics by cargo ubiquitination. Dev Cell *23*, 519-532.

Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol *9*, 107-112.

Hirano, S., Kawasaki, M., Ura, H., Kato, R., Raiborg, C., Stenmark, H., and Wakatsuki, S. (2006). Double-sided ubiquitin binding of Hrs-UIM in endosomal protein sorting. Nat Struct Mol Biol *13*, 272-277.

Hobbs, H.H., Russell, D.W., Brown, M.S., and Goldstein, J.L. (1990). The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. Annu Rev Genet *24*, 133-170.

Hofmann, K., and Falquet, L. (2001). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. Trends Biochem Sci *26*, 347-350.

Horton, J.D., Goldstein, J.L., and Brown, M.S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest *109*, 1125-1131.

Huang, F., Khvorova, A., Marshall, W., and Sorkin, A. (2004). Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. The Journal of biological chemistry *279*, 16657-16661.

Innerarity, T.L., and Mahley, R.W. (1978). Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. Biochemistry *17*, 1440-1447.

Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001). Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. Science *291*, 1047-1051.

Jakobsson, J., Gad, H., Andersson, F., Low, P., Shupliakov, O., and Brodin, L. (2008). Role of epsin 1 in synaptic vesicle endocytosis. Proc Natl Acad Sci U S A *105*, 6445-6450.

Kamath, R.S., and Ahringer, J. (2003). Genome-wide RNAi screening in Caenorhabditis elegans. Methods *30*, 313-321.

Kamikura, D.M., and Cooper, J.A. (2003). Lipoprotein receptors and a disabled family cytoplasmic adaptor protein regulate EGL-17/FGF export in C. elegans. Genes Dev *17*, 2798-2811.

Kamikura, D.M., and Cooper, J.A. (2006). Clathrin interaction and subcellular localization of Ce-DAB-1, an adaptor for protein secretion in Caenorhabditis elegans. Traffic *7*, 324-336.

Kang, Y.L., Yochem, J., Bell, L., Sorensen, E.B., Chen, L., and Conner, S.D. (2013). Caenorhabditis elegans reveals a FxNPxY-independent low-density lipoprotein receptor internalization mechanism mediated by epsin1. Molecular biology of the cell *24*, 308-318.

Kazazic, M., Bertelsen, V., Pedersen, K.W., Vuong, T.T., Grandal, M.V., Rodland, M.S., Traub, L.M., Stang, E., and Madshus, I.H. (2009). Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits. Traffic *10*, 235-245.

Keyel, P.A., Mishra, S.K., Roth, R., Heuser, J.E., Watkins, S.C., and Traub, L.M. (2006). A single common portal for clathrin-mediated endocytosis of distinct cargo governed by cargo-selective adaptors. Molecular biology of the cell *17*, 4300-4317.

Klapisz, E., Sorokina, I., Lemeer, S., Pijnenburg, M., Verkleij, A.J., and van Bergen en Henegouwen, P.M. (2002). A ubiquitin-interacting motif (UIM) is essential for Eps15 and Eps15R ubiquitination. The Journal of biological chemistry *277*, 30746-30753.

Lajoie, P., and Nabi, I.R. (2010). Lipid Rafts, Caveolae, and Their Endocytosis *282*, 135-163.

Legendre-Guillemin, V., Wasiak, S., Hussain, N.K., Angers, A., and McPherson, P.S. (2004). ENTH/ANTH proteins and clathrin-mediated membrane budding. Journal of cell science *117*, 9-18.

Lockman, A.R., Tribastone, A.D., Knight, K.V., and Franko, J.P. (2005). Treatment of cholesterol abnormalities. Am Fam Physician *71*, 1137-1142.

Maldonado-Baez, L., and Wendland, B. (2006). Endocytic adaptors: recruiters, coordinators and regulators. Trends Cell Biol *16*, 505-513.

Martin, P.J., Ledbetter, J.A., Clark, E.A., Beatty, P.G., and Hansen, J.A. (1984). Epitope mapping of the human surface suppressor/cytotoxic T cell molecule Tp32. J Immunol *132*, 759-765.

Marzolo, M.P., and Farfan, P. (2011). New insights into the roles of megalin/LRP2 and the regulation of its functional expression. Biol Res *44*, 89-105.

Maurer, M.E., and Cooper, J.A. (2006). The adaptor protein Dab2 sorts LDL receptors into coated pits independently of AP-2 and ARH. Journal of cell science *119*, 4235-4246.

Mettlen, M., Loerke, D., Yarar, D., Danuser, G., and Schmid, S.L. (2010). Cargo- and adaptor-specific mechanisms regulate clathrin-mediated endocytosis. The Journal of cell biology *188*, 919-933.

Michaely, P., Zhao, Z., Li, W.P., Garuti, R., Huang, L.J., Hobbs, H.H., and Cohen, J.C. (2007). Identification of a VLDL-induced, FDNPVY-independent internalization mechanism for the LDLR. EMBO J *26*, 3273-3282.

Mishra, S.K., Keyel, P.A., Hawryluk, M.J., Agostinelli, N.R., Watkins, S.C., and Traub, L.M. (2002a). Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor. EMBO J *21*, 4915-4926.

Mishra, S.K., Watkins, S.C., and Traub, L.M. (2002b). The autosomal recessive hypercholesterolemia (ARH) protein interfaces directly with the clathrin-coat machinery. Proc Natl Acad Sci U S A *99*, 16099-16104.

Miyake, Y., Tajima, S., Funahashi, T., and Yamamoto, A. (1989). Analysis of a recycling-impaired mutant of low density lipoprotein receptor in familial hypercholesterolemia. The Journal of biological chemistry *264*, 16584-16590.

Morris, S.M., and Cooper, J.A. (2001). Disabled-2 colocalizes with the LDLR in clathrin-coated pits and interacts with AP-2. Traffic *2*, 111-123.

Motley, A., Bright, N.A., Seaman, M.N., and Robinson, M.S. (2003). Clathrin-mediated endocytosis in AP-2-depleted cells. The Journal of cell biology *162*, 909-918.

Mulkearns, E.E., and Cooper, J.A. (2012). FCH domain only-2 organizes clathrin-coated structures and interacts with Disabled-2 for low-density lipoprotein receptor endocytosis. Molecular biology of the cell *23*, 1330-1342.

Murphy, S.L., and Xu, J. (2012). Deaths: Preliminary Data for 2010. National Vital Statistics Reports *60*, 1-52.

Naoumova, R.P., Tosi, I., Patel, D., Neuwirth, C., Horswell, S.D., Marais, A.D., van Heyningen, C., and Soutar, A.K. (2005). Severe hypercholesterolemia in four British families with the D374Y mutation in the PCSK9 gene: long-term follow-up and treatment response. Arterioscler Thromb Vasc Biol *25*, 2654-2660.

Ness, G.C., Kohlruss, N., and Gertz, K.R. (2003). Association of the low-density lipoprotein receptor with caveolae in hamster and rat liver. Biochemical and Biophysical Research Communications *303*, 177-181.

Norman, D., Sun, X.M., Bourbon, M., Knight, B.L., Naoumova, R.P., and Soutar, A.K. (1999). Characterization of a novel cellular defect in patients with phenotypic homozygous familial hypercholesterolemia. J Clin Invest *104*, 619-628.

Oldham, C.E., Mohney, R.P., Miller, S.L., Hanes, R.N., and O'Bryan, J.P. (2002). The ubiquitin-interacting motifs target the endocytic adaptor protein epsin for ubiquitination. Curr Biol *12*, 1112-1116.

Overstreet, E., Chen, X., Wendland, B., and Fischer, J.A. (2003). Either Part of a Drosophila Epsin Protein, Divided after the ENTH Domain, Functions in Endocytosis of Delta in the Developing Eye. Current Biology *13*, 854-860.

Pasula, S., Cai, X., Dong, Y., Messa, M., McManus, J., Chang, B., Liu, X., Zhu, H., Mansat, R.S., Yoon, S.J., Hahn, S., Keeling, J., Saunders, D., Ko, G., Knight, J., Newton, G., Luscinskas, F., Sun, X., Towner, R., Lupu, F., Xia, L., Cremona, O., De Camilli, P., Min, W., and Chen, H. (2012). Endothelial epsin deficiency decreases tumor growth by enhancing VEGF signaling. J Clin Invest *122*, 4424-4438.

Polo, S., Confalonieri, S., Salcini, A.E., and Di Fiore, P.P. (2003). EH and UIM: endocytosis and more. Sci STKE *2003*, re17.

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M.R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P.P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature *416*, 451-455.

Rader, D.J., Cohen, J., and Hobbs, H.H. (2003). Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. J Clin Invest *111*, 1795-1803.

Reusch, U., Bernhard, O., Koszinowski, U., and Schu, P. (2002). AP-1A and AP-3A lysosomal sorting functions. Traffic *3*, 752-761.

Robinson, M.S. (2004). Adaptable adaptors for coated vesicles. Trends Cell Biol *14*, 167-174.

Rosenthal, J.A., Chen, H., Slepnev, V.I., Pellegrini, L., Salcini, A.E., Di Fiore, P.P., and De Camilli, P. (1999). The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. The Journal of biological chemistry *274*, 33959-33965.

Shafaq-Zadah, M., Brocard, L., Solari, F., and Michaux, G. (2012). AP-1 is required for the maintenance of apico-basal polarity in the C. elegans intestine. Development *139*, 2061-2070.

Sharrock, W.J. (1983). Yolk proteins of Caenorhabditis elegans. Dev Biol *96*, 182-188.

Shekhtman, A., and Cowburn, D. (2002). A ubiquitin-interacting motif from Hrs binds to and occludes the ubiquitin surface necessary for polyubiquitination in monoubiquitinated proteins. Biochem Biophys Res Commun *296*, 1222-1227.

Shih, S.C., Katzmann, D.J., Schnell, J.D., Sutanto, M., Emr, S.D., and Hicke, L. (2002). Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. Nat Cell Biol *4*, 389-393.

Shih, S.C., Sloper-Mould, K.E., and Hicke, L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. EMBO J *19*, 187-198.

Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P.P., and Polo, S. (2005). Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci U S A *102*, 2760-2765.

Sirinian, M.I., Belleudi, F., Campagna, F., Ceridono, M., Garofalo, T., Quagliarini, F., Verna, R., Calandra, S., Bertolini, S., Sorice, M., Torrisi, M.R., and Arca, M. (2005). Adaptor protein ARH is recruited to the plasma membrane by low density lipoprotein (LDL) binding and modulates endocytosis of the LDL/LDL receptor complex in hepatocytes. The Journal of biological chemistry *280*, 38416-38423.

Sorensen, E.B., and Conner, S.D. (2010). gamma-secretase-dependent cleavage initiates notch signaling from the plasma membrane. Traffic *11*, 1234-1245.

Soria, L.F., Ludwig, E.H., Clarke, H.R., Vega, G.L., Grundy, S.M., and McCarthy, B.J. (1989). Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. Proc Natl Acad Sci U S A *86*, 587-591.

Soutar, A.K., and Naoumova, R.P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. Nat Clin Pract Cardiovasc Med *4*, 214-225.

Strickland, D.K., Gonias, S.L., and Argraves, W.S. (2002). Diverse roles for the LDL receptor family. Trends Endocrinol Metab *13*, 66-74.

Sugiyama, S., Kishida, S., Chayama, K., Koyama, S., and Kikuchi, A. (2005). Ubiquitin-interacting motifs of Epsin are involved in the regulation of insulin-dependent endocytosis. J Biochem *137*, 355-364.

Swanson, K.A., Kang, R.S., Stamenova, S.D., Hicke, L., and Radhakrishnan, I. (2003). Solution structure of Vps27 UIM-ubiquitin complex important for endosomal sorting and receptor downregulation. EMBO J *22*, 4597-4606.

Teckchandani, A., Mulkearns, E.E., Randolph, T.W., Toida, N., and Cooper, J.A. (2012). The clathrin adaptor Dab2 recruits EH domain scaffold proteins to regulate integrin beta1 endocytosis. Molecular biology of the cell *23*, 2905-2916.

Tian, X., Hansen, D., Schedl, T., and Skeath, J.B. (2004). Epsin potentiates Notch pathway activity in Drosophila and C. elegans. Development *131*, 5807-5815.

Timsit, Y.E., Miller, S.L., Mohney, R.P., and O'Bryan, J.P. (2005). The U-box ligase carboxyl-terminus of Hsc 70-interacting protein ubiquitylates Epsin. Biochem Biophys Res Commun *328*, 550-559.

Truong, T.Q., Aubin, D., Bourgeois, P., Falstrault, L., and Brissette, L. (2006). Opposite effect of caveolin-1 in the metabolism of high-density and low-density lipoproteins. Biochim Biophys Acta *1761*, 24-36.

van Driel, I.R., Davis, C.G., Goldstein, J.L., and Brown, M.S. (1987). Self-association of the low density lipoprotein receptor mediated by the cytoplasmic domain. The Journal of biological chemistry *262*, 16127-16134.

Wang, W., and Struhl, G. (2004). Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development *131*, 5367-5380.

Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1978). Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. The Journal of biological chemistry *253*, 9053-9062.

Wendland, B. (2002). Epsins: adaptors in endocytosis? Nat Rev Mol Cell Biol *3*, 971-977.

Wendland, B., Steece, K.E., and Emr, S.D. (1999). Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. EMBO J *18*, 4383-4393.

Willnow, T.E., Hammes, A., and Eaton, S. (2007). Lipoproteins and their receptors in embryonic development: more than cholesterol clearance. Development *134*, 3239-3249.

Willnow, T.E., Nykjaer, A., and Herz, J. (1999). Lipoprotein receptors: new roles for ancient proteins. Nat Cell Biol *1*, E157-162.

Woelk, T., Oldrini, B., Maspero, E., Confalonieri, S., Cavallaro, E., Di Fiore, P.P., and Polo, S. (2006). Molecular mechanisms of coupled monoubiquitination. Nat Cell Biol *8*, 1246-1254.

Xie, X., Cho, B., and Fischer, J.A. (2012). Drosophila Epsin's role in Notch ligand cells requires three Epsin protein functions: the lipid binding function of the ENTH domain, a single Ubiquitin interaction motif, and a subset of the C-terminal protein binding modules. Dev Biol *363*, 399-412.

Xu, J., Toops, K.A., Diaz, F., Carvajal-Gonzalez, J.M., Gravotta, D., Mazzoni, F., Schreiner, R., Rodriguez-Boulan, E., and Lakkaraju, A. (2012). Mechanism of polarized lysosome exocytosis in epithelial cells. Journal of cell science.

Yochem, J., and Greenwald, I. (1993). A gene for a low density lipoprotein receptor-related protein in the nematode Caenorhabditis elegans. Proc Natl Acad Sci U S A *90*, 4572-4576.

Yochem, J., Tuck, S., Greenwald, I., and Han, M. (1999). A gp330/megalin-related protein is required in the major epidermis of Caenorhabditis elegans for completion of molting. Development *126*, 597-606.

Yoshida, H., Yokode, M., Yamamoto, A., Masaki, R., Murayama, T., Horiuchi, H., and Kita, T. (1999). Compensated endocytosis of LDL by hamster cells co-expressing the two distinct mutant LDL receptors defective in endocytosis and ligand binding. J Lipid Res *40*, 814-823.

Yun, M., Keshvara, L., Park, C.G., Zhang, Y.M., Dickerson, J.B., Zheng, J., Rock, C.O., Curran, T., and Park, H.W. (2003). Crystal structures of the Dab homology domains of mouse disabled 1 and 2. The Journal of biological chemistry *278*, 36572-36581.

Zelcer, N., Hong, C., Boyadjian, R., and Tontonoz, P. (2009). LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science *325*, 100-104.

Zhang, H., Kim, A., Abraham, N., Khan, L.A., Hall, D.H., Fleming, J.T., and Gobel, V. (2012). Clathrin and AP-1 regulate apical polarity and lumen formation during C. elegans tubulogenesis. Development *139*, 2071-2083.

Zou, P., and Ting, A.Y. (2011). Imaging LDL receptor oligomerization during endocytosis using a co-internalization assay. ACS Chem Biol *6*, 308-313.