# LGD-1 regulation of ESCRT-III during multivesicular endosome biogenesis

By

Aryel Lynn Clarke

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The dissertation is approved by the following members of the Final Oral Committee:

Anjon Audhya, Associate Professor, Biomolecular Chemistry

Deane Mosher, Professor, Biomolecular Chemistry

Sebastian Bednarek, Professor, Biochemistry

Marisa Otegui, Professor, Botany

*Dedicated to Mimi, Jim, Lynna, Harry, Sina,*

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## ABSTRACT

The Endosomal Sorting Complexes Required for Transport (ESCRT) machinery facilitates several membrane remodeling events that occur throughout a cell which are essential for protein signaling and degradation, repair of organelle membranes, and cell division. One example of an ESCRT-mediated membrane remodeling event is the formation of a multivesicular endosome (MVE) within the endolysosomal pathway, where ESCRT complexes act sequentially to cluster protein cargoes embedded in the endosomal membrane and package them into intralumenal vesicles (ILVs). Although the proper timing and localization of the ESCRT machinery in MVE biogenesis as well as other ESCRT-mediated membrane remodeling events are critical to the cell, many open questions remain about how ESCRT recruitment and assembly are regulated. In this thesis, I will focus on one ESCRT regulation strategy, the stabilization of the ESCRT-III complex by Lgd/CC2D1 proteins during MVE biogenesis.

The Lgd/CC2D1 protein family is evolutionarily conserved across higher eukaryotes known to play a role in ESCRT regulation during MVE biogenesis. In this thesis, I present evidence that Lgd/CC2D1 stabilizes the major ESCRT-III subunit, CHMP4/Vps32, on the endosomal membrane, and that this stabilization is required for efficient ESCRT function. In Chapter 1, I describe the ESCRT machinery and its role in MVE biogenesis as well as provide necessary background on what is known about Lgd/CC2D1 proteins in relation to the ESCRT machinery and in other biological contexts. In the second chapter, I present the results of my investigation of Lgd/CC2D1-mediated ESCRT regulation at the MVE. I show that the *C. elegans* Lgd/CC2D1 homolog LGD-1 localizes to endosomes with similar distribution to members of the

ESCRT machinery, and that this endosomal localization is at least partially dependent on early acting ESCRT complexes and the interaction of LGD-1 with phospholipids. Additionally, I demonstrate that loss of LGD-1 dramatically decreases recruitment of VPS32/CHMP4 to endosomes, ultimately leading to a decrease in ILV formation. In the third chapter, I discuss my journey to and through graduate school more broadly and give a general audience overview of my work as part of a collaboration with the Wisconsin Initiative for Science Literacy.

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**LIST OF ABBREVIATIONS**

Aki1, Akt kinase (PDK1) interacting protein 1

ALIX, ALG-2 interacting protein X

ASD, autism spectrum disorder

ATP, adenosine triphosphate

BROX, BRO1-domain containing protein

CC2D1, Coiled-coil and C2 domain-containing protein 1

CHMP, charged multivesicular body protein

DM14, *Drosophila melanogaster* 14

DRD2, dopamine D2 receptor

DUIM, double ubiquitin-interacting motif

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

EMR-1, emerin homolog 1

ESCRT, endosomal sorting complex required for transport

Freud-1/-2, Five prime Repressor Under Dual repression -1/-2

FYVE, Fab1p, YOTB, Vac1p, and EEA1

HD-PTP, His domain-containing protein tyrosine phosphatase

ILV, intralumenal vesicle

INM, inner nuclear membrane

LEM, LAP2, emerin, MAN1 domain

Lgd, lethal (2) giant discs

MDD, major depressive disorder

MIM, MIT-interacting motif

MIT, microtubule interacting and transport

MVE, multivesicular endosome

NE, nuclear envelope

PDK1, pyruvate dehydrogenase kinase 1

PI3P, phosphatidylinositol 3-phosphate

RIG-I, Retinoic acid-inducible gene I

RLR, RIG-I-like receptor

TAPE, TBK1-associated protein in endolysosomes

TLR4, Toll-like receptor 4

UEV, ubiquitin E2 variant

UIM, ubiquitin-interacting motif

VHS, Vps27, HRS, STAM

VPS, vacuolar protein sorting

VSL, Vta1/SBP1/LIP5

# Chapter 1. ESCRT function in the endolysosomal pathway and the role of Lgd/CC2D1

**ABSTRACT**

The Endosomal Sorting Complexes Required for Transport (ESCRT) machinery is required for many essential membrane remodeling events that occur throughout a cell and is comprised of five protein complexes, ESCRT-0,-I,-II,-III, and the VPS4 AAA-ATPase. ESCRT-dependent membrane remodeling events include multivesicular endosome (MVE) biogenesis, nuclear envelope reformation and repair, cytokinetic abscission, and repair of the plasma membrane and lysosomal membrane. Among ESCRT-dependent remodeling events, only ESCRT-III and VPS4 are universally required, as these complexes perform the final membrane constriction and scission steps. Although ESCRT-III is required for membrane remodeling, the composition of the complex varies between contexts and ESCRT-III assembly is regulated remains an open area of study. One recently described regulation strategy occurs through the action of the evolutionarily conserved Lgd/CC2D1 protein family, which interacts with CHMP4/Vps32, one of the core ESCRT-III subunits. Here, I discuss how ESCRT machinery remodels cellular membranes and outline a role for Lgd/CC2D1 proteins in ESCRT regulation, with a focus on MVE biogenesis.

## 1. Introduction

The Endosomal Sorting Complex Required for Transport (ESCRT) machinery is composed of five complexes (ESCRT-0-III) and the Vps4 AAA-ATPase. The ESCRT machinery was initially defined in the context of multivesicular endosome (MVE) biogenesis, where early acting complexes (ESCRT-0,-I, and -II) recognize and cluster ubiquitinated integral membrane cargoes on the endosomal limiting membrane, then recruit ESCRT-III and Vps4 to generate an intralumenal vesicle (ILV). Cargo clustering on the limiting membrane leads to initial membrane deformation, followed by ESCRT-III and Vps4-mediated constriction of the bud neck, leading to scission. Internalized cargoes include growth receptors from the plasma membrane and integral membrane proteins from the Golgi apparatus, which proceed from the MVE either to be degraded by fusion with the lysosome, secreted via exocytosis, or recycled.

Since its initial characterization in the context of the endolysosomal pathway, the ESCRT machinery has been shown to play a role in several topologically similar membrane remodeling events including cytokinetic abscission, viral budding, and repair of organellar membranes including the nuclear envelope, plasma membrane, and lysosome[1,2]. In each of these contexts, ESCRT-III is assembled on the target membrane, where it is subsequently re-organized by Vps4 to mediate membrane scission or sealing. In this chapter, I will focus on the canonical function of ESCRT proteins, MVE biogenesis, and the regulation of ESCRT at the MVE by Lgd/CC2D1 proteins.

## 2. ESCRT-mediated membrane remodeling during MVE biogenesis

The early acting ESCRT complexes, ESCRT-0, -I, and -II, are thought to assemble sequentially on the endosomal limiting membrane, functioning together to cluster ubiquitinated cargoes and recruit ESCRT-III. ESCRT-0, a 1:1 heterodimer of Hrs and STAM, is targeted to the limiting membrane of early endosomes through interactions between the Hrs Fab1p, YOTB, Vac1p, and EEA1 (FYVE) domain and phosphatidylinositol 3-phosphate (PI3P) as well as a clathrin-interacting domain in Hrs[3]. Both Hrs and STAM contain ubiquitin-binding domains, a double ubiquitin-interacting motif (DUIM) domain in Hrs and UIM and Vps27, HRS, STAM (VHS) domains in STAM, which function to cluster ubiquitinylated cargoes. Although the affinity between ESCRT-0 subunits and ubiquitin, as measured by isothermal titration calorimetry, are relatively weak, ubiquitinated cargoes are anchored in the endosomal membrane and therefore have limited ability to diffuse, suggesting biological relevance of weak solution-based measurements[3,4].

Beyond recognizing ubiquitinated cargoes, ESCRT-0 also functions to recruit subsequent ESCRT complexes to the membrane. At the Hrs carboxyl terminus, a PxxP motif serves to recruit the ESCRT-I heterotetramer via interaction with the ubiquitin E2 variant (UEV) domain of Tsg101[5]. ESCRT-I can possess several distinct compositions that include Tsg101, Vps28, one Vps37 isoform, and either one Mvb12 or UBAP1 isoform[6–8]. Like ESCRT-0, ESCRT-I can also recognize ubiquitinated cargoes, through domains in Tsg101, Mvb12, and UBAP1, and exhibits weak affinity for acidic phospholipids[8,9]. A carboxyl terminal domain within Vps28 functions to recruit the final early-acting ESCRT complex, ESCRT-II[10]. ESCRT-II is a Y-shaped heterotetramer

made up of Vps22, Vps36, and two Vps25 subunits[11]. GLUE domains within Vps36 mediate interactions with ESCRT-I, phospholipids, and ubiquitin[12]. A conserved basic helical domain within Vps22 mediates additional contacts with the endosomal membrane and is required for ESCRT-II endosomal association[11]. Finally, each of the two Vps25 subunits interact with the ESCRT-III subunit Vps20 to initiate assembly of two ESCRT-III filaments simultaneously[13–15].

Although ESCRT-III and Vps4 are universally required for ESCRT-mediated membrane remodeling, many early acting ESCRT complexes are dispensable outside of their canonical role in MVE biogenesis[1]. In addition to recruitment by ESCRT-II, ESCRT-III can also be recruited to membranes through direct interaction with ESCRT-I subunit Tsg101 and/or BRO1 domain proteins such as ALIX, HD-PTP, or BROX[1,16]. This pathway bypasses ESCRT-II and is used in several ESCRT-mediated remodeling events including viral budding, cytokinetic abscission, and others. Finally, in the context of the reforming nuclear envelope (NE), ESCRT-III is recruited via interactions between CHMP7 and LEM domain proteins LEM-2 and EMR-1 anchored in the inner nuclear membrane (INM)[17–19].

The ESCRT-III filament can be made up of eight structurally homologous subunits, CHMP6/Vps20, CHMP4/Vps32, CHMP3/Vps24, CHMP2/Vps2, CHMP5/Vps60, CHMP1/Did2, Ist1, and CHMP7[2]. CHMP6/Vps20, CHMP4/Vps32, CHMP3/Vps24, and CHMP2/Vps2 are traditionally thought of as the four core ESCRT-III subunits[20]. CHMP6/Vps20 is considered the nucleator of ESCRT-III, as in the context of MVE biogenesis it is recruited to the endosomal membrane via its previously described interaction with the ESCRT-II subunit Vps25. CHMP6/Vps20 in turn recruits

CHMP4/Vps32, which is thought to make up the majority of ESCRT-III filaments. CHMP3/Vps24 and CHMP2/Vps2 are associated with the end of the filament[15]. CHMP5/Vps60, CHMP1/Did2, Ist1, and CHMP7 play unique accessory roles in specific ESCRT-mediated membrane remodeling events. For example, Ist1 is required for recruiting spastin to sever microtubules during both cytokinetic abscission and nuclear envelope sealing[21].

ESCRT-III subunits are characterized by their high degree of structural similarity, comprised of at least five alpha helices and one or two microtubule interacting motif (MIM) domains[22]. The amino-terminal helices α1-4 form a four-helix bundle which makes up the ESCRT-III core[22]. The carboxyl-terminal α5 helix plays a regulatory role and can be folded back onto the core in a 'closed' conformation or extended away in an 'open' conformation[23]. Many ESCRT-III subunits are predicted to have a sixth helix based on amino acid sequence, although this helix has not been observed in crystallographic studies, likely due to high flexibility. The conformational state of ESCRT-III subunits varies depending on experimental conditions such as salt concentration and the presence of other ESCRT proteins or membranes. For instance, biochemical studies have suggested that the presence of membrane is required for interaction between Vps25 of ESCRT-II and CHMP6/Vps20[15,24]. Moreover, open and closed conformations do not dictate whether an ESCRT-III subunit is able to form polymers. Truncations of CHMP4/Vps32 containing only α1-4 are constitutively open and form homopolymers in solution[25]. In contrast, Ist1 forms polymers with CHMP1/Did2 in the closed state[26]. At the carboxyl terminus of ESCRT-III subunits, MIM domains mediate interaction with MIT domains on Vps4 and its cofactor, Vta1.

The final ESCRT complex, Vps4, is a hexameric AAA-ATPase which remodels ESCRT-III filaments, recycles ESCRT-III subunits from membranes, and is the only complex that requires input of ATP. Vps4 contains one amino-terminal MIT domain, which can independently associate with MIM1 and MIM2 domains present on ESCRT-III subunits[27,28]. The interaction between Vps4 and its cofactor, Vta1, is mediated by the β-domain of Vps4 and the VSL domain of Vta1[29]. This interaction is important for stabilizing Vps4 oligomerization and may increase enzymatic activity[30]. Vta1 also possesses two amino-terminal MIT domains which mediate additional interactions with ESCRT-III subunits[31].

Although Vps4 is required for ESCRT-III mediated membrane remodeling, a mechanistic understanding of how Vps4 contributes to membrane constriction via interaction with ESCRT-III remains to be resolved. One proposed model is the buckling model, where constriction and scission occur as a result of ESCRT-III filaments buckling to relieve tension of over- and underbent regions of the spiral[22,32,33]. In the buckling model, the ESCRT-III filament exists as a flat spiral filament, resembling structures observed by electron microscopy of ESCRT-III subunits reconstituted on membrane bilayers. Within the spiral are regions where the polymer is either overbent (near the center), underbent (near the periphery), or in a relaxed state. Strain in the spiral is released through spontaneous extension of the spiral in a third dimension, creating a helical tube. Vps4 then facilitates the transition from a tubular confirmation back to a planar conformation, bringing the membrane closer together. This model is supported by evidence that Vps4 can assemble on the inner surface of ESCRT-III-coated membrane nanotubes and facilitate tubule scission in an ATP-dependent manner [23,34].

Alternatively, the dynamic exchange model hypothesizes the ESCRT-III filament is gradually constricted by Vps4 mediated subunit exchange, where subunits with lower preference for curvature are replaced with subunits with higher preference for increased curvature[22]. Notably, this model is supported by recent observation that Vps4 and ESCRT-III subunits dissociate from HIV bud necks ~20s prior to virion release[35].

**3. Role of Lgd/CC2D1 proteins in ESCRT function**

ESCRT-III subunits must be rapidly recruited and assembled into filaments on diverse cellular membranes to perform the membrane remodeling tasks critical for cell viability and division. An aspect of ESCRT function which remains poorly understood is how ESCRT-III assembly is spatiotemporally regulated. Past work has illuminated several strategies to modulate ESCRT-III polymerization including specific protein-protein interactions which determine ESCRT-III recruitment and/or polymerization competency. One major protein-protein interaction that has been shown to have an important regulatory role in ESCRT-III assembly is that which occurs between CHMP4/Vps32 and Lgd/CC2D1 proteins.

Lgd/CC2D1 homologs are conserved across higher eukaryotes including *D. melanogaster* (Lgd), mammals (CC2D1A/B), and *C. elegans* (LGD-1), but absent from *S. cerevisiae*. Originally identified in fruit flies, Lgd mutants were found to cause embryonic lethality and dramatic expansion in wing imaginal disc cells[36]. Later evidence demonstrated these phenotypes were induced by misactivation of Notch, a master regulator of development and an endolysosomal cargo[37,38]. Upon ligand binding, Notch is trafficked through the endolysosomal pathway, where it undergoes a series of

proteolytic cleavages to become activated. Additionally, full-length, inactive Notch is continuously trafficked through MVEs to be degraded in the lysosome. Upon loss of function of several ESCRT components including Tsg101, Vps25, or CHMP4/Vps32, Notch can become activated independently of ligand binding, resulting in defective developmental growth phenotypes[38–40]. Together, these data suggested a role for Lgd/CC2D1 proteins in ESCRT-mediated endolysosomal transport.

Lgd/CC2D1 homologs are characterized by the presence of four N-terminal DM14 (*D. melanogaster*-14) domains (DM14-1:DM14-4) followed by a C-terminal C2 domain. The DM14 domains have been shown to mediate protein-protein interactions including with ESCRT-III subunits CHMP4/Vps32 (DM14-3) and CHMP7 (DM14-4)[41,42]. Lgd interaction with CHMP4/Vps32 inhibits polymerization in solution[41]. A crystal structure of a fusion between Lgd DM14-3 and the CHMP4/Vps32 core shows the DM14-3 domain forming a helical hairpin that associates with a basic region of CHMP4/Vps32[43]. This association physically masks the CHMP4/Vps32 homopolymerization surface, demonstrating how Lgd inhibits CHMP4/Vps32 polymerization structurally. At the carboxyl terminus, the Lgd/CC2D1 C2 domain is likely involved in structural stability of the protein and localization via interaction with phospholipids. Lgd has been shown to interact with several phospholipids, including PI(3)P, PI(4)P, and PI(5)P, with dramatic reduction in lipid interaction when the C2 domain is removed[37]. Mammalian CC2D1A and CC2D1B have also been shown to associate with cellular membranes[44].

Several studies have demonstrated a role for Lgd/CC2D1 in overall ESCRT function. In *D. melanogaster*, null *lgd* mutants result in defective endosomal sorting of

cargoes such as Notch and epidermal growth factor receptor (EGFR) and exhibit lethality in a pupal stage[37]. This defect is exacerbated by partial CHMP4/Vps32 loss of function and can be rescued by exogenous expression of either CC2D1A or CC2D1B or constructs minimally including DM14-3 and a C2 domain[38,43,45]. In mammalian cell models, depletion of both CC2D1A and CC2D1B results in enlarged MVEs and irregular sorting of endosomal cargoes such as EGFR and Toll-like receptor 4 (TLR4)[44,46]. Deletion of CC2D1B has also been shown to affect NE integrity[42]. In mouse models, both systemic and central nervous system-specific deletion of CC2D1A result in death of pups at birth due to asphyxiation, although no gross morphological defects in the respiratory system are observed[44,47]. Systemic CC2D1B deletion, however, has no apparent impact on mouse viability[44]. Electron microscopy studies of mouse embryonic fibroblasts derived from these mice show enlarged MVEs in CC2D1A deletion but not CC2D1B deletion[44]. This data suggests Lgd/CC2D1 proteins serve overlapping but functionally distinct roles in mammalian organisms.

In addition to MVE biogenesis, Lgd/CC2D1 proteins have also been shown to play a role in another ESCRT-mediated membrane remodeling event, NE reformation. Both CC2D1B and *C. elegans* LGD-1 have been shown to interact with CHMP7 *in vitro*[42]*.* As a non-core ESCRT-III subunit, CHMP7 has been shown to be largely dispensable for MVE biogenesis but plays a larger role in ESCRT-mediated NE reformation and repair[19]. Although few studies have been published on the role of Lgd/CC2D1 proteins in NE reformation and repair, it was recently reported that depletion of CC2D1B results in defective NE sealing during cell division, and that under these conditions recruitment of ESCRT-III becomes uncoordinated[42]. In Chapter 3,

further evidence supporting a role for Lgd/CC2D1 at the NE in *C. elegans* will be discussed.

Together, these data support a role for Lgd/CC2D1 in regulating ESCRT-III assembly, although the precise mechanism is yet to be understood. This is significant because Lgd/CC2D1 regulation of ESCRT-III represents a novel regulatory strategy for the ESCRT machinery. While Lgd/CC2D1 has been shown to act in only two ESCRT-mediated membrane remodeling contexts—MVE biogenesis and NE reformation—the interaction between Lgd/CC2D1 and CHMP4/Vps32 raises the possibility of a wider role in ESCRT-III regulation.

**4. Lgd/CC2D1 functions outside of ESCRT machinery**

In addition to their role in regulating ESCRT-III assembly, Lgd/CC2D1 proteins have been studied in several other areas of biology. Mammalian Lgd/CC2D1 proteins CC2D1A and CC2D1B have been described to have several unique functions, including modulating transcription of the serotonin receptor 5-HT1A, serving as a scaffold for the PDK1/Akt pathway, and regulation of innate immune response pathways.

Loss of Lgd/CC2D1 function has been implicated in several neurological and mental health disorders, including intellectual disability (ID), autism spectrum disorder (ASD), schizophrenia, and major depressive disorder (MDD) in humans and rodent models[48–50]. These disorders are generally thought to be linked to Lgd/CC2D1 proteins' role as a transcriptional repressor of both the serotonin receptor 5-HT1A and dopamine D2 receptor DRD2. This role is primarily mediated by CC2D1A, also called Freud-1 in this context. CC2D1A/Freud-1 binds to a dual repressor element (DRE) in the *Htr1a*

promoter and within the second intron of *DRD2 and* has been shown to mediate transcriptional repression via either histone deacetylase (HDAC)-dependent or HDAC-independent pathways in non-neuronal and neuronal cell types, respectively[48,51,52]. Null mutations in *Cc2d1a* associated with ID, ASD, and seizures, which have been reported in several families[49,50,53]. Post-mortem analysis of prefrontal cortices from male MDD patients have found a significant decrease in CC2D1A protein levels[49,50,53]. Although decrease in CC2D1A/Freud-1 was also observed in female MDD patients, this decrease has not been found to be significant[53]. In rodent models, loss of CC2D1A results in changes in behavior that resemble anxiety, depression, and defects in other social behaviors[54]. CC2D1B/Freud-2 has also been shown to bind the *Htr1a* promoter DRE[55]. Unlike CC2D1A/Freud-1, loss of CC2D1B/Freud-1 in mice is not associated with changes in social behaviors or anxiety-like behaviors. Instead, CC2D1B/Freud-1 deletion was shown to negatively affect memory, with the strongest impact observed in males[56]. Differences in CC2D1A/B-mediated transcriptional regulation of 5-HT1A and DRD2 expression may explain sex-differences observed in neurological disorders such as MDD. Future work is needed to better understand the interplay between 5-HT1A, DRD2, CC2D1A, and CC2D1B in contributing to neurological disorders.

CC2D1A has also been described as a scaffold protein for the EGFR/PDK1/Akt signaling pathway, where it is referred to as Akt interacting protein 1 (Aki1). In this pathway, PDK1 activates Akt in response to EGF-stimulation, leading to phosphorylation of downstream targets and promoting cell survival and proliferation[57]. PDK1 is a constitutively active kinase and is thought to be regulated by proximity to its targets. CC2D1A/Aki1 can interact with PDK1 independently of EGF stimulation[57].

However, in EGF-stimulated conditions, CC2D1A/Aki1 forms a complex with EGFR and Akt, and interaction is required for PDK1-mediated activation of Akt[57]. Importantly, PDK1/Akt signaling is implicated in tumorigenesis. CC2D1A/Aki1 has been shown to interact with constitutively active EGFR mutants identified in lung cancers independently of EGF stimulation[58]. Moreover, increased expression of CC2D1A/Aki1 has been observed in both lung and pancreatic cancer patient samples[58,59].

A third alternative function of CC2D1A is in the regulation of innate immune responses mediated by a subset of Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). TLRs present in the plasma membrane and endolysosome recognize common microbial components. CC2D1A directly interacts with factors downstream of innate immune receptors including the TLR3/TLR4 adaptor Trif and IKK-related kinases TBK1 and IKK-epsilon, which link TLRs with RLRs to stimulate production of Type I interferons[60]. CC2D1A has been shown to activate several pathways downstream of TLR3 and TLR4 innate immune receptors including TBK1, NF-κB, and ERK, resulting in production of inflammatory cytokines and interferon beta[60]. In the context of viral infection, RLRs recognize viral RNA in the cytosol and stimulate expression of Type I interferons and other pro-immune factors. Two RLRs, RIG-I and MDA5, have each been shown to form tri-protein complexes with CC2D1A and IPS-1, a mitochondrial RLR adaptor[61]. Loss of CC2D1A results in impaired RLR-mediated interferon response, which is independent of Trif[61]. Like its role in the PDK1/Akt pathway, CC2D1A may act as a scaffold for innate immune receptors and their downstream signaling factors.

## 5. Concluding perspectives

ESCRT-mediated membrane remodeling is a critical process that underlies many of the basic functions of a cell. Yet, many gaps in our fundamental understanding of how ESCRT machinery works remain. These include how Vps4 enzymatic activity remodels and recycles ESCRT-III subunits off membranes, and how various ESCRT-III subunits and Vps4 mediate constriction and final scission of membranes. Other open questions in the field highlighted here are how conformation of ESCRT-III subunits is modulated, how different conformations affect ESCRT-III assembly, and how ESCRT-III subunits localize and assemble on target membranes with the necessary precision of timing. The answers to many of these questions may include the action of Lgd/CC2D1 proteins, and potentially additional factors yet to be identified.

At this time, it is unclear how the many diverse functions of Lgd/CC2D1 proteins are interconnected, if at all. Some roles, such as bridging TLR and RLR immune pathways, theoretically are more directly connected to ESCRT function in that both occur on endosomal membranes and because the ESCRT machinery plays a role in the lifecycle of intracellular pathogens such as *Coxiella burnetii*[62]. Further work is needed to fully understand how Lgd/CC2D1 proteins function across the cell and the degree to which these roles influence each other.

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## Chapter 2. LGD-1 regulates ESCRT-III polymer assembly at multivesicular endosomes to control intralumenal vesicle formation

The work presented in this chapter is under review:

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**ABSTRACT**

Membrane remodeling mediated by heteropolymeric filaments composed of ESCRT-III subunits is an essential process that occurs at a variety of organelles to maintain cellular homeostasis. Members of the evolutionarily conserved Lgd/CC2D1 protein family have been suggested to regulate ESCRT-III polymer assembly, although their specific roles, particularly *in vivo*, remain unclear. Using the *Caenorhabditis elegans* early embryo as a model system, we show that Lgd/CC2D1 localizes to endosomal membranes, and its loss impairs endolysosomal cargo sorting and degradation. At the ultrastructural level, the absence of Lgd/CC2D1 results in the accumulation of enlarged endosomal compartments that contain a reduced number of intralumenal vesicles (ILVs). However, unlike aberrant endosome morphology caused by depletion of other ESCRT components, ILV size is only modestly altered in embryos lacking Lgd/CC2D1. Instead, loss of Lgd/CC2D1 impairs normal accumulation of ESCRT-III polymers on endosomal membranes, likely slowing the kinetics of ILV formation. Together, our findings suggest a role for Lgd/CC2D1 in the recruitment and/or stable assembly of ESCRT-III subunits on endosomal membranes to facilitate efficient ILV biogenesis.

Keywords: multivesicular endosome, CC2D1A, CC2D1B, endolysosomal trafficking

## INTRODUCTION

The Endosomal Sorting Complex Required for Transport (ESCRT) machinery has been implicated in membrane remodeling events at numerous intracellular sites, including endosomes, lysosomes, autophagosomes, plasma membrane, endoplasmic reticulum, and the nuclear envelope (Olmos, 2022; Vietri et al., 2020; Gatta and Carlton, 2019). In particular, ESCRT-III complexes facilitate both membrane bending and scission, likely through their ability to form dynamic filaments that are capable of binding to lipid bilayers with high affinity, while simultaneously undergoing constriction (Migliano et al., 2022; Pfitzner et al., 2021). Unlike other molecular machines that promote membrane severing reactions, ESCRT-III has been suggested to act in two distinct orientations. At multivesicular endosomes (MVEs), ESCRT-III functions within the necks of nascent intralumenal vesicles (ILVs) to mediate their narrowing, eventually resulting in the deposition of ILVs into the endosome lumen for degradation (Frankel and Audhya, 2018). In a similar manner, recruitment of ESCRT-III to small holes in the plasma membrane created by extracellular damage enables lipid bilayer resealing and synchronized shedding of wounded membrane away from the cytoplasm (Zhen et al., 2021; Jimenez et al., 2014). Acting in an opposite orientation, ESCRT-III has also been implicated in the fission of membrane tubules, both on endosomes and the inner nuclear membrane, which are released into the cytoplasm and nucleoplasm, respectively (Shankar et al., 2022; McCullough et al., 2015; Allison et al., 2013). How the recruitment and dynamics of ESCRT-III complexes are regulated at these different sites remains poorly understood, but likely relies upon upstream components of the

ESCRT machinery, as well as accessory factors that have been shown to interact with ESCRT-III subunits (Olmos, 2022).

In mammals, twelve ESCRT-III subunits (CHMP1A-CHMP7 and Ist1) have been identified, some of which possess overlapping roles at membrane remodeling sites. All exhibit structural similarities to one another, including the presence of multiple alpha helices, which have been shown to mediate homo- and hetero-polymerization between individual subunits. In some cases, regulation of this assembly process has been suggested to rely on conformational changes that expose binding interfaces (Olmos, 2022; Migliano, 2022). As an example, inactive CHMP3 appears to exist in a closed state, where five of its alpha helices are packed against one another to prevent polymerization (Lata et al., 2008; Muziol et al., 2006). Transition to an open conformation relieves autoinhibition, enabling interaction with CHMP2 family members (Bajorek et al., 2009). Repeating units of CHMP2-CHMP3 heterodimers co-assemble to form helical filaments on lipid bilayers, which can additionally interact with other ESCRT-III polymers, as well as the Vps4 ATPase, to ultimately drive membrane remodeling (Azad et al., 2022; Alqabandi et al., 2021; Bertin et al., 2020; Mierzwa et al., 2017). However, it remains unknown how conformational changes in individual ESCRT-III subunits are triggered to promote this cascade of events, nor is it well understood how their local concentrations are maintained at membrane surfaces to sustain progressive bilayer bending.

An alternative model suggests that ESCRT-III subunits exist in a dynamic equilibrium between open and closed states and leverage membrane binding and/or interactions with other factors to stimulate polymerization, while precocious filament

assembly at inappropriate sites including the cytoplasm is prevented by a distinct set of regulators (Pfitzner et al., 2021; McMillan et al., 2017; Tang et al., 2016; Schuh et al., 2015). In particular, members of the conserved Lgd/CC2D1 (Lgd) family have been demonstrated to bind directly to CHMP4 isoforms, occluding the interface used normally to form homo-polymers, without impacting their conformational states (Baeumers et al., 2020; McMillan et al., 2017; Martinelli et al., 2012). All Lgd proteins exhibit a similar architecture, with a series of DM14 (*D. melanogaster*-14) motifs in their amino-termini, which bind to a subset of ESCRT-III subunits, followed by a C2 domain that directs localization and stability (Baeumers et al., 2022; McMillan et al., 2017; Drusenheimer et al., 2015; Gallagher et al., 2012). Loss of function mutations in Lgd cause defects in endosomal cargo sorting, leading to lethality at the early pupal stage in *Drosophila*, which has been attributed, at least in part, to excessive activation of the Notch signaling pathway (Gallagher et al., 2012; Jaekel and Klein, 2006; Childress et al., 2006). Notably, overexpression of CHMP4 could partially rescue this phenotype, enabling animals to reach adulthood, although the mechanism underlying this suppression remains unclear (Baeumers et al., 2020). In mammals, simultaneous deletion of both genes encoding Lgd isoforms (CC2D1A and CC2D1B) causes early embryo lethality, and depletion studies indicate that they possess partially overlapping roles in regulating degradative cargo sorting within the endolysosomal system (Zamarbide et al., 2018; Deshar et al., 2016; Drusenheimer et al., 2015; Zhao et al., 2011). Additionally, CC2D1B has also been shown to regulate the timing of ESCRT-III assembly at the nuclear envelope as it reforms following mitosis. Specifically, loss of CC2D1B results in the premature polymerization of CHMP4 protomers at gaps in the nuclear envelope,

leading to defects in its morphology and function (Ventimiglia et al., 2018). Together, these findings suggest that Lgd isoforms serve as molecular timers for ESCRT-III filament formation, helping to ensure the proper spatiotemporal activity of ESCRT-mediated membrane remodeling, although our mechanistic understanding of this regulatory role remains incomplete.

Here, we take advantage of the newly fertilized *C. elegans* zygote as a model system to further define the roles of Lgd in regulating ESCRT-III assembly and function. We demonstrate that Lgd localizes to early endosomes that harbor components of the ESCRT machinery rapidly after the oocyte-to-embryo transition, when de novo MVE biogenesis is initiated. Additionally, similar to prior findings (Baeumers et al., 2020), we find that loss of Lgd slows the rate of degradative cargo sorting and reduces the kinetics of ILV biogenesis, resulting in the dilation of endosomes. Surprisingly, the few ILVs that form in the absence of Lgd are morphologically similar to those found in control animals, exhibiting only a modest increase in diameter, suggesting that ESCRT-III function remains largely intact under these conditions. However, we find that loss of Lgd reduces the concentration of CHMP4 on endosomes, providing a potential explanation for the kinetic delay in cargo degradation. Our data support a model in which Lgd acts to stabilize levels of CHMP4 at endosomal microdomains from which ILV formation occurs.

## RESULTS

### Lgd localizes to endosomes following the oocyte-to-embryo transition

Based on sequence analysis, the nematode *C. elegans* expresses a single member of the Lgd/CC2D1 protein family that is encoded by Y37H9A.3, which we refer to as Lgd. To determine its distribution during early development, we used CRISPR/Cas9-mediated genome editing to append a GFP tag onto the amino-terminus of the endogenous protein. Live imaging of the resulting strain (PHX1889) using spinning disk confocal microscopy revealed a mainly cytoplasmic localization of GFP-Lgd, although soon after fertilization, we found that it accumulated at numerous punctate structures that were highly dynamic near the periphery of zygotes, which was reminiscent of the distribution of early endosomes (Wang and Audhya, 2014) (**Figure 2.1A and Supplemental Video 2.1**). To investigate this idea further, we examined the relative localizations of Lgd and the Rab5 GTPase, which marks early endosomes (Chavrier et al., 1990), and found that on average, more than 50% of Lgd-positive structures also harbored Rab5 (**Figure 2.1, B and C**). These data indicate that Lgd is at least in part an endosomal protein.

Early-acting components of the ESCRT machinery, including the ESCRT-I subunit Tsg101, that function upstream of ESCRT-III in the formation of MVEs similarly associate with Rab5-positive endosomes (Audhya et al., 2007a) (**Supplemental Figure S2.1, A and B**). To examine the degree of overlap between Lgd and the ESCRT machinery, we generated animals co-expressing GFP-Lgd and mCherry-STAM, a component of the ESCRT-0 complex. On average, we found that Lgd co-localizes with STAM more than 60% of the time, consistent with a role for Lgd in regulating ESCRT

activity on endosomes (**Figure 2.1, D and E**). These findings raised the question of whether the ESCRT machinery itself may be involved in recruiting Lgd to membranes. To test this possibility, we conducted a series of RNA interference (RNAi)-mediated depletion studies targeting key components of the ESCRT-0, -I, -II, and -III complexes, as well as the Vps4 ATPase. In most cases, Lgd retained an ability to associate with endosomes, at least in part, suggesting that additional factors, such as phospholipids that have been suggested to associate with its C2 domain (Gallagher and Knoblich, 2006), are also involved in directing Lgd to these compartments (**Figure 2.2, A-D**). Consistent with this idea, we found that recombinant Lgd, which forms elongated monomers in solution (**Figure 2.2E**), binds directly to liposomes in vitro, in a manner that is dependent on the presence of acidic phospholipids, which are abundant in early endosomes (Arumugam and Kaur, 2017) (**Figure 2.2F**).

Notably, following penetrant depletion of the ESCRT-III subunit CHMP4, we found that Lgd hyperaccumulates on the nuclear envelopes of daughter cells (**Figure 2.3A**). Additionally, upon closer inspection of control embryos, we found that Lgd associates weakly with the periphery of the nucleus as the nuclear envelope begins to disassemble ahead of each mitotic division (**Figure 2.3B**). We speculated that exposure to CHMP7, which we demonstrated previously to localize to the inner nuclear membrane during interphase in *C. elegans* embryos (Shankar et al., 2022), enables transient recruitment of Lgd during nuclear envelope breakdown. Such a scenario is predicated on the existence of a direct interaction between Lgd and CHMP7. To test this idea, we conducted a series of GST pull-down assays, which revealed that Lgd binds CHMP7 specifically through its amino-terminal ESCRT-III-like region (**Figure 2.3C**),

similar to their mammalian homologs (Ventimiglia et al., 2018). We additionally found that Lgd binds directly to the *C. elegans* CHMP4 isoform (**Supplemental Figure S2.1C**), also consistent with previous studies (Martinelli et al., 2012; McMillan et al., 2017). However, we failed to detect interactions between Lgd and any other ESCRT-III subunit (**Supplemental Figure S2.1D**). Taken together, our findings suggest that binding to CHMP4 facilitates retention of Lgd within the cytoplasm. Moreover, in the absence of CHMP4, an interaction with CHMP7 drives redistribution of Lgd to the inner nuclear membrane. Consistent with this idea, deletion of CHMP7 prevents targeting of Lgd to the nuclear envelope, even when CHMP4 levels are reduced via RNAi. Instead, under these conditions, Lgd accumulates at the plasma membrane, suggesting that additional binding partners for Lgd likely exist there (**Supplemental Figure S2.1E**).

**Lgd is required for rapid ESCRT-mediated cargo degradation**

To further investigate the role of Lgd in embryonic development, we used CRISPR/Cas9-mediated editing to create a deletion allele (*hz24*), which lacks coding regions for all four of its DM14 motifs, as well as a significant portion of its C2 domain (**Supplemental Figure 2.1F**). Animals heterozygous for this mutation were allowed to propagate under normal feeding conditions, which revealed that approximately 25% of all progeny failed to mature beyond the L1 larval stage. Sequencing results indicated that these developmentally arrested animals were homozygous for the deletion allele. We also examined growth under a second feeding condition, using bacterial strain HT115 as opposed to OP50 as a food source. To our surprise, this relatively modest change enabled homozygous mutant animals to reach adulthood, which was confirmed

using immunoblot analysis (**Figure 2.4A**). However, homozygous mutant hermaphrodites were all sterile, exhibiting a defect in spermatogenesis, similar to a phenotype observed previously in animals lacking the ESCRT-0 subunit STAM (Govindan et al., 2006). By mating these mutant hermaphrodites with control males, we were able to study the first zygotic divisions in the absence of Lgd, as expression of the male-derived genome does not initiate for several embryonic cell cycles (Seydoux and Dunn, 1997).

Previous studies have identified numerous ubiquitin-modified, membrane-associated cargoes that are substrates for ESCRT-dependent sorting into MVEs (Raiborg et al., 2003). In *C. elegans* embryos, a GFP fusion to caveolin-1 (Cav1) serves as an ideal model cargo, with inactivation of GFP fluorescence serving as a proxy for the timing of ESCRT-mediated degradation (Frankel et al., 2017; Audhya et al., 2007a; Sato et al., 2006). To determine how loss of Lgd affects endosomal cargo sorting, we examined the rate at which Cav1 fluorescence was terminated during early embryogenesis in control and mutant animals. Our findings consistently showed that the absence of Lgd dramatically slowed the kinetics of Cav1 degradation relative to control (**Figure 2.4B**). Instead, Cav1 localized to enlarged membrane compartments in mutant animals, similar to those seen in animals depleted of CHMP4 (Frankel et al., 2017).

To confirm the identity of the sites where Cav1 accumulated in embryos lacking Lgd, we conducted a series of immunofluorescence studies. Consistent with the idea that the loss of Lgd impairs endosomal protein sorting, we found that Cav1 co-localized with components of the ESCRT-0 and ESCRT-I complexes on abnormally large endosomes in mutant animals (**Figure 2.4, C and D**). In contrast, we failed to identify

CHMP4 at the majority of these sites (**Figure 2.4, E and F**), suggesting a defect in its recruitment to endosomal microdomains in the absence of Lgd. Even under conditions where we co-depleted Vps4, which blocks recycling of ESCRT-III subunits from membranes (McCullough et al., 2018), we did not find that CHMP4 hyperaccumulated at the abnormally swollen endosomes that form in embryos lacking Lgd (**Figure 2.4G**). Instead, CHMP4 remained largely cytoplasmic under these conditions. Our findings suggest that Lgd functions in the stable recruitment of CHMP4 to endosomes, as opposed to acting only as an inhibitor of CHMP4 polymerization.

**Lgd regulates the formation of ILVs**

To better define the impact of removing Lgd on the formation of MVEs, we conducted high pressure freezing of intact control and mutant animals, and following freeze substitution and embedding in plastic, negatively-stained thin sections of early one-cell stage embryos were analyzed by electron microscopy. These studies revealed significant differences in the sizes of endosomes and ILVs that form in the presence and absence of Lgd (**Figure 2.5, A-D**). Specifically, we found that the average diameter of endosomes increased by more than 30% when Lgd was eliminated, with a concomitant decrease in the number of ILVs that were identified within them (**Figure 2.5, B and C**). The vast majority of endosomes in embryos lacking Lgd harbored no internal vesicles (more than 75%), with the remainder containing fewer than 5 ILVs in any individual section (**Figure 2.5D**). In contrast, analysis of control embryos showed most MVEs contained at least 1 ILV (more than 75%), and greater than 30% possessed more than 6 ILVs, a population that was entirely absent in mutant embryos (**Figure 2.5D**).

Rigorous analysis of ILVs demonstrated that loss of Lgd causes an approximately 20% increase in their diameter (**Figure 2.5B**). Our previous studies similarly showed that partial depletion of CHMP4 also results in the formation of larger ILVs (Frankel et al., 2017), consistent with a model in which Lgd facilitates its stable recruitment to the endosomal limiting membrane (**Figure 2.6**). Together, our findings suggest that Lgd-mediated regulation of CHMP4 polymerization promotes efficient ILV formation at MVEs to sort and ultimately degrade cargoes.

## DISCUSSION

Since the first descriptions of the ESCRT machinery were published over two decades ago (Katzmann et al., 2001; Babst et al., 2002a, Babst et al., 2002b), substantial efforts have been invested to define the structure and function of individual subunits and complexes to understand how its ability to manipulate membranes is achieved. We now have a comprehensive list of the key players involved, including broad consensus in the field that the ESCRT-III complex promotes membrane bending and scission (Olmos, 2022). However, at a mechanistic level, how ESCRT-III assembly on membranes is orchestrated and properly tuned remains unclear. Given the large number of membrane remodeling processes in which ESCRT-III has been implicated, there likely exist numerous forms of regulation to ensure its timely action at each site. Our studies highlight the importance of ESCRT-III regulation at endosomes, which involves the CHMP4-binding protein Lgd. Despite the ability of Lgd to obscure the key interface necessary for CHMP4 homo-polymerization, we find that it is surprisingly required for promoting CHMP4 association with the endosomal limiting membrane, potentially by maintaining a local pool of subunits for rapid assembly necessary to scaffold the membrane bending process to create ILVs.

Although several studies have attempted to specify the distribution of Lgd in cells, most have depended upon the use of overexpression, making it difficult to resolve Lgd association with cellular membranes above its presence in the cytoplasm (Reiff et al., 2021). Recently, by driving overexpression under its native promoter in Drosophila, a small fraction of Lgd was found to associate with endosomes (Baeumers et al., 2020). In contrast, by leveraging CRISPR/Cas9-mediated genome editing, we have found that

a substantial fraction of Lgd localizes with the early endosome markers Rab5 and STAM in one-cell stage *C. elegans* embryos. At this stage of development, a large number of MVEs are generated de novo, as membrane proteins associated with the oocyte plasma membrane must be rapidly downregulated and replaced (Wang and Audhya, 2014). Elevated flux through the ESCRT pathway during this period likely enhanced our ability to resolve Lgd with other components of the ESCRT machinery at endosomes. Similarly, Lgd association with the nuclear envelope has been challenging to resolve under normal conditions, due to its transient accumulation there during mitosis (Ventimiglia et al., 2018). Strikingly, by depleting CHMP4, we found that Lgd accumulated in a CHMP7-dependent manner at the inner nuclear envelope during interphase. This was surprising, since several lines of evidence suggest that Lgd can associate with both CHMP7 and CHMP4 simultaneously (Ventimiglia et al., 2018; McMillan et al., 2017). The necessity to deplete CHMP4 to visualize stable localization of Lgd to the nuclear envelope in *C. elegans* embryos suggests that in vivo, there exists competition between ESCRT-III subunits for binding to Lgd, at least during mitosis, when CHMP7 enters the cytoplasm and localizes throughout the endoplasmic reticulum (Vietri et al., 2015; Olmos et al., 2016). Further studies will be necessary to understand how Lgd binding to different ESCRT-III factors is regulated, although our biochemical studies suggest that only CHMP4 and CHMP7 are substrates of Lgd.

An additional localization determinant for Lgd lies within its carboxyl-terminal C2 domain. Several studies have shown that the C2 domain of Lgd is necessary for its function and ability to bind membranes, both at endosomes and the nuclear envelope (Gallagher et al., 2006; Troost et al., 2012; Ventimiglia et al., 2018). Consistent with

these studies, we found that *C. elegans* Lgd minimally requires the presence of acidic phospholipids to bind liposomes in vitro, indicative that the role of the C2 domain in membrane binding is likely conserved. Additionally, our depletion studies suggest that Lgd remains capable, albeit weakly, of binding to endosomes, even when upstream components of the ESCRT machinery are absent. These studies suggest that coincident detection of protein- and lipid-binding partners, a common theme in phosphoinositide signaling pathways (Carlton and Cullen, 2005), are necessary to specify the membranes on which Lgd acts. By leveraging coincident detection, Lgd may achieve highly restricted localization patterns in cells, preventing its action at other sites, which could interfere with ESCRT function as opposed to facilitating it. Notably, when both CHMP4 and CHMP7 are absent, Lgd is redistributed in part to the plasma membrane. These studies suggest that additional, potentially lower affinity binding partners for Lgd exist, which await discovery.

Based on previous studies, in concert with our current findings, a role for Lgd at MVEs to regulate degradative cargo sorting via stimulation of ILV biogenesis is clear (Baeumers et al., 2020). Our data show that normal CHMP4 accumulation at the limiting membrane requires Lgd, suggesting that targeting of ESCRT-III subunits likely relies on additional factors beyond canonical upstream components of the ESCRT machinery. Although several mechanisms exist to promote CHMP4 accumulation on membranes, including key roles for CHMP6 at MVEs, CHMP7 at the nuclear envelope, and ALIX at sites of HIV budding, the efficiencies of these processes may be further enhanced by molecules like Lgd (Vietri et al., 2020). One possibility is that a low concentration of Lgd present on cellular membranes serves to maintain a local pool of CHMP4 subunits,

which can rapidly co-assemble into spiral filaments upon demand. In light of our prior work demonstrating that nearly 800 subunits of CHMP4 can homo-polymerize into flat spiral filaments (Shen et al., 2014), it is perhaps unsurprising that additional regulatory mechanisms act in cells to retain individual CHMP4 protomers at sites of filament assembly. In the absence of Lgd, recruitment of CHMP4 to MVEs is reduced, but not eliminated, compromising the rate of ILV formation. Under conditions where rapid downregulation of membrane-bound receptors is necessary, including embryonic and larval development, the loss of Lgd is lethal, as has been observed in mice, *Drosophila*, and now in *C. elegans* (Reiff et al., 2021). Future studies aimed at testing the idea of Lgd serving as a “concentrator” for CHMP4 on membranes will be important to fully understand the mechanisms underlying regulation of ESCRT-mediated membrane remodeling.

**ACKNOWLEDGEMENTS**

This work was supported in part by National Institute of Health Grants R35 GM134865 (to A.A.) and T32 GM07215, as well as National Science Foundation Graduate Research Fellowship (to A.L.C.). Support was also provided by the UW School of Medicine and Public Health Electron Microscope Core facility and the 3D Cell Electron Microscopy Core Facility (supported by S10OD026769). The model figure presented in this manuscript was prepared using BioRender. We thank Siyu Li and Gordon Thompson for their contributions to generating preliminary data and members of the Audhya lab for critically reading this manuscript.

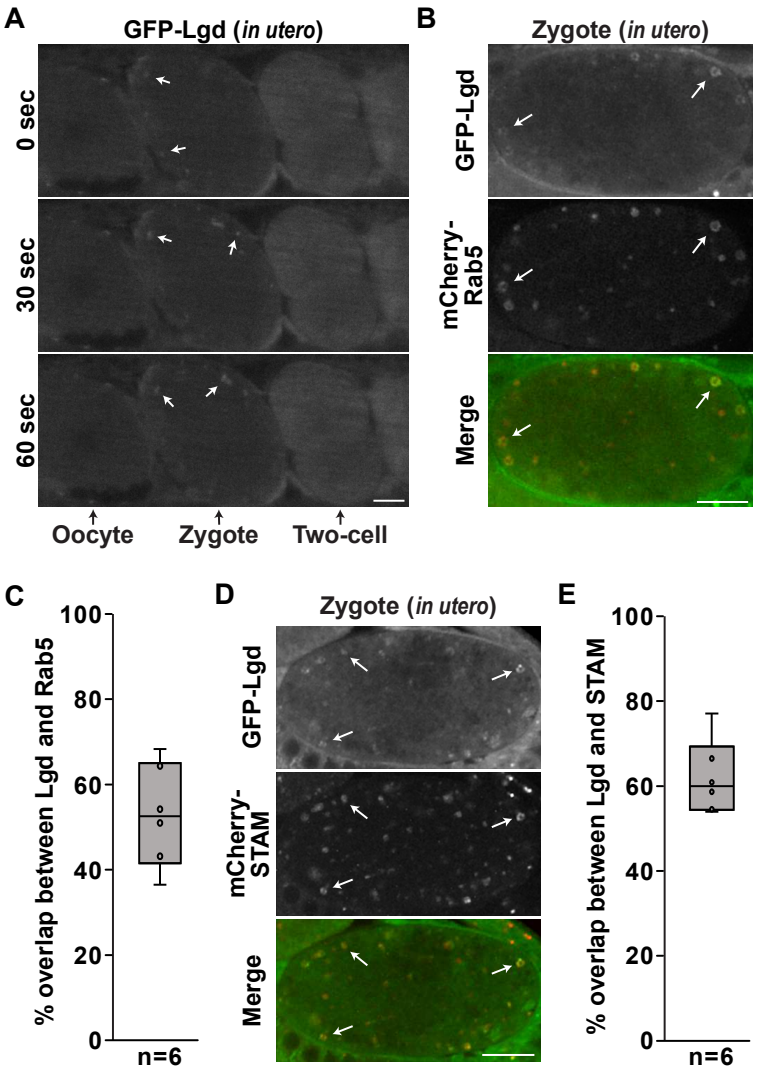

A
GFP-Lgd (in utero)
0 sec
30 sec
60 sec
Oocyte
Zygote
Two-cell
B
Zygote (in utero)
GFP-Lgd
mCherry-Rab5
Merge
C
% overlap between Lgd and Rab5
100
80
60
40
20
0
n=6
D
Zygote (in utero)
GFP-Lgd
mCherry-STAM
Merge
E
% overlap between Lgd and STAM
100
80
60
40
20
0
n=6

**Figure 2.1. Lgd localizes to endosomes during early *C. elegans* embryogenesis.** (A) Representative images taken *in utero* of animals natively expressing a GFP fusion to Lgd using swept-field confocal microscopy are shown at 30-sec intervals (n = 6 animals). Bar, 10 μm. (B and D) Representative images of zygotes co-expressing a GFP fusion to Lgd and a mCherry fusion to Rab5 (B) or STAM (D) were acquired live using swept-field confocal microscopy (n = 6 animals each). Bars, 10 μm. (C and E) Quantification showing the percentage of Lgd-labeled structures that also harbor Rab5 (C) or STAM (E), calculated using Imaris.

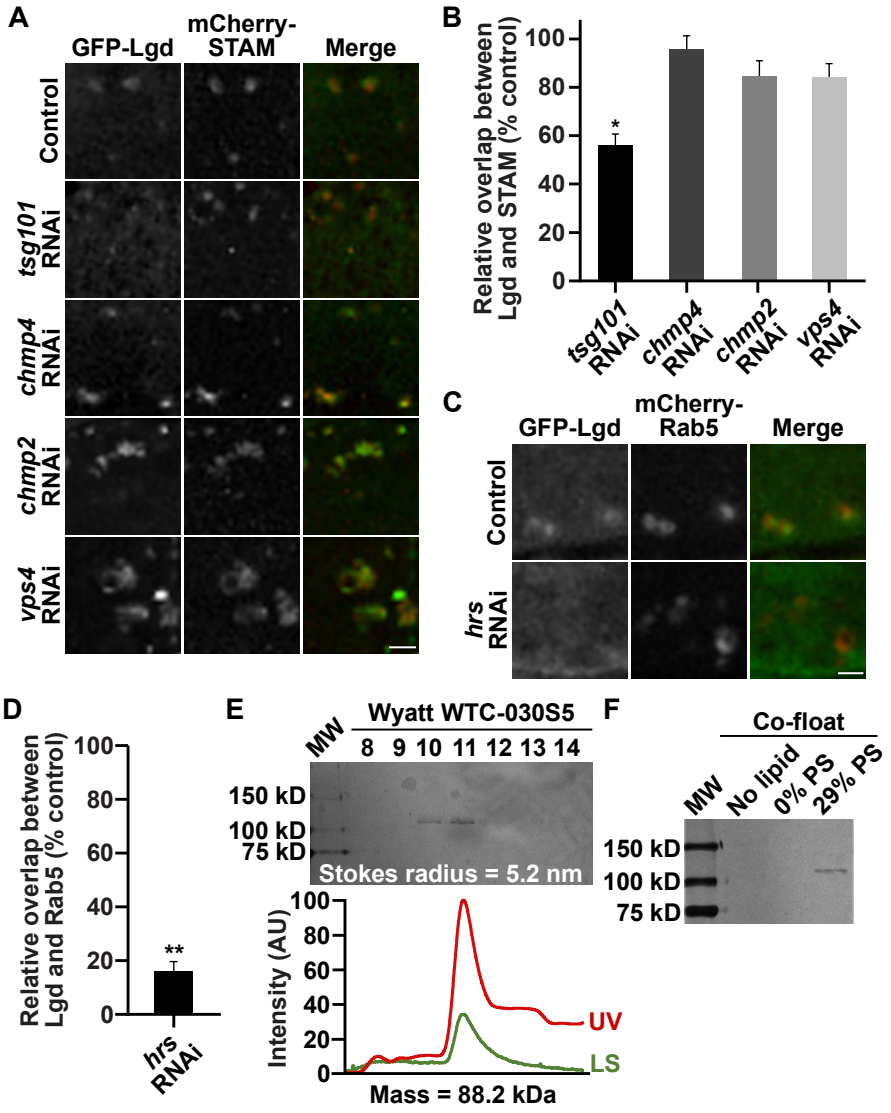

A
GFP-Lgd
mCherry-STAM
Merge
Control
tsg101 RNAi
chmp4 RNAi
chmp2 RNAi
vps4 RNAi
B
Relative overlap between Lgd and STAM (% control)
100
80
60
40
20
0
*
tsg101 RNAi
chmp4 RNAi
chmp2 RNAi
vps4 RNAi
C
GFP-Lgd
mCherry-Rab5
Merge
Control
hrs RNAi
D
Relative overlap between Lgd and Rab5 (% control)
100
80
60
40
20
0
**
hrs RNAi
E
Wyatt WTC-030S5
MW
8 9 10 11 12 13 14
150 kD
100 kD
75 kD
Stokes radius = 5.2 nm
Intensity (AU)
100
80
60
40
20
0
UV
LS
Mass = 88.2 kDa
F
Co-float
MW
No lipid
0% PS
29% PS
150 kD
100 kD
75 kD

**Figure 2.2. Lgd localization to endosomes is dependent on multiple factors.** (A and C) Representative images of zygotes co-expressing a GFP fusion to Lgd and a mCherry fusion to STAM (A) or Rab5 (C) were acquired live using swept-field confocal microscopy (n = more than 5 animals each; zoomed portions of embryos are shown), in the presence and absence of various ESCRT proteins. Bar, 2 μm. (B and D) Quantification showing the relative level of overlap between Lgd and STAM (B) or Rab5 (D) under various depletion conditions as compared to control animals. **$p < 0.01$ and *$p < 0.05$, based on an ANOVA test. (E) Purified recombinant Lgd was separated over a Wyatt gel filtration column coupled to a multi-angle light scattering system and fractions were separated by SDS-PAGE, followed by silver staining, to calculate its Stokes radius (top). UV absorbance (red) and light scattering (green) profiles are also shown (bottom), which were used to calculate the molecular mass of Lgd (n = 3). (F) Recombinant Lgd (4 μM) was resuspended in a buffer containing Accudenz in the presence or absence of liposomes with two distinct lipid compositions and subjected to centrifugation. Protein-lipid complexes that floated to the surface were recovered by hand and subjected to SDS-PAGE, followed by silver staining (n = 3).

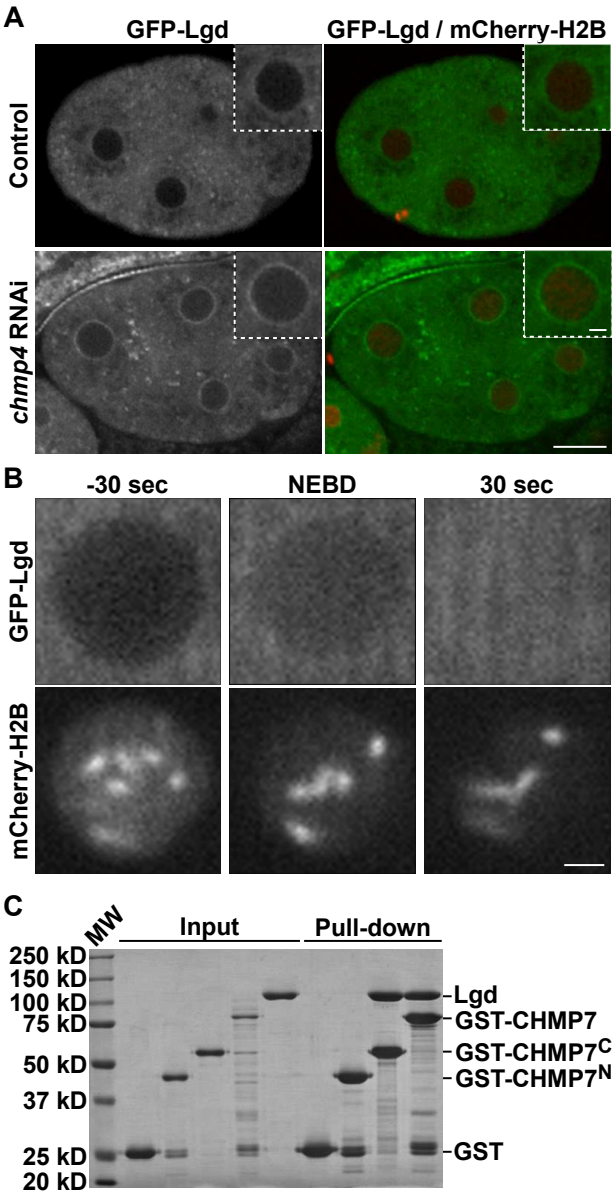

A
GFP-Lgd
GFP-Lgd / mCherry-H2B
Control
chmp4 RNAi
B
-30 sec
NEBD
30 sec
GFP-Lgd
mCherry-H2B
C
MW
Input
Pull-down
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
Lgd
GST-CHMP7
GST-CHMP7C
GST-CHMP7N
GST

**Figure 2.3. Lgd binds to CHMP7 at the inner nuclear membrane.** (A) Representative images of four-cell stage embryos co-expressing a GFP fusion to Lgd and a mCherry fusion to histone H2B were imaged live using spinning disk confocal microscopy in the presence and absence of CHMP4 (n = 5 animals each). Zoomed regions of nuclei are also shown. Bar, 10 μm; inset bar, 2 μm. (B) Representative images of embryos (zoomed to visualize nuclei) co-expressing a GFP fusion to Lgd and a mCherry fusion to histone H2B during nuclear envelope breakdown are shown over time. Bar, 2 μm. (C) GST and GST fusions to CHMP7 (full-length or fragments) bound to glutathione agarose were incubated with full-length recombinant Lgd, and after extensive washing, proteins were eluted and separated by SDS-PAGE followed by staining using Coomassie (n = 3).

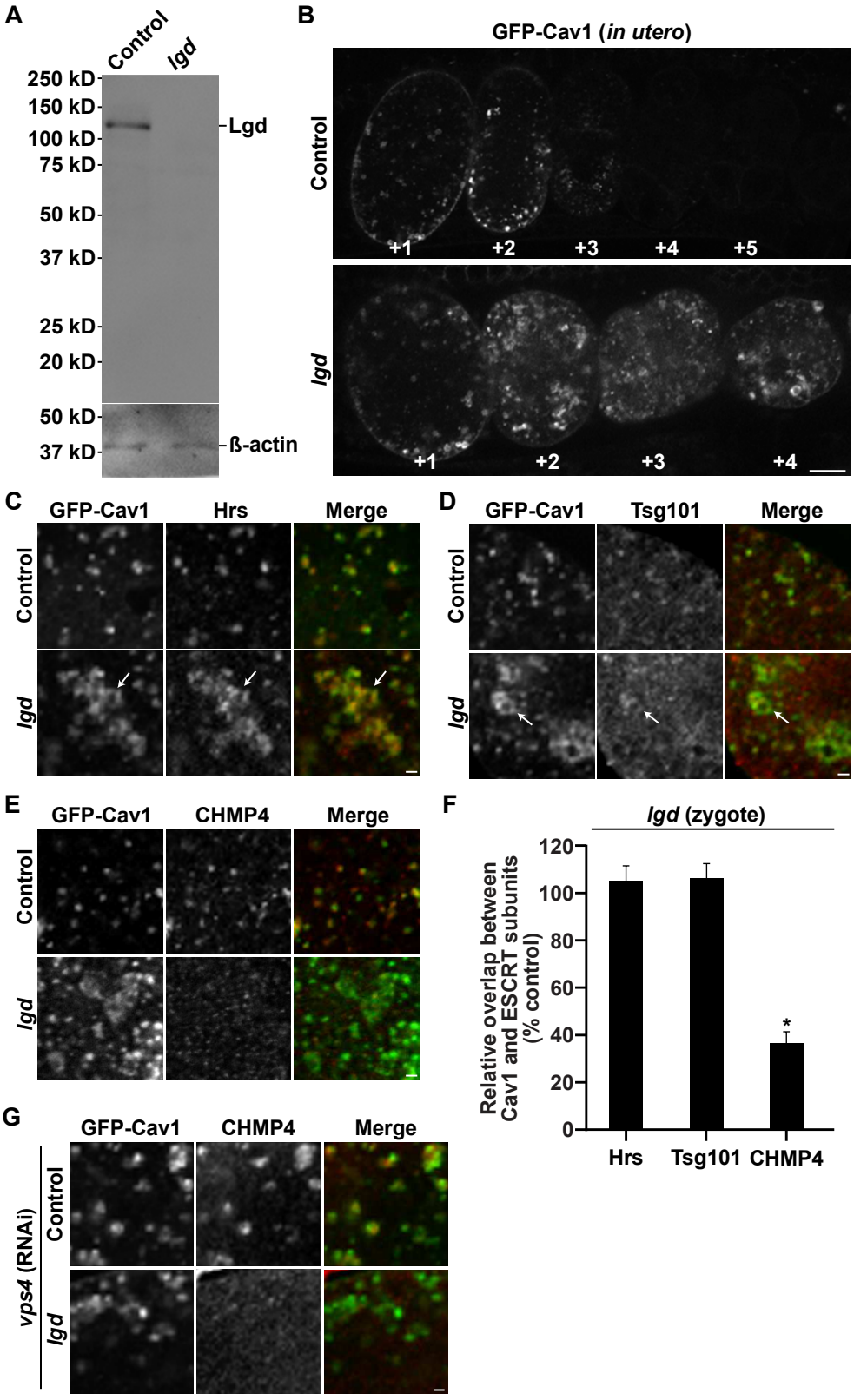
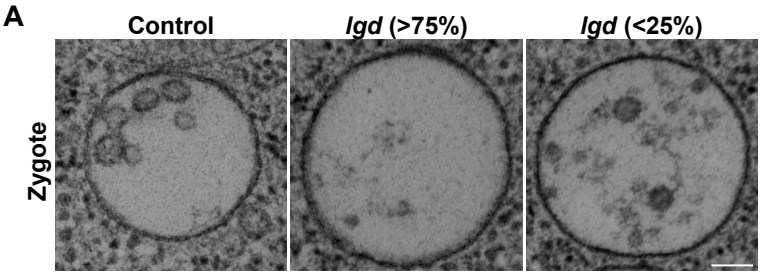

A
Control
lgd
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
50 kD
37 kD
Lgd
ß-actin
B
GFP-Cav1 (in utero)
Control
lgd
+1
+2
+3
+4
+5
+1
+2
+3
+4
C
GFP-Cav1
Hrs
Merge
Control
lgd
D
GFP-Cav1
Tsg101
Merge
Control
lgd
E
GFP-Cav1
CHMP4
Merge
Control
lgd
F
lgd (zygote)
Relative overlap between Cav1 and ESCRT subunits (% control)
120
100
80
60
40
20
0
*
Hrs
Tsg101
CHMP4
G
GFP-Cav1
CHMP4
Merge
vps4 (RNAi)
Control
lgd

**Figure 2.4. Lgd regulates CHMP4 recruitment to MVEs.** (A) Representative immunoblot using antibodies directed against Lgd (top) or β-actin (bottom) of extracts generated from control and *lgd* mutant animals, following separation by SDS-PAGE (n = 3). (B) Representative images taken *in utero* of control and *lgd* mutant animals expressing a GFP fusion to Cav1 using spinning disk confocal microscopy are shown (n = 10 animals each). Bar, 10 μm. (C-E) Representative images of control and *lgd* mutant zygotes expressing a GFP fusion to Cav1 and immunostained using antibodies directed against Hrs (C), Tsg101 (D), or CHMP4 (E) are shown (n = 5 animals each; zoomed portions of embryos are shown). Bars, 2 μm. (F) Quantification showing the relative level of overlap between Cav1 and various ESCRT subunits in animals lacking Lgd as compared to control animals. *$p < 0.05$, based on an ANOVA test. (G) Representative images of control and *lgd* mutant zygotes expressing a GFP fusion to Cav1 and immunostained using antibodies directed against CHMP4 are shown following depletion of Vps4 (n = 5 animals each; zoomed portions of embryos are shown). Bar, 2 μm.




B

| Strain | MVE diameter | ILV diameter |
|---|---|---|
| Control | 364.9 (±17.3) nm (n=72) | 49.2 (±0.6) nm (n=516) |
| *lgd1* | 484.8 (±50.9) nm (n=66) | 59.3 (±5.7) nm (n=28) |

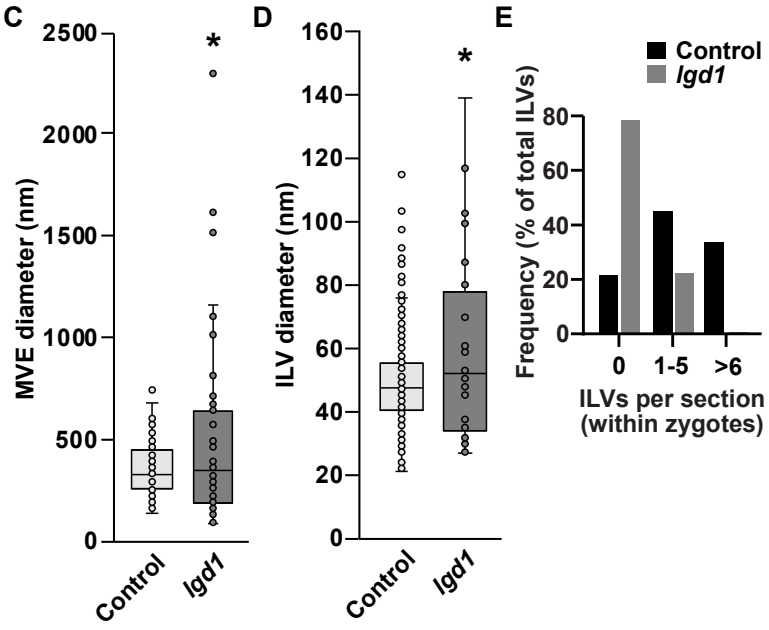


**Figure 2.5. Loss of Lgd impairs ILV formation within MVEs.** (A) Representative images of MVEs in control and *lgd* mutant zygotes following negative staining electron microscopy (n = more than 60 MVEs, each). Bar, 100 nm. (B-D) Quantification of MVE (C) and ILV (D) diameter in control and *lgd* mutant zygotes. *$p < 0.05$, based on a t test. (E) Histogram showing the relative number of ILVs identified in thin sections from control and *lgd* mutant zygotes.

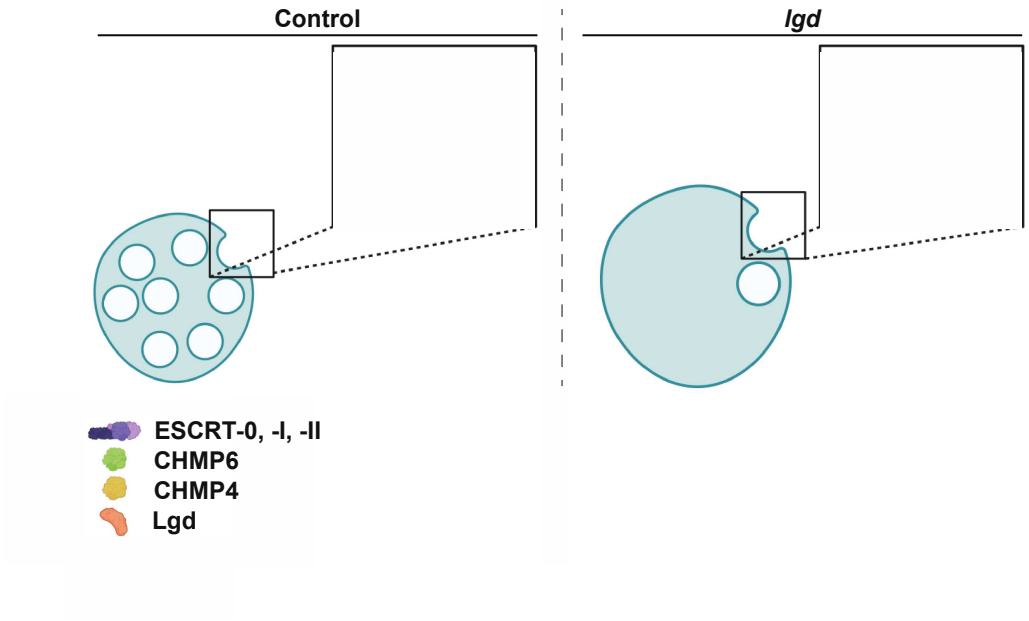
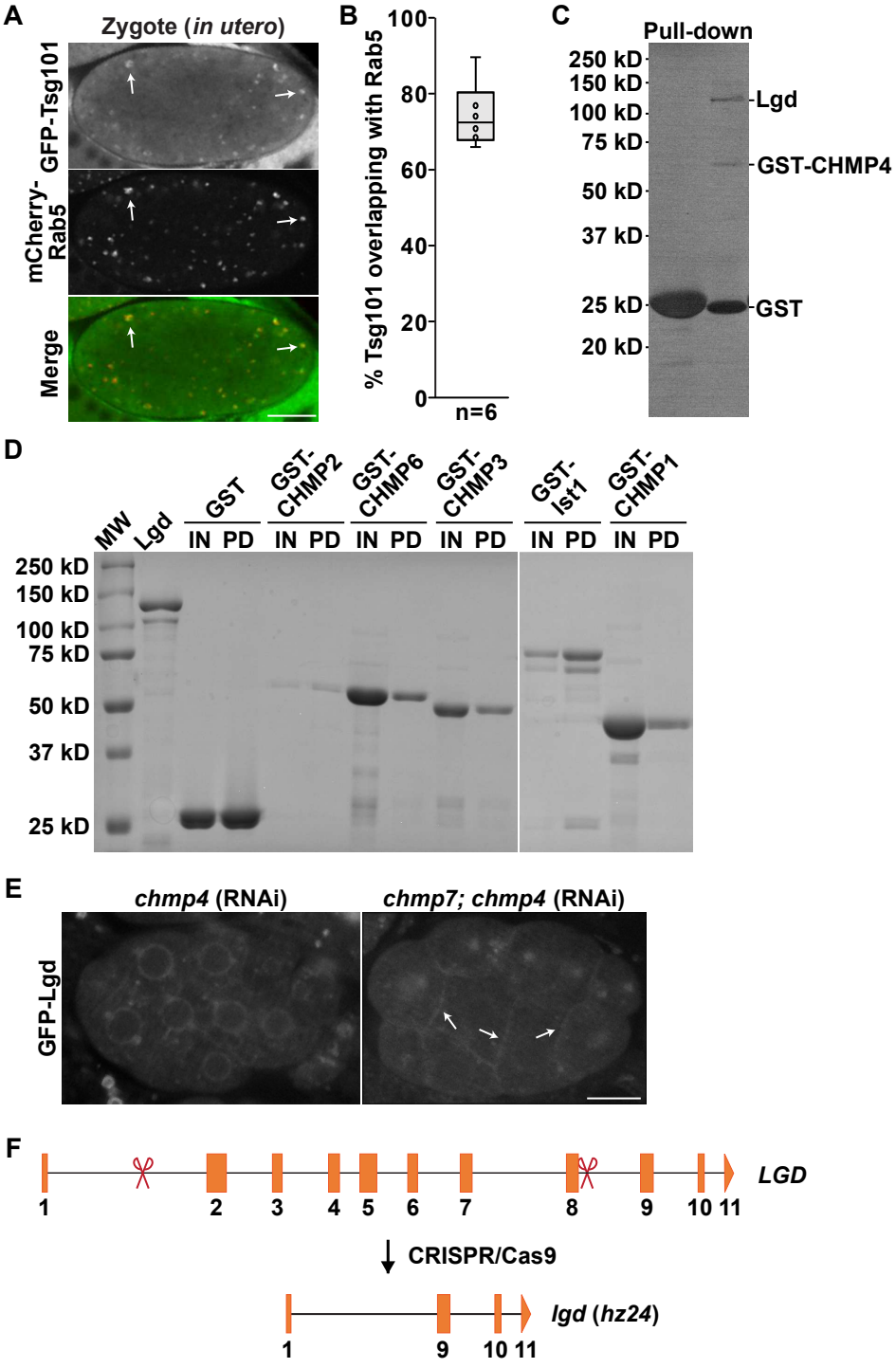

Control
lgd
ESCRT-0, -I, -II
CHMP6
CHMP4
Lgd

**Figure 2.6. Model highlighting a role for Lgd during ILV formation.** Based on our findings, we propose that Lgd functions to stabilize CHMP4 at the limiting membrane of endosomes to facilitate ILV formation (left). In the absence of Lgd, CHMP4 recruitment to MVEs is diminished, slowing the biogenesis of ILVs and decreasing the rate of cargo degradation (right).


A
Zygote (in utero)
GFP-Tsg101
mCherry-Rab5
Merge
B
% Tsg101 overlapping with Rab5
100
80
60
40
20
0
n=6
C
Pull-down
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
Lgd
GST-CHMP4
GST
D
MW
Lgd
GST
GST-CHMP2
GST-CHMP6
GST-CHMP3
GST-Ist1
GST-CHMP1
IN PD
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
E
chmp4 (RNAi)
chmp7; chmp4 (RNAi)
GFP-Lgd
F
LGD
1 2 3 4 5 6 7 8 9 10 11
CRISPR/Cas9
lgd (hz24)
1 9 10 11

**Supplemental Figure 2.1. Lgd interacts with CHMP4 at MVEs.** (A) Representative images of zygotes co-expressing a GFP fusion to Tsg101 and a mCherry fusion to Rab5 were acquired live using swept-field confocal microscopy (n = 6 animals each). Bar, 10 μm. (B) Quantification showing the percentage of Tsg101-labeled structures that also harbor Rab5, calculated using Imaris. (C) GST and a GST fusion to CHMP4 bound to glutathione agarose were incubated with full-length recombinant Lgd, and after extensive washing, proteins were eluted and separated by SDS-PAGE followed by silver staining (n = 3). (D) GST and GST fusions to different ESCRT-III subunits bound to glutathione agarose were incubated with full-length recombinant Lgd, and after extensive washing, proteins were eluted and separated by SDS-PAGE followed by staining using Coomassie (n = 3). IN, input; PD, pull-down. (E) Representative images of control and *chmp7* mutant animals expressing a GFP fusion to Lgd using swept-field confocal microscopy are shown following depletion of Chmp4 (n = 5 animals each). Bar, 10 μm. (F) Cartoon illustrating the targeting approach used to create a deletion allele of *LGD*. The locations of guide RNAs are indicated by scissors.

**Supplemental Video 2.1. Lgd dynamics during early embryogenesis.** Timelapse confocal imaging taken *in utero* of a GFP fusion to Lgd during early zygotic development. Playback rate, 48x.

## MATERIALS AND METHODS

### *CRISPR/Cas9-mediated genome editing and RNAi*

Genome editing to create a deletion within *C. elegans* Lgd (*hz24*) was carried out in the Bristol strain N2 (or a derivative) as described previously (Paix et al., 2015). Generation of an allele encoding an amino-terminal GFP fusion to endogenous Lgd (*syb1889*) was outsourced (SunyBiotech). All other strains used have been characterized previously (Sato et al., 2006; McNally et al., 2006; Audhya et al., 2007b; Frankel et al., 2017). Standard genetic crosses were performed to combine alleles, and all strains were maintained at either 20°C or 15°C. Templates for generating double stranded RNAs used in RNAi experiments were synthesized by PCR using N2 genomic DNA or cDNA. Depletion studies were conducted as described previously (Frankel et al., 2017).

### *Fluorescence and electron microscopy imaging studies*

Confocal imaging was conducted either using a swept-field confocal scanhead (distributed by Bruker) mounted onto a Nikon Eclipse Ti-E microscope stand, equipped with a 60x Planapo oil immersion objective and a CoolSNAP HQ2 CCD camera or a Yokogawa W1 confocal scanhead mounted onto a Nikon Ti2 microscope with a Hamamatsu Orca Flash 4 camera. For immunofluorescence studies, embryos were fixed using cold methanol and stained as described previously (Frankel et al., 2017; Crittenden and Kimble, 2009). For live imaging, animals were mounted onto 10% agarose pads and imaged under minimal compression in polystyrene beads (Polysciences, 2.5% by volume, 0.1 μm diameter). Fluorescence intensity measurements and colocalization analyses were performed using Imaris.

For electron microscopy, worms were loaded into 100-μm deep sample holders (Technotrade, Manchester, NH) coated with 1-hexadecene, and a suspension of bacteria was used as a cryoprotectant. High pressure freezing was carried out using a Leica EM ICE system. After freezing, samples were substituted into a solution of 1% OsO4 and 1% $H_20$ in acetone, as described previously (Shankar et al., 2022), followed by centrifugation into increasing concentrations of epoxy EMbed 812 resin (Electron Microscopy Sciences, Hatfield, PA). Animals were mounted and sectioned longitudinally (~80 nm). Samples were post-stained as described previously and imaged using a Philips CM120 TEM operated at 80kV and equipped with an AMT Biosprint 12 series digital camera.

***Antibody production and recombinant protein purification and analysis***

Production of rabbit polyclonal antiserum directed against Lgd was outsourced (Pacific Immunology, Ramona, CA). Rabbits were immunized with a fragment of Lgd (residues 345-792), and serum was affinity purified as described previously (Audhya et al., 2007a). Antibodies directed against other *C. elegans* ESCRT proteins have been described elsewhere (Mayers et al., 2013; Shen et al., 2014; Frankel et al., 2017). All antibodies were used at a final concentration of 1 μg/mL, with the exception of Lgd antibodies (used at 10 μg/mL) and CHMP4 antibodies (used at 5 μg/mL). Antibodies directed against β-actin were obtained commercially (Sigma).

Recombinant proteins were expressed using BL21-T1R (DE3) bacteria, as described previously (Schuh et al., 2015). Full-length Lgd, as well as truncation mutants, were cloned into the pET-28a vector, which encodes a cleavable 6xHis-SUMO

tag at the amino-terminus. Initial purifications from bacterial lysates were performed using Ni-nitrilotriacetic acid (NTA) agarose, and eluted proteins were applied onto a MonoS 5/50 GL cation exchange column to increase purity. ESCRT-III subunits were expressed as GST fusion proteins and purified on glutathione agarose beads. In cases where the GST tag was cleaved, eluted proteins were applied onto a SRT-C SEC-300 size exclusion column (Sepax Technologies, Neward, DE) for further purification.

For GST pull-down assays, 100 μg of purified GST or a GST fusion protein was bound to 8 μL of glutathione agarose beads and washed extensively. 80 μg of a potential binding partner was then incubated with the beads for 1 hour, followed by washing. Bound proteins were recovered by boiling in sample buffer and separated by SDS-PAGE, followed by Coomassie or silver staining. For multiangle light scattering studies, purified proteins were applied onto a size exclusion chromatography column (WTC-030S5; Wyatt Technology Corporation, Santa Barbara, CA), coupled to a three-angle light scattering detector (miniDAWN TREOS; Wyatt Technology Corporation). Data were collected at a flow rate of 0.5 mL/min and analyzed using ASTRA software (Wyatt Technology Corporation). The Stokes radius of each protein or protein complex was calculated from its elution volume, based on the elution profiles of characterized globular standards on the Wyatt column, as described previously (Schuh et al., 2015).

***Liposome generation and co-floatation assays***

Liposomes were generated as described previously (Schuh et al., 2015; Hanna et al., 2016), composed of either 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 99% and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)

(ammonium salt) (Rho-PE), 1%, or DOPC, 70%; 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 29%; Rho-PE, 1%. Lipid mixtures were dried, resuspended in buffer to a final concentration of 15 mM, and subjected to extrusion through a nitrocellulose filter (Whatman) with 0.03 μm pores.

For liposome co-floatation assays, 4 μM of purified Lgd was incubated with 2 mM charged or neutral liposomes at room temperature for 30 min, then mixed with an equal volume of 80% Accudenz (Accurate Chemical & Scientific Corporation, Carle Place, NY) to form a 40% Accudenz-liposome-protein solution. 35%, 30%, and 0% Accudenz layers were added on top and centrifuged at 50,000 rpm for 2.5 hr. The top 40 μL were collected and analyzed by SDS-PAGE followed by silver staining.

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# Chapter 3. ESCRTs, LGD-1, and worms: exploring basic cell biology through biochemistry and *Caenorhabditis elegans*

## INTRODUCTION

*Why I wrote this chapter*

Defining themes I have maintained throughout my scientific training are science communication, science literacy, and community building. These themes stem from my experiences with mentors and from the missions of programs which have supported my journey. As a former low-income student and the first in my family to earn bachelor's and doctoral degrees, I have seen firsthand the need for strong science education and critical thinking. I would not be writing this dissertation without support from programs throughout my education aimed at helping disadvantaged students succeed—from the Early College program in high school, to the McNair Scholars program during my undergraduate years, and finally the National Institutes of Health and National Science Foundation training grants that supported me while completing this work. The missions of these programs, which center around increasing diversity in professional settings, have been extremely influential to me as I progressed in my career and instilled a deep commitment to promoting a diverse, inclusive, and equitable environment wherever I go. Finally, as a future science educator, I believe it is important to always strive to make information accessible through effective communication. To me, this must start with my own work. To this end, I partnered with staff at the Wisconsin Initiative for Science Literacy (WISL) to write this chapter of my thesis to describe my scientific journey, experience in graduate school, and my thesis work for a non-scientist audience.

*My journey into science*

I had my first taste of scientific research in high school, when I started working in the Kestler lab at Lorain County Community College. I was attending a dual high school and community college program when my freshman biology teacher offered me the opportunity to work with Dr. Harry Kestler studying HIV after school with a small group of students. We worked collaboratively in groups of high school and traditional community college students, studying mutations in one of the receptors that HIV uses to infect cells. Dr. Kestler taught us how to do experiments and read research articles, but more importantly he instilled a deep appreciation for science communication and the value of using humor to connect with people. I could fill an entire chapter on its own about my time in the Kestler lab. I still think of Dr. Kestler as my science dad, and I am forever grateful that he inspired me to ‘persist like a lentivirus’.

As an undergraduate, I originally intended to continue on the path I had started studying viruses by majoring in microbiology. However, after taking Dr. Sina Ghaemmaghami’s honors biochemistry class, I became enamored with proteins. Proteins are the building blocks of biology; they make up the structure of cells, transform nutrients into energy, and protect and replicate DNA, among other essential roles. While DNA and RNA are the instructions, proteins are what those instructions build; and they perform nearly all tasks cells and viruses need in order to survive and thrive. I was especially captivated by the elegance of the relationship between protein structure and function. In biochemistry, we can use various tools to map protein structure, and that visualization can reveal an amazing amount of information about how a protein works and interacts with other biological components.

Although I did not end up directly studying protein structure, I did declare my major in biochemistry and worked in Dr. Ghaemmaghami's lab throughout college. In the Ghaemmaghami lab, instead of infectious viruses I studied infectious proteins, called prions. Prion proteins are somewhat unique in that they can change their structure dramatically, in a process known as misfolding, which induces other copies of prion proteins to misfold as well. These misfolded proteins stick together to form large aggregates which are often associated with cell death. In humans and many other mammals including cows, sheep, and deer, misfolded prion proteins cause severe neurodegenerative diseases, such as Creutzfeldt-Jakob Disease, bovine spongiform encephalopathy ('mad cow disease'), or Chronic Wasting Disease. One of my projects in the Ghaemmaghami lab centered on exploring how different pathways for breaking down proteins might impact misfolded aggregate formation.

During one summer in college, I participated in a summer research program at the University of Wisconsin in Madison, where I worked in Dr. Thomas Martin's lab. In the Martin lab, my project was less focused on disease than on answering questions about basic cell biology. Namely, I studied how cellular compartments called endosomes are made and how they are moved along with their protein cargoes both inside and outside of the cell. This work intersected with my growing interest in the endosome-mediated processes that break down proteins from the Ghaemmaghami lab. Ultimately, these experiences gave me a strong foundation in biochemistry and cellular biology, from which I launched into my thesis work in the Audhya lab.

**MY THESIS**

*Membranes – the unsung heroes of cell biology*

At its most basic level, a cell is a compartment enclosed by a membrane. Within this larger membrane compartment, there are many sub compartments, all with their own specialized roles and with membranes of their own. These sub compartments are called organelles, and their specialized roles include essential tasks such as transporting proteins (endosomes), generating energy (mitochondria), protecting DNA (nucleus), and breaking down cellular materials to be recycled (lysosome), among others (Fig. 3.1). For a cell and its organelles to function, it is critical that their membranes remain intact, because they create an inside environment separate from the outside environment. Thus, membranes define an organelle's identity as a distinct compartment. As an example, if the nucleus was no longer closed, DNA could be damaged by cellular components normally kept out of the nucleus, resulting in mutations and possibly cell death.

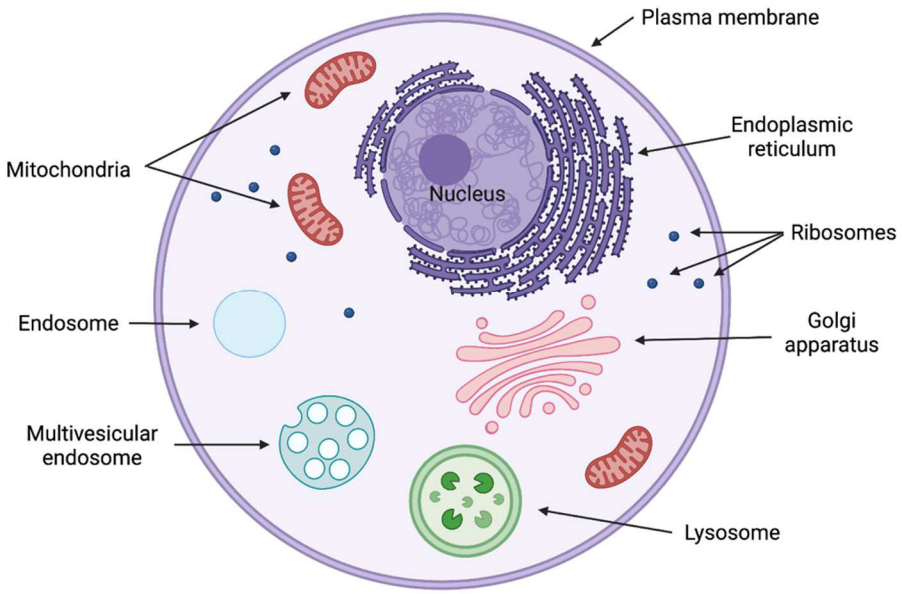


**Figure 3.1 Diagram of an animal cell.** Cells contain many different types of membrane-bound organelles and other structures which work together to carry out processes necessary for cell survival including energy production (mitochondria), protein transport (endosomes and Golgi apparatus), protein synthesis (endoplasmic reticulum and ribosomes), and protein degradation (lysosomes). Figure made with BioRender.

When looking at a diagram of a cell and its organelles, it's easy to assume that scientists for the most part have the basics of cell biology all worked out. However, these diagrams are an oversimplification. This is in part due to the need to reduce the cell's vast complexity down to an easily representable and digestible cartoon, but also because scientists don't have all the fine details worked out yet. While it is true that we have a very good general understanding of how the cell works, many gaps in the depth of our knowledge remain. For example, although we know a lot about the proteins that remodel or restructure cell membranes, how those proteins 'know' to go to specific locations in the cell at specific times or how they perform the final steps of membrane remodeling remain unclear. That we can know so much about basic cell biology without

fully understanding the fundamentals of how cellular membranes are remodeled and maintained is one of the main ideas that initially drew me into the Audhya lab and the world of ESCRT biology.

*ESCRTs & LGD-1:*

As discussed above, membranes are critical for a cell to maintain its identity, carry out essential functions like metabolism and signaling, and to protect genetic material from damage that could lead to cancerous growth or cell death. My thesis work broadly aims to understand how cellular membranes are remodeled and maintained by a series of protein complexes called the Endosomal Sorting Complexes Required for Transport (ESCRT, pronounced ‘escort’). The ESCRT machinery is composed of five protein complexes, called ESCRT-0, -I, -II, -III, and Vps4. ESCRT-0, -I, and -II are considered early acting ESCRTs, which are responsible for recruiting ESCRT-III, among other functions. ESCRT-III and Vps4 can be conceptualized as the ‘business end’ of the ESCRT machinery because together they perform the main membrane remodeling steps. The ESCRT-III complex is a spiral filament made up of up to 8 different protein subunits, which assemble on membranes (Figure 3.2). Vps4 uses cellular energy to remodel ESCRT-III filaments in order to constrict membranes. Ultimately, this process leads to membrane separation to either generate a budded vesicle (a small membrane-bound sac) or to seal a membrane bilayer (Figure 3.2).

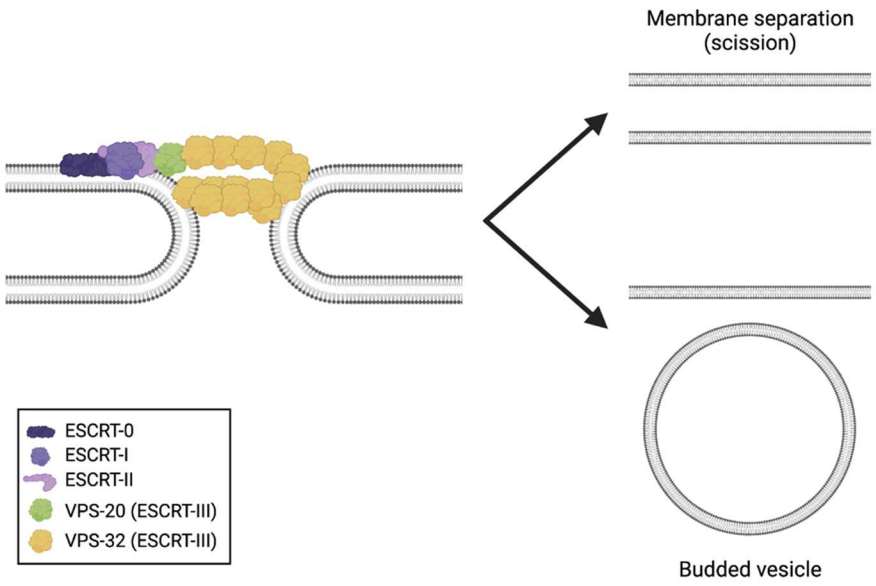


**Figure 3.2 ESCRT-mediated membrane remodeling.** Early acting ESCRT complexes (purple) recruit ESCRT-III component VPS-20, which recruits VPS-32/CHMP4, the main component of the ESCRT-III spiral filament. The result of ESCRT-mediated constriction of membranes can either be membrane separation (as in nuclear membrane sealing) or a budded vesicle (as in multivesicular endosome formation). Figure made with BioRender.

As their name implies, ESCRT proteins were originally discovered and studied for their role in transporting proteins through endosomes, which transport proteins along the endolysosomal pathway (Figure 3.3). In the endolysosomal pathway, membrane proteins are first brought into the cell from the plasma membrane to early endosomes. Then, the ESCRT machinery remodels the early endosomal membrane to bud vesicles inside of the endosome, ultimately generating an endosome containing many vesicles, called a multivesicular endosome. Finally, multivesicular endosomes can fuse to lysosomes so that their contents can be broken down and recycled by the cell.

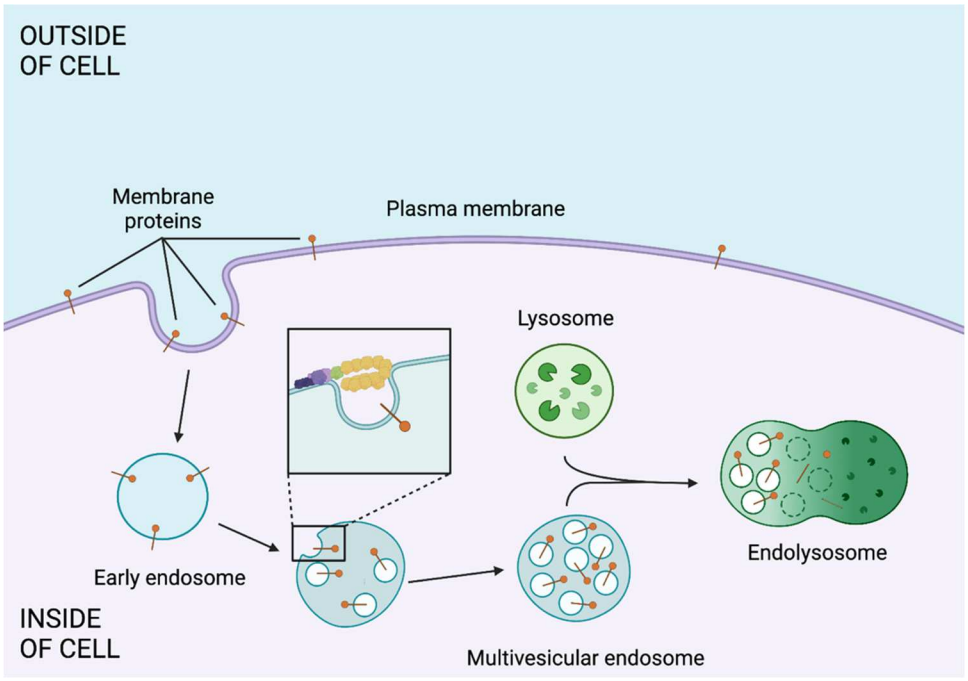


**Figure 3.3 The endolysosomal pathway.** Membrane proteins in the plasma membrane such as growth receptors are brought into the cell on an early endosome, which matures through ESCRT-mediated remodeling to become a multivesicular endosome. Internalized membrane proteins are budded inside of the endosome on vesicles. Ultimately, multivesicular endosomes can fuse with lysosomes to degrade their contents and recycle cellular nutrients. Figure made with BioRender.

ESCRT machinery is also involved in many other membrane remodeling and repairing processes throughout the cell, including repair of the outer cell membrane (plasma membrane), lysosome, and nucleus. To underline their importance, ESCRT proteins are highly evolutionarily conserved, meaning they are found across many species of organisms, including mammals and other vertebrates, plants, fungi, and other unicellular organisms. For many reasons, ESCRTs have been historically hard to study and therefore the finer details about how they function and how they are regulated have remained elusive.

My thesis work aims to address the question of how ESCRT machinery is targeted to the various membranes it must remodel at the appropriate time. Membranes

within a cell such as the nucleus, endosome, and plasma membrane have similar but distinct compositions, and precise timing of ESCRT recruitment is critical to proper remodeling. Specifically, my project explores how ESCRT machinery is regulated by another protein, called LGD-1, using protein biochemistry and the worm model *Caenorhabditis elegans*.

LGD-1 is a member of the Lethal giant discs/Coiled-coil Domain 1 (Lgd/CC2D1) family of proteins. The Lgd/CC2D1 protein family includes Lgd in fruit flies, LGD-1 in worms, and CC2D1A and CC2D1B in humans and other mammals. In fruit flies, mutations in Lgd result in developmental defects in the tissue destined to become a fly wing, called wing imaginal discs, hence the name ‘lethal giant discs’, and CC2D1A/B were named for their predicted protein structure. Early work on Lgd/CC2D1 proteins demonstrated their importance in regulating ESCRT-mediated membrane remodeling specifically along the endolysosomal pathway, although exactly how remained unclear. Lgd/CC2D1 proteins are known to prevent the assembly of the major ESCRT complex, ESCRT-III, through interaction with ESCRT-III component VPS-32/CHMP4. However, these experiments were carried out with purified proteins outside of the cell, without the presence of membranes or other biologically relevant factors.

*C. elegans as an ideal model:*

While artificial experimental environments are often crucial to gain a basic understanding of protein function, it remains essential to build upon knowledge gained from such experiments in a biological context. For my work, I chose the nematode worm model *C. elegans. C. elegans* are very small (~1mm in length as adults) transparent

worms that eat bacteria and other microbes in soil and rotting plant matter and are found in many parts of the world. In the lab, worms are grown on agarose plates seeded with *Escherichia coli* bacteria (Figure 3.4). The *C. elegans* lifecycle is relatively short, with a maturation time of 3-4 days. From a general scientific perspective, *C. elegans* are an ideal model for several reasons. First, many techniques are available for genetically modifying worms, and mutant worms are easily maintained. *C. elegans* primarily exist as hermaphrodites, meaning that an adult worm contains both oocytes (eggs) and sperm. This allows for easy maintenance of mutant strains. A small population of male worms also exist, making genetic crosses possible to combine mutations. Second, *C. elegans* are transparent, making them practical for imaging experiments, and their regular lifespan and cell division allow for precise timing of cellular events. There are also several reasons *C. elegans* are ideal for my project in particular. *C. elegans* possess many proteins similar to humans. In the case of ESCRT machinery and Lgd/CC2D1 proteins, worms have single copies of each of the ESCRT proteins, and one Lgd/CC2D1 protein, LGD-1. Compared to humans, which possess multiple copies of several ESCRT components and two Lgd/CC2D1 proteins, worms are a simplified system. Additionally, worms naturally undergo a programmed wave of trafficking through endosomes in the one-cell embryo just after fertilization. This is unlike other popular models such as human cells grown in a petri dish (cell culture) which rely on non-physiological treatments like treatment with growth factors to study ESCRT function in the endolysosomal pathway.

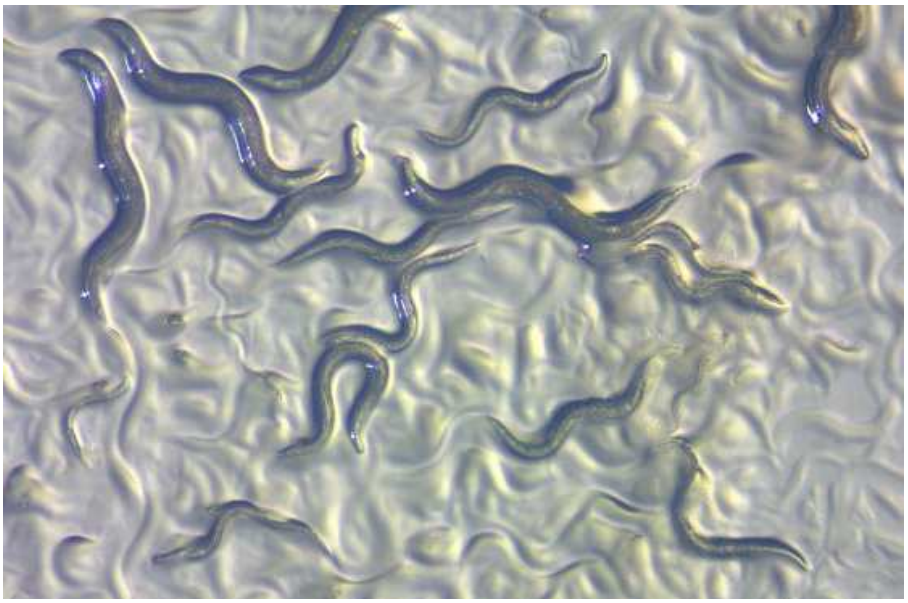

**Figure 3.4 *Caenorhabditis elegans* growing on bacterial plate.** Image credit: Carl Zeiss Microscopy, https://bit.ly/3B2FHeq

*LGD-1 stabilizes ESCRT-III assembly at the endosome:*

Though much of my work was performed in *C. elegans*, it was also important to gain a biochemical understanding of LGD-1 and its interactions with components of the ESCRT machinery. To do this, I purified LGD-1 and ESCRT proteins from bacteria engineered to produce these proteins in large quantities. I used these purified proteins to carry out experiments to test whether LGD-1 could physically interact with different proteins that make up the ESCRT-III complex. I found that out of 7 tested ESCRT-III proteins, LGD-1 only interacts with the major ESCRT-III subunit, VPS-32/CHMP4, and an another ESCRT-III protein, CHMP7. This agrees with previous studies of fruit fly and mammalian proteins. I also demonstrated LGD-1 can bind to membranes directly. These results suggested LGD-1 might play a specific role in VPS-32/CHMP4 recruitment or function on membranes.

In parallel to biochemical studies, I also generated several mutant worm strains to study LGD-1 in live *C. elegans* embryos. These included a strain expressing LGD-1 fused to green fluorescent protein and a strain containing a large deletion mutation, resulting in complete loss of LGD-1. I also combined these strains with others available in the lab through genetic crosses to add other fluorescent markers and mutations. Through microscopy experiments using these strains, I found that LGD-1 is present on endosomes during the programmed wave of trafficking through the endolysosomal pathway that occurs in single-cell embryos, similar to ESCRT proteins. Somewhat surprisingly, I found that the presence of LGD-1 on endosomes is at least in part dependent on early acting ESCRT complexes (ESCRT-0 & ESCRT-I), but not on VPS-32/CHMP4, even though we know LGD-1 directly interacts with VPS-32/CHMP4. This is an interesting result because no interaction between LGD-1 and ESCRT-0 or ESCRT-I proteins has been reported. On the other hand, loss of LGD-1 results in swollen endosomes containing very few vesicles, indicative of ESCRT dysfunction, but not total loss of function (Figure 3.5). I observed VPS-32/CHMP4 did not build up on endosomes in the absence of LGD-1, providing an explanation for the abnormal endosome structure. Taken together, my results suggest LGD-1 stabilizes ESCRT-III as it assembles on the endosomal membrane through its interaction with VPS-32/CHMP4 (Figure 3.5).

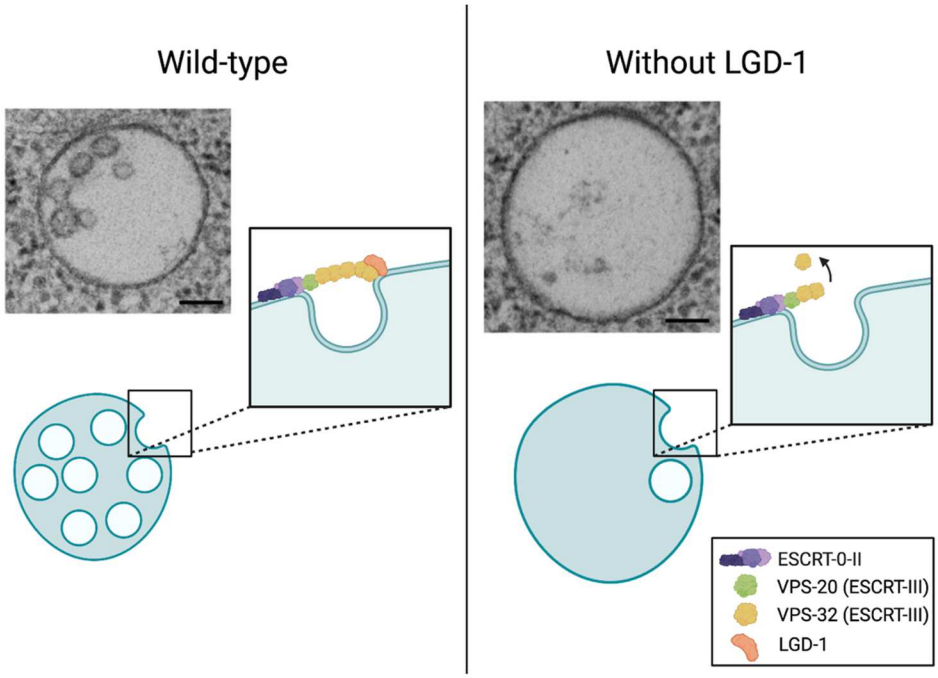


**Figure 3.5 Loss of LGD-1 impacts multivesicular endosome formation by destabilizing VPS32/CHMP4 in ESCRT-III assembly.** Electron microscopy images of multivesicular endosomes from either wild-type (control) or LGD-1 loss of function mutant worms. Scale bar = 100 microns. Also shown are cartoon schematics of what I hypothesize is happening at the protein level. Figure made with BioRender.

In summary, I have characterized LGD-1 and its interaction with components of the ESCRT machinery biochemically, described the cellular distribution of LGD-1 in the *C. elegans* embryo, and demonstrated how loss of LGD-1 affects ESCRT function at the endosome. My work is the first study of LGD-1's role in ESCRT function using the *C. elegans* model, an ideal system to study naturally occurring ESCRT-mediated membrane remodeling. While my thesis focused on ESCRT function at the endosome, because of its interaction with the major ESCRT-III subunit VPS-32/CHMP4 and CHMP7, it is likely that LGD-1 plays a role in other ESCRT-mediated membrane remodeling events such as those occurring at the nucleus.

**REFLECTIONS**

The previous section is a neat summary which might lead you to believe that my thesis work was straightforward. However, I can assure you that much like the oversimplified cell diagram discussed earlier, the reality is much more complicated. Scientists often joke that around 90% of experiments fail, and prospective graduate students are often asked in interviews about their resilience for a reason.

During the first few years in the lab, I generated very little data. In fact, for the first few months after joining the lab, I worked on an entirely different project using traditional cell culture models. After making the switch to studying LGD-1 in worms, it took many months just to learn how to handle worms and the basic techniques to study them. For example, to move worms from one plate to another, or to use worms for experiments, you use a small piece of platinum wire with a scoop fashioned at the end to pick up the worms while viewing them under a microscope. Moreover, it is often critical for experiments to pick single worms at specific lifecycle stages. Early on, it was difficult for me to gently pick up worms without squishing them or damaging the agarose plate and I spent hours a day practicing identifying specific larval stage worms and males.

Once I was comfortable with the practical skills needed to perform my experiments, I encountered another barrier to progress. Because Lgd/CC2D1 proteins have not been extensively studied by our lab or others, especially not in worms, there was a lack of ready-made and validated molecular tools to study them. Without this experimental infrastructure, it was up to me to build the tools I needed to study LGD-1 myself. These tools included both the LGD-1 worm strains mentioned previously, as

well as an antibody against LGD-1 and many, many DNA constructs to make modified proteins for biochemical experiments. Some tools were relatively straightforward to make. Others, like the LGD-1 mutant worm strains and antibody, I struggled with for at least a year, with several mishaps along the way. For example, I nearly lost all of my LGD-1 antibody on my second purification attempt. Antibodies are used to detect specific proteins in several types of experiments. They are typically generated by injecting a rabbit or other animal with your protein of interest and collecting the antibodies the animal produces through an immune response from their serum (blood). The process of purifying antibody from serum involves circulating the serum through tubing and over a column bound with the target protein of interest overnight and eluting the antibody off the column the next day. My first attempt to purify LGD-1 antibody from serum failed because I had not bound enough LGD-1 to the column, so very little antibody stuck to the column. The second attempt, I had included much more protein and was feeling confident until I came into the lab the following day only to find the tubing had sprung a leak and my precious rabbit serum was drying in a pool on my bench. Luckily, I had reserved a small amount of serum in the freezer, and eventually was successful.

Along the way, especially after my tools were built, I did make some exciting discoveries that bolstered my confidence and kept me going. My first major discovery was that LGD-1 and CHMP7 directly interact using purified proteins. Prior to this experiment, no published data existed to suggest Lgd/CC2D1 proteins interacted with CHMP7 in other organisms. I was so elated to have data, I texted pictures of the gel to my partner and family. A few weeks later and the morning before I debuted this result in

lab meeting, Jon forwarded me a newly published paper showing this exact experiment using mammalian proteins, and I was scooped. Although it stung quite a bit, it was also comforting to see another group corroborate my result, and ultimately, CHMP7 factored much less into my thesis than I originally expected.

A second breakthrough happened just before the COVID-19 pandemic closed down the lab. I had successfully made a mutation in *lgd-1* in *C. elegans*, *lgd-1Δ*, but homozygous mutant worms (having two copies of the mutation) died at an early larval stage. I hypothesized a partial depletion of VPS-32/CHMP4 might at least partially rescue these worms. While testing this hypothesis, I accidentally used a different reagent by grabbing the wrong tube out of the freezer. This proved to be a happy accident, because this reagent allowed *lgd-1Δ* homozygotes to grow into adult worms. Partial depletion of VPS-32/CHMP4, on the other hand, did not. Without this discovery, I would have been severely limited in my ability to study LGD-1 function without an adult mutant worm to study the consequences of losing LGD-1.

The *lgd-1Δ* worm made the third major success I want to highlight possible. As I was starting out in graduate school, I attended a presentation by a senior student in the Audhya lab. She showed electron microscopy (EM) images of endosomes and their internal vesicles, and I was immediately enamored. After I had joined the lab and needed to find a new project, the promise of doing my own EM experiments enticed me to make the switch to studying LGD-1 in worms. However, it was only years later once I had the *lgd-1Δ* strain, I finally had a reason to do EM for my project. It was so worth the wait; the first time seeing the swollen, empty endosomes in *lgd-1Δ* embryos by EM was a dream come true and this data became the highlight of my publication.

Despite all the ups and downs throughout my graduate school journey, I am thankful for the opportunity to contribute my work to the scientific community and for everything I have learned along the way. Beyond all the science and research-related knowledge I have gained throughout graduate school, I also learned a lot about myself and what I want to do after graduation, which is to teach. When I was a teenager starting out in science, I was driven by a desire to make a positive impact on my community. At the time, I envisioned this taking place on a grand scale, by making scientific discoveries that helped fight human diseases through treatments and cures. Later, reflecting on my experience in research, I realized I could make a more immediate impact through science communication and education. I was also influenced by the COVID-19 pandemic, and how misinformation and lack of trust in scientists and health officials contributed to the enormous human cost of the pandemic, which is still ongoing today. I used my time out of the lab during pandemic shutdowns to begin training for a career in education through the Delta Program here at UW Madison. I finished earlier this year, completing a teaching certificate through the program. Shortly after submitting this dissertation, I will move into a year-long position as a Lecturer in the Department of Chemistry and Biochemistry at the University of Wisconsin – La Crosse, where I will teach a mix of biochemistry and chemistry lab courses and a biochemistry lecture. Following my year at UW La Crosse, I hope to find a tenure-track position at a primarily undergraduate institution or community college teaching biochemistry and molecular biology. Although I will be leaving the bench behind for now as I transition into teaching, I will absolutely miss research and the sweetness of generating new data.

# Chapter 4. Discussion and Future Directions

**Lgd/CC2D1 regulation of ESCRT-III at the multivesicular endosome**

Lgd, the first described Lgd/CC2D1 homolog, was initially described as a *Drosophila* mutant displaying aberrant wing development and larval lethality in 1971[1]. Thirty years later, this developmental defect was linked to dysfunction in the Notch signaling pathway, which depends on endolysosomal trafficking to constitutively degrade the Notch receptor in a ligand-independent manner[2]. Over the past two decades, there have been several advances in our knowledge about Lgd/CC2D1's role in endolysosomal transport. As discussed in Chapter 1, these advances have demonstrated Lgd/CC2D1 plays a role in regulating Endosomal Sorting Complex Required for Transport-III, affecting ESCRT-mediated multivesicular endosome (MVE) formation. In Chapter 2, I present work that further defines the function of Lgd/CC2D1 and its role in regulating ESCRT-mediated MVE biogenesis.

Past investigations of Lgd/CC2D1 and on ESCRT-mediated MVE biogenesis have often relied upon overexpression or stimulation with growth factors in order to monitor endolysosomal trafficking[3–10]. Instead, by observing endogenously tagged LGD-1 during post-fertilization MVE biogenesis in the *C. elegans* embryo, we were able to define the subcellular distribution of LGD-1 under physiological conditions. In agreement with previous work, we observe LGD-1 is primarily cytosolic[3]. Additionally, we observe a population of LGD-1 associated with newly formed endosomes in the one-cell stage embryo. This population substantially overlaps with markers for early acting ESCRT complexes, ESCRT-0 (HRS) and ESCRT-I (TSG-101). Further, we show that LGD-1 endosomal recruitment is partially dependent on the presence of these early acting complexes, in addition to charged phospholipids. The exact mechanism of Lgd/CC2D1

targeting to endosomes, including whether Lgd/CC2D1 directly interacts with ESCRT-0 or ESCRT-I, the specific lipid species involved, and the potential presence of additional factors remain to be worked out.

Lgd/CC2D1 regulation of ESCRT-III has long been thought to involve the interaction between Lgd/CC2D1 and CHMP4/Vps32, which is known to prevent homopolymerization of CHMP4/Vps32[8,11]. Surprisingly, we found that loss of the *C. elegans* Lgd/CC2D1 homolog, LGD-1, prevented CHMP4/Vps32 association with the endosomal membrane. This result was highlighted by the failure of CHMP4/Vps32 to accumulate on endosomes in the absence of LGD-1 even under depletion of Vps4, the AAA-ATPase required for recycling ESCRT components off membranes. It may be possible that interaction with Lgd/CC2D1 on the endosomal membrane generates an increased local concentration of CHMP4/Vps32 necessary for rapid ESCRT-III assembly. Future studies will be needed to address the mechanism by which Lgd/CC2D1 facilitates ESCRT-III assembly, such as how CHMP4/Vps32 transitions from interaction with Lgd/CC2D1 to participating in the ESCRT filament and what factors are required for this transition.

Finally, I found that in the absence of LGD-1, MVE biogenesis is impaired. Typically, during the post-fertilization wave of endocytosis in the *C. elegans* embryo, cargoes are rapidly internalized and degraded via MVE intermediate[12]. In contrast, we found loss of LGD-1 results in the accumulation of undegraded endolysosomal cargoes that persist in multicellular embryos, indicating decreased flux through the endolysosomal pathway. At the ultrastructural level, loss of LGD-1 resulted in enlarged MVEs containing few ILVs, which are also slightly enlarged. Combined with the

previously discussed finding that CHMP4/Vps32 recruitment is decreased but not abolished, these data suggest Lgd/CC2D1 is important for efficient ESCRT-III assembly needed to facilitate rapid cargo sorting into ILVs. Importantly, although my work indicates Lgd/CC2D1 is not absolutely required for ILV formation, it demonstrates how precise timing of ESCRT-III assembly is critical for proper membrane remodeling.

**Lgd/CC2D1 regulation of ESCRT-III at the nuclear envelope**

Similar to their role in regulating ESCRT-mediated MVE biogenesis, Lgd/CC2D1 proteins have also been implicated in ESCRT-mediated nuclear envelope sealing[8]. Recent work has shown CC2D1B, one of the two Lgd/CC2D1 homologs found in mammals, controls timing of ESCRT assembly at holes in the newly formed nuclear envelope following mitosis[8]. In Chapter 2, I describe evidence LGD-1 localizes to the nuclear envelope in a cell-cycle dependent manner and interacts with CHMP7, an ESCRT-III subunit which has previously been described by our lab and others to have a primary role in nuclear resealing[13–17].

While previous work has described CC2D1B associating with the nuclear envelope during reformation, I primarily observed LGD-1 association with the nuclear envelope coincident with nuclear envelope breakdown. This is presumably due to exposure of cytosolic LGD-1 to CHMP7 present on the inner nuclear membrane (INM). However, due to the significant cytosolic population of LGD-1 and the transient nature of nuclear envelope recruitment, LGD-1 may also associate with the nuclear envelope during reformation. Using recombinant purified proteins, I also demonstrated that LGD-1 interacts with CHMP7, consistent with previous reports of interaction between CC2D1B

and CHMP7 in mammalian cells[8]. Further, I found this interaction is mediated by the DM14-4 domain of LGD-1 and the ESCRT-III-like domain of CHMP7.

Several open questions remain about how Lgd/CC2D1 regulates ESCRT-III assembly at the nuclear envelope, and how this process is similar to or differs from its role at the MVE. First, further work is needed to determine whether Lgd/CC2D1 proteins are recruited to the INM by CHMP7 or whether interactions with phospholipids also contribute to recruitment. Another open question is how Lgd/CC2D1 is recycled off the nuclear envelope following mitosis. One hypothesis is that CHMP4/Vps32 outcompetes CHMP7 to bring Lgd/CC2D1 back into the cytosol. However, the relative affinity of each of these interactions is not yet known, and the potential for Lgd/CC2D1 to interact with both CHMP4/Vps32 and CHMP7 simultaneously has not been explored.

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