DISSECTING THE ROLE OF THE LIPODYSTROPHY PROTEIN SEIPIN IN THE BIOGENESIS OF THE LIPID DROPLET ORGANELLE

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DEDICATION

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by

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Long thought to be little more than inert storage depots, lipid droplets have recently become recognized as unique, dynamic, regulated organelles that play an essential role in fat storage. Despite this increased interest, much remains unknown. Lipid droplets have been observed to emerge from the endoplasmic reticulum, but the available models for lipid droplet biogenesis are largely conceptual, with little to no evidence for specific mechanisms of droplet formation. Debate even continues within the field as to whether lipid droplet formation is a spontaneous process, driven by physicochemical and hydrophobic forces, or a regulated process driven by protein factors.

The Goodman laboratory previously found evidence to suggest that seipin, mutated in the most severe cases of congenital generalized lipodystrophy, may be a key factor in the early stages of lipid droplet formation. Seipin resides at the junction between lipid droplets and the endoplasmic reticulum, and deletion of seipin results in both a drastic impediment to de novo droplet formation and a striking disorganization of droplet morphology.

For my thesis work, I have explored several aspects of seipin's role at the lipid droplet. I have studied the effects of seipin deletion on protein targeting to abnormal lipid droplets, through which I identified a unique effect of seipin on the regulation of lipase targeting. I have also analyzed the topology of the seipin complex itself through a series of deletion mutants, identifying regions that contribute to the localization, membrane association, and stability of the seipin complex. Furthermore, these studies have led to novel insights on the function of seipin, through the characterization of a remarkable N-terminal seipin mutation that presents with defects in droplet initiation but homogenous droplet morphology. I have therefore concluded that seipin plays two dissectible roles in lipid droplet formation: 1) promoting lipid droplet initiation and 2) regulating subsequent droplet morphology. Finally, I suggest hypotheses on the mechanisms by which seipin exerts these effects, proposing that the N-terminus of seipin may regulate lipin, a mouse lipodystrophy protein, to effect droplet initiation, while the bulk of the protein may serve to regulate the access of phospholipids to the lipid droplet surface.

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ADE2 – phosphoribosylaminoimidazole carboxylase; an adenine synthesis enzyme

AGL – Acquired generalized lipodystrophy

AGPAT2 – 1-acylglycerol-3-phosphate O-acyltransferase 2

ANOVA – analysis of variance

ARE1 – acyl-CoA:sterol acyltransferase 1

ATGL – adipose triglyceride lipase

ATP – adenosine triphopshate

BODIPY – 4,4-difluoro-1,3,5,7,8-pentomethyl-4-bora-3a,4a-diaza-s-indacene; an NL dye

BSCL – Berardinelli-Seip congenital lipodystrophy

CAV1 – caveolin-1

CFP – cyan fluorescent protein

CGL – congenital generalized lipodystrophy

CTDNEP-1 – C-terminal domain nuclear envelope phosphatase 1; human homolog of NEM1

Cterm – cytosolic carboxy-terminus of seipin

CTM – carboxy-terminal transmembrane domain of seipin

DAG – diacylglycerol

DGA1 – diacylglycerol acyltransferase

DMSO – dimethyl sulfoxide

DPM1 – dolichol phosphate mannose synthase; an integral ER membrane protein

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

ER – endoplasmic reticulum

ERG1 – squaline epoxidase; a sterol synthesis enzyme

ERG6 - Δ24-sterol C-methyltransferase; a sterol synthesis enzyme

ERPO – ER-derived protective organelle

FA – fatty acid

FAA4 – long chain fatty acyl-CoA synthetase

FFA – free fatty acid

FIT – fat storage-inducing transmembrane protein

FLD1 – fewer lipid droplets 1; yeast homolog of seipin

GAL – galactose-inducible promoter

GFP – green fluorescent protein

HDEL – ER retention sequence

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIS3 – imidazoleglycerol-phosphate dehydratase; a histidine synthesis enzyme

HIV – human immunodeficiency virus

HSL – hormone-sensitive lipase

KAR2 – ER protein import ATPase

LD – lipid droplet

LDB16 – low dye binding protein 16

LEU2 – beta-isopropylmalate dehydrogenase; a leucine synthesis enzyme

LRO1 – lecithin cholesterol acyl transferase related protein 1; a PL:DAG acyltransferase

NEM1 – nuclear envelope morphology 1; an activator of PAH1

NEP1-R1 – nuclear envelope phosphatase 1 regulatory subunit 1; human homolog of SPO7

NL – neutral lipid

Nterm – cytosolic amino-terminus of seipin

NTM – amino-terminal transmembrane domain of seipin

OD_{600} – optical density at 600nm wavelength

ORF – open reading frame

PA – phosphatidic acid

PAH1 – Mg^{2+}-dependent phosphatidate phosphatase

PC – phosphatidylcholine

PCR – polymerase chain reaction

PE – phosphatidylethanolamine

PGK – promoter of the 3-phosphoglycerate kinase gene

PI – phosphatidylinositol

PIPES – piperazine-N,N'-bis(2-ethanesulfonic acid)

PL – phospholipid

PPARγ - peroxisome proliferator-activated receptor gamma

PS – phosphatidylserine

PTRF – polymerase I and transcript release factor; encodes cavin-1

REEP1 – receptor accessory protein 1

SE – steryl ester

SEM – standard error of the mean

SERCA – sarco/endoplasmic reticulum calcium transporting ATPase

SLD – supersized lipid droplet; defined as >1μm diameter

SPO7 – sporulation defect 7; an activator of PAH1

TAG – triacylglycerol

TGL1 – triglyceride lipase 1; actually a steryl ester hydrolase

TGL3, 4, 5 – triglyceride lipases

TLC – thin layer chromatography

TZDs – thiazolidinediones; agonists of PPARγ

UBXD8 – UBX domain-containing protein 8

URA3 – orotidine-5'-phosphate decarboxylase; a uracil synthesis enzyme

YEH1 – yeast steryl ester hydrolase 1

CHAPTER ONE
Introduction

Obesity, once a rare disease of affluence, has become perhaps the defining illness of the early 21st century. After startlingly rapid increases in the 1980s and 1990s[1], obesity prevalence in the United States has now reached 34.9% of adults and 16.9% of children[2]. Far from affecting only the wealthy, recent data indicates the most alarming increases in recent decades have occurred in developing countries, leading the World Health Organization to formally classify obesity as a global epidemic[3]. This phenomenon has produced costly and deadly consequences: the co-morbidities associated with obesity (type II diabetes, fatty liver disease, coronary artery disease, etc.; collectively known as metabolic syndrome) have produced estimated annual medical expenses of $147 billion per year in the United States (10% of all medical spending)[4] and are projected to result in flattened or even decreased life expectancy for children born this century[1].

Treatment of obesity and associated metabolic syndrome has clearly been problematic. The "simple" solution of improving lifestyle through diet and exercise has found little traction: low-intensity clinical counseling was found to have no clinically meaningful effect on weight loss[5], and even high-intensity efforts effective in weight loss[6] are highly susceptible to weight regain[7], leading to a need for effective drug therapy in many cases[8]. Most medical intervention takes place to treat metabolic complications once they have already arisen; in the United States, only 2.74 million patients (of approximately 100 million obese adults) are estimated to use medication targeted at the underlying obesity[9].

Patients taking such drugs must successfully achieve at least 5% weight loss after 12 weeks to justify the risk of side effects[8]; because of this and other factors, less than 5% of these patients continue long-term anti-obesity medication longer than one year[9]. Clearly we are in dire need of more effective and safer treatment strategies.

Unfortunately, drug discovery has become increasingly problematic in recent decades as well. Attrition of potential drugs during clinical trials has been a significant problem; during the 2006-2008 period only 5% of drugs tested in clinical trials were approved for market distribution. Failure most often occurs late in clinical trials due to failures of safety and efficacy, resulting in a tremendous loss of funds (the estimated cost of full development of a single drug is $900 million)[10]. Even clinical trials have been known to miss significant side effects due to small sample size and short trial periods, leading to withdrawal of multiple anti-obesity drugs; a field where large patient populations and long-term treatment are of particular concern[8, 11]. Late, costly failures like these are often due to a lack of basic knowledge concerning the mechanism of a drug's action or toxicity[10].

From the intractable rates of obesity and metabolic syndrome and the discouraging outcomes of treatment, it has become apparent that we do not know nearly enough about how best to combat this disease. In the words of Dr. Huda Zoghbi at Baylor College of Medicine, "The challenge in translational medicine is that scientists are trying to translate a text with the sophistication and depth of Shakespeare using a first-grader's vocabulary and experience, because our knowledge about the functions of most pathways … is still rudimentary and piecemeal."[12] If we do not understand the most fundamental processes of the cell, how can

we hope to accurately, effectively, and safely manipulate such processes in the treatment of disease?

The obesity epidemic has therefore presented a particular need for basic science research in lipid biology, a field made especially enigmatic by limited experimental tools and only recent intense interest. The work presented here was conducted to address a particularly mysterious area of research, namely the mechanism of fat storage within cells via the biogenesis of the lipid droplet organelle. I will discuss the importance of a protein called seipin in this process, notably a protein discovered in rare genetic cases of lipodystrophy, a disease which also produces metabolic syndrome. I will then present experimental data in yeast demonstrating multiple roles for seipin in lipid droplet biology, and finally I will present hypotheses based on this data for the mechanism of seipin action. It is my hope that the findings and ideas presented in this dissertation will contribute, even if only in a small way, to a better understanding of the cellular forces underlying lipid-related diseases.

CHAPTER TWO
Review of the Literature

THE LIPID DROPLET ORGANELLE

The Role and Nature of the Lipid Droplet

Lipid droplets (LDs) comprise the sub-cellular compartment that stores fats within the cell. Fat storage is one of the most fundamental adaptations for survival in an environment that fluctuates between feast and famine, enabling the storage of energy-dense molecules during nutrient excess for utilization during nutrient scarcity. The collection of lipid into distinct droplets serves the added function of protecting the cell from lipotoxicity, the adverse effects of excess hydrophobic material on the largely aqueous cellular environment[13]. In addition to these roles in lipid regulation, evidence is now accumulating suggesting that the lipid droplet may also function in providing a unique hydrophobic milieu for protein regulation[14, 15].

Studies of lipid droplets once focused on cells with specialized lipid functions, such as plant seed and fruit tissue and mammalian liver and adipose tissue[16]. Within these fields, lipid droplets were known by a remarkable plethora of names (such as lipid globules, lipid bodies, lipid particles, oil bodies, liposomes, microscomes, spherosomes, and adiposomes), and not necessarily recognized to represent a conserved structure [16-18]. It has become increasingly clear, however, that the functions of the lipid droplet are much more highly conserved; no eukaryotic species or cell type (other than erythrocytes) has yet been observed

to lack lipid droplets[16, 17, 19], and even some prokaryotes produce them[20]. After this universality became apparent, and lipid droplets were found to represent a relatively conserved composition and structure (see below), a more unified field emerged, and the term "lipid droplet" gained consensus[17].

Lipid droplets were once thought of as inert cytoplasmic inclusions that served as passive storage depots[21]. As Dr. Richard Anderson of U.T. Southwestern once described, "I've been in cell biology for more than 30 years, and lipid droplets have always been this bag of lipid."[22] However, the universality and conserved structure of the lipid droplet, early findings on the unique protein composition of lipid droplets[23, 24], the discovery of the lipid droplet's unique phospholipid monolayer[18, 25], and the increasingly complex and regulated processes and interactions observed at the lipid droplet[21, 26] have all led to a unified view of lipid droplets as a unique, dynamic organelle in its own right[17, 27].

Structure and Composition of the Lipid Droplet

Lipid droplets appear as generally spherical globules within the cytoplasm (Fig. 1). The core of the lipid droplet is composed of neutral lipid, the most hydrophobic type of lipid (as opposed to at least partially amphipathic polar lipids), often colloquially generalized as "fat"[28]. Neutral lipid refers to polar lipids that have been esterified into hydrophobic, inert storage forms, the major classes being triacylglycerol (TAG; three fatty acids esterified to glycerol) and steryl esters (SE; a single fatty acid esterified to a sterol)[29]. This neutral lipid

forms the bulk of the volume of the lipid droplet, and its hydrophobic nature accounts for the unique structure and functions of this organelle.

The composition of this neutral lipid core can vary widely based on the type of cell analyzed. While lipid droplet fractions from the unicellular yeast *Saccharomyces cerevisiae* were found to be composed of equal parts TAG and SE[30-32], specialized mammalian cells often display a predominance of one type of neutral lipid, such as TAG in adipocytes, SE in steroidogenic cells[19], and even retinyl ester in retinal pigment epithelium[33]. Specialization may even occur among lipid droplets within a cell; heterogenous lipid droplets containing primarily TAG or SE have been observed in two reports, one in mammalian cells[34] and one in plants[35], although it is not yet clear how universal or significant such segregated lipid droplets may be.

Even within the core of an individual lipid droplet, different types of neutral lipid may be organized or segregated: while lipid droplets containing only triacylglycerol appear to be packed in a generally disordered manner, analysis of lipid droplets containing only steryl esters displayed ordered, layered shells[36]. This is consistent with the concentric "onion-skin" appearance often seen in freeze-fracture electron microscopy of lipid droplets[37, 38], suggesting that lipid droplets containing both TAG and SE may contain a core of TAG surrounded by ordered layers of SE. It has also been suggested that different TAG species could segregate based on the different melting temperatures of their component fatty acids[39]. What implications such intra-droplet organization might have in vivo and whether these effects are strongly temperature-dependent have yet to be determined.

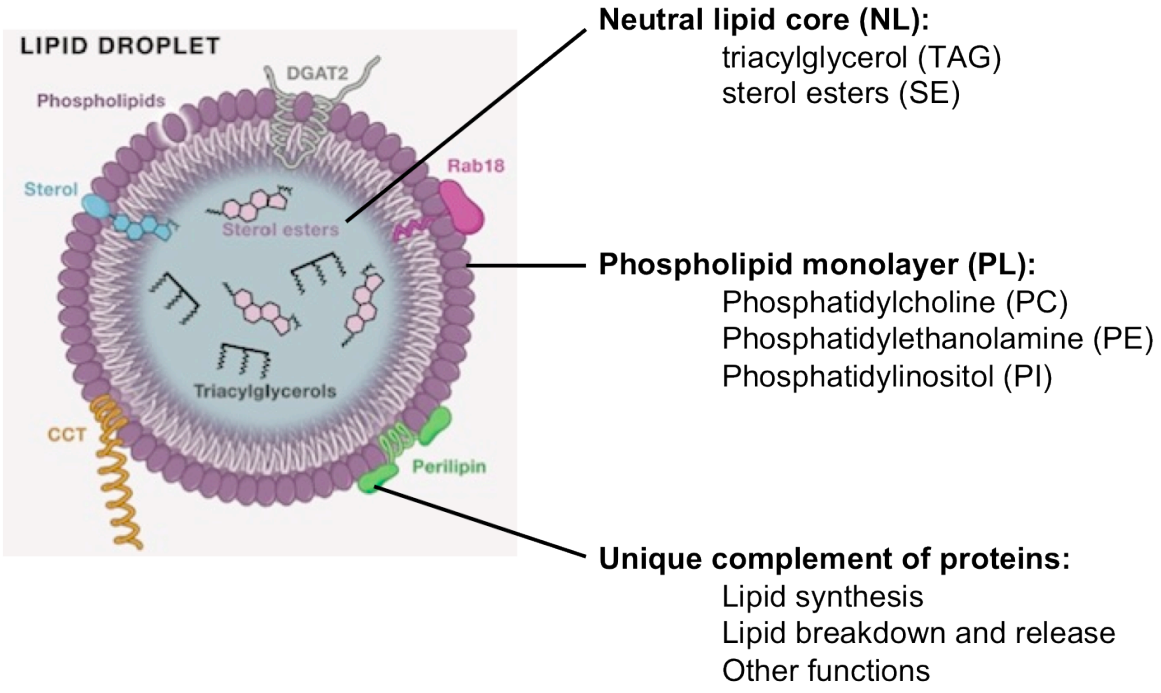

Figure 1. Lipid droplet structure. The lipid droplet is composed of a hydrophobic neutral lipid core volume surrounded by an amphipathic phosphoilipid surface monolayer. The phospholipid composition of LDs is unique among organelles, as is the complement of proteins associated with the surface monolayer. Figure adapted from Krahmer et al.[40]

The lipid droplet core is surrounded by a surface phospholipid monolayer[18, 25]; this structure is unique, as the vast majority of phospholipid within the cell is organized into bilayers. The amphipathic phospholipid monolayer is critical for the stability of the lipid droplet; hydrophobic phospholipid tails are immersed within the neutral lipid core, while hydrophilic phospholipid heads interact with the surrounding aqueous cytosol. In this fashion, the phospholipid monolayer effectively acts as an emulsifier to solubilize the lipid droplet[41]. Disruption of this monolayer, expected to expose the underlying hydrophobic core, has been shown to cause unusual reactivity and fusion of the lipid droplet with nearby proteins, membranes, and other LDs[42-44], highlighting the importance of the phospholipid surface in lipid droplet stability. The lipid droplet surface has also been shown to contain

free sterol[45], although whether it significantly participates in the structure or surface tension of the monolayer is not clear.

The composition of phospholipids within the monolayer is unique among organelles, even compared to the endoplasmic reticulum (ER) from which lipid droplets are derived[25] (see Section 2-2). In both mammalian and yeast LDs, phosphatidylcholine (PC) is the dominant phospholipid (at 36.4% of total droplet phospholipid in yeast[32] and up to 60% in mammals[46, 47]), while phosphatidylethanolamine (PE) is more abundant in insect lipid droplets (reflecting an overall higher PE/PC ratio in insect cells[48]). PE is the second most abundant lipid droplet phospholipid in mammalian cells (up to 24%), followed by low percentages of phosphatidylinositol (PI), phosphatidylserine (PS), and some lyso-phospholipids[46, 47]. In *S. cerevisiae* yeast, however, PI is the second-most abundant droplet phospholipid at 31.6%, nearly equal to the abundance of PC, and PE follows at 20% of droplet phospholipids[32]. Phosphatidic acid (PA) has so far been undetectable in mammalian lipid droplets[49] and detected at very low percentage in yeast lipid droplets (2.7%, the least abundant of the phospholipids analyzed[32]), likely reflecting its nature as an intermediate precursor in phospholipid synthesis. The implications of such variances across species have not yet been determined, nor has lipid droplet composition across mammalian cell types been rigorously analyzed.

Compared to the yeast endoplasmic reticulum, the lipid droplet has higher PI at the apparent expense of both PC and PE[32, 50]; fatty acid composition of phospholipids is also different between the two organelles[25]. Importantly, because of its large head group, PI forms an inverse conical shape that is expected to conform well to the curved surface of a

small yeast lipid droplet. (This could explain the difference in PI between yeast and mammalian lipid droplets; whether PI is preferentially enriched on small nascent LDs compared to very large LDs has not yet been tested in mammalian cells). In fact, the unique phospholipid monolayer is predicted so energetically favorable in the shape of a small, newly-formed lipid droplet that one group has proposed that phospholipid de-mixing into these ratios may participate in energetically driving lipid droplet budding from the ER[51] (see the following section).

Finally, in addition to the lipids described above, lipid droplets also contain a host of associated proteins that regulate and carry out many of the organelle's functions. The first of these to be discovered was perilipin[23], a discovery which revolutionized the lipid droplet field not only by demonstrating the specific association of proteins with lipid droplets, but also through the eventual identification of a family of related proteins, often termed perilipins or PAT domain proteins, all of which associate specifically with the lipid droplet. These proteins have proved to play significant roles in processes such as the regulation of lipid release from the droplet[52], interaction of lipid droplets with mitochondria[53, 54] (one of the many organelle interactions in which lipid droplets participate[26, 55]), and even a potential role in lipid droplet biogenesis[56-59]. However, while perilipin proteins are well conserved in animals, they are absent in fungi and plants, indicating relatively non-essential, specialized roles in lipid droplet manipulation.

The remaining protein complement of the lipid droplet is unique, diverse, and dynamic, including enzymes involved in lipid synthesis[24] and degradation[60, 61] as well as membrane trafficking proteins[62]. These proteins generally associate with the lipid droplet

surface; although some lipid droplet proteins have been detected within the lipid droplet core in one freeze-fracture electron microscopy study[63], it is difficult to imagine how proteins could be significantly functional in such an extremely hydrophobic environment. While there is no canonical LD-targeting domain or sequence yet identified, many of these proteins (and the perilipins described above) have been found to localize to lipid droplets via amphipathic or hydrophobic helices[64-68]. Due to the topological constraints of the extremely hydrophobic lipid droplet core, proteins with a single N-terminal transmembrane domain or a hairpin configuration can localize to the lipid droplet surface, while multi-spanning integral membrane proteins do not appear to be compatible with this environment[15, 69]. Often these proteins can also localize to the ER; many lipid droplet proteins can shuttle between the ER and the LD, and several re-localize to the ER in the absence of lipid droplets[69-72]

Lipid droplet proteomics studies have further identified a number of "refugee proteins" that co-purify with lipid droplets but are known to have functions that lie apparently outside of expected roles in lipid metabolism[73]. These have suggested a potential role for lipid droplets in the sequestration, regulation, and degradation of such proteins. For example, excess histones are sequestered on the lipid droplet surface during fly embryo development[74], and researchers have postulated a role for this sequestration in the response to intra-embryonic bacterial infection[75]. Furthermore, shuttling of UBXD8 between the ER and the lipid droplet appears to participate in regulating the degradation of UBXD8 target proteins[76]. Some such proteins targeted for degradation may transition through lipid droplets during extraction from the ER membrane, utilizing the hydrophobic environment to unfold or displace transmembrane domains[77], however this is not likely to be an essential component of

membrane protein degradation, as extraction and degradation can occur in yeast strains lacking lipid droplets[78]. Such regulation, sequestration, and ER-exit functions may also be exploited by certain pathogens[14], such as the hepatitis C virus, components of which interact intimately with lipid droplets and the ER; lipid droplets are required for the virus's maturation and release from the cell[14, 79]. Lipid droplets and lipid droplet proteins thus participate in a wide variety of cellular functions, although molecular details are often lacking.

LIPID DROPLET BIOGENESIS

Derivation from the Endoplasmic Reticulum

The first apparent identification of the origin of lipid droplets resulted from ultrastructural studies of seed oil bodies in corn and rapeseed[18]. Although these conclusions were debated at the time[80], this model was strongly adopted in later decades, as the ER was found to be the site of neutral lipid synthesis activity[31], and freeze-fracture electron microscopy demonstrated the lipid droplet surface to be occasionally continuous with the ER membrane[81]. Indeed, most neutral lipid synthetic enzymes localize to the ER (unless they translocate to the LD surface; discussed in Section 2-2-3), and de novo lipid droplets have recently been directly observed to arise in the vicinity of the endoplasmic reticulum[69, 72].

Furthermore, while lipid droplets were once believed to completely bud off from the ER after their synthesis, several proteins have been found to shuttle between the ER and the

lipid droplet surface[69-71, 76], suggesting a maintained connection; indeed, lipid droplets have been observed to maintain close proximity to the ER throughout their lifetime in yeast[82, 83], and a subset of mammalian droplets have been shown to develop new LD-ER connections during droplet growth[69]. Notably, the finding that LD phospholipid composition is distinct from that of the ER has led researchers to conclude that the connection between the lipid droplet and the endoplasmic reticulum cannot be entirely free-flowing and continuous: "the lipid droplet may be connected to the ER, but some molecular mechanism may demarcate the lipid droplet surface from the bulk ER membrane as postulated for other ER domains."[25]

Models of Lipid Droplet Biogenesis

Because the endoplasmic reticulum was demonstrated to be the site of neutral lipid synthesis, it has long been assumed that neutral lipid accumulates within the ER, often described in the form of a coalesced "lens" between the two membrane leaflets, before budding into a discrete droplet. Known as the "lens" or "budding" model, it is the most widely accepted model of lipid droplet biogenesis, although little direct experimental information has been collected to support or refute this hypothesis.

Neutral lipid is indeed capable of accumulating within a membrane bilayer up to 3-7% (by mass) before becoming unstable[84-87], consistent with calculated predictions[88, 89], and blisters within the bilayer of at least 17nm diameter have been predicted[89]. However due to their small, transient, and relatively scarce nature experimental techniques have thus far lacked the temporal and spatial resolution to directly visualize these hypothetical lens

structures[51]. One group did identify lipid accumulations within the ER bilayer arrested by tight binding of an abnormal apolipoprotien[90], however due to the non-physiological conditions of this study, it has not been determined whether these structures are artefactually derived or whether they represent valid, trapped intermediates in lipid droplet biogenesis. Until such a lens structure is finally captured in vivo, it remains possible that neutral lipid accumulating within the ER normally does so diffusely, without coalescing until it enters the lipid droplet environment; however evidence of transition structures for this version of the model face the same technical difficulties. Although far from proven, the budding model is the longest and most widely-held model of lipid droplet biogenesis, and it has shown to be highly adaptable to multiple hypotheses for the mechanism and driving factors behind lipid droplet budding (discussed in Section 2-2-4).

The primary alternative model of lipid droplet formation was largely based on freeze-fracture electron microscopy studies demonstrating extensive regions of close proximity between the lipid droplet surface and the ER membrane, forming an "egg-cup" shape where much of the lipid droplet sits nestled in a layer of ER[91]. This "egg-cup" or "vesicular" model suggests that lipid droplets form entirely separate from, though adjacent to, the endoplasmic reticulum, within vesicles that are derived from the ER via some of the typical secretory pathway machinery[21, 92]. In this model, neutral lipid accumulates within the bilayer of the vesicle until neutral lipid fills the vesicle, dispersing or displacing the small inner leaflet of the vesicle and retaining the outer leaflet as the LD monolayer.

While some components of membrane trafficking in the secretory pathway have indeed been implicated in lipid droplet function[42, 43, 62], and neutral lipid can be synthesized

directly on lipid droplets, bypassing the ER[69], it is difficult to reconcile this model with the known ER origin of a substantial portion of LD neutral lipids and proteins and the demonstrated exchangeability of these components over the lifetime of many lipid droplets[69-71, 76]. Neutral lipid not synthesized on the lipid droplet surface would have to be extracted from the ER membrane, cross at least a thin layer of aqueous cytosol and be specifically inserted into the phospholipid bilayer or monolayer of the targeted vesicle/LD before reaching the hydrophobic core. This model has gained considerably less traction than the budding model because of these topological constraints and its resulting lack of parsimony; furthermore, the egg-cup shape that forms the inspiration for this model is not inconsistent with a lipid droplet that has budded from the ER but maintains close proximity and communication with the endoplasmic reticulum after its maturation.

A third model, largely a variation of the lens model, was once proposed wherein, rather than bulging or budding outward from the ER, the nascent lens-shaped lipid droplet would be excised from the ER as a bicelle with both cytosolic and luminal leaflet membrane components[14]. Although this "bicelle" model seemed attractive in explaining the extraction of integral membrane proteins from the ER through excision with the lipid droplet, it has not been favored due to the inherent problem of such scission being likely to generate holes within the ER membrane and release luminal contents into the cytoplasm[21].

Lipid Droplet Initiation vs. Growth

Once a lipid droplet is formed from the endoplasmic reticulum, it must then grow to accumulate lipid, and in mammalian cells, especially adipocytes, it must often mature and specialize as it does so[58, 93]. It has become increasingly apparent within the lipid droplet field that there is a distinct mechanistic switch between the de novo biogenesis, or initiation, of a lipid droplet and the subsequent growth of pre-existing lipid droplets[69, 94, 95]. It is important not to conflate these two processes; upon review, many early descriptions of models and experiments implicating certain proteins in "lipid droplet biogenesis" are found to more accurately describe the partitioning of lipid into pre-existing droplets during lipid droplet expansion. This is further complicated by the fact that most direct observations of lipid droplets, even during de novo lipid droplet formation, are of droplets that are already fairly well established at >100nm diameter as a result of resolution constraints, and by the lack of a defined threshold for what size of lipid droplet constitutes an early, initiating LD as opposed to a mature, growing LD[95, 96]. While much has been learned from the study of how lipid is packaged into pre-existing lipid droplets, the mechanism of lipid droplet biogenesis, a term which I use to mean the initial formation of the LD structure, has remained much less understood. It is therefore important not to confound these processes when interpreting experimental results, especially considering the likelihood of separate mechanisms for each stage.

Lipid droplet growth after initiation was initially thought to occur through fusion of small, newly synthesized lipid droplets into larger LDs[97]. While growth by LD fusion certainly appears to occur in the generation of a large unilocular droplet during adipocyte differentiation[98], LD fusion in non-adipose or yeast cells is at best a rare event: fusion events

have never been observed in wild-type yeast[99], and the study that initially proposed SNARE-mediated fusion of lipid droplets as a common growth mechanism[100] was largely discounted by a experiments demonstrating that only severe chemical disruption of the LD phospholipid monolayer could induce LD fusion[44]. Therefore, fusion of lipid droplets is unlikely to represent a conserved mechanism of lipid droplet growth.

The mechanistic switch between lipid droplet initation and growth that has since emerged relies on the differential localization of neutral lipid synthesis proteins[95]. While some isoforms of enzymes in the neutral lipid synthesis pathway localize exclusively to the ER, other isoforms have been found to translocate to the surface of a subset of pre-existing LDs[69, 94], and neutral lipid synthesis activity has been detected in isolated lipid droplets[101]. Therefore, it appears that neutral lipid synthesized within the ER is largely packaged into newly-forming lipid droplets, while additional neutral lipid can be synthesized directly on the LD surface for droplet growth. These experiments were conducted in mammalian cells; yeast lack the multiple enzyme isoforms and the heterogenous LD populations described in these studies. However, the differential localization of the two yeast triacylglycerol synthases (Lro1p in the ER, Dga1p in the ER and LDs[72, 102]) and observations in this study (Chapter Four) suggest that some form of this mechanistic switch between LD initiation and growth may be highly conserved.

Drivers of Lipid Droplet Biogenesis

Unfortunately, many of the models described above for lipid droplet biogenesis remain largely descriptive; the mechanisms driving such processes are not yet well established. An open question exists as to whether lipid droplet formation is a spontaneous process driven by the physicochemical properties of the hydrophobic molecules involved, by active construction by protein machinery, or by a combination of these two forces, the manipulation and regulation of physicochemical factors by proteins. Of note, all of the proposed mechanisms discussed below are at least partially based on the budding model of lipid droplet biogenesis described in Section 2-2-2, as it is the most widely discussed and intuitive model.

The notion of hydrophobic forces acting as the primary driver in lipid droplet budding has gained popularity quite recently, culminating in a very thorough model presented by Thiam et al. this year[41]. This model is based on the biophysical view of lipid droplets as an emulsion within the cytosol, utilizing phospholipid as a surfactant. This "spontaneous emulsification" model posits that neutral lipid undergoes spontaneous de-wetting from a lens-shaped accumulation into a spherical shape as a result of hydrophobic forces optimizing the surface area of the globule. Subsequent budding of the spherical lipid drop then relies on decreased surface tension of the monolayer; this surface tension can be influenced by phospholipid composition and by the presence of free fatty acids (FFA) or diacylglycerol (DAG) as co-surfactants, resulting in potentially highly variable bud size. This model therefore proposes that lipid droplets are formed spontaneously in the presence of sufficient neutral lipid and surfactant; Thiam et al. suggest that this explains the lack of any single gene deletion found to be incapable of generating lipid droplets[41]. While the biophysical

considerations of this model appear sound, this final argument is flawed – proteins may be critical for the regulation, catalysis, or suppression of this mechanism, in which case even in the absence of such proteins, spontaneous formation of lipid drops would naturally be expected to occur in a dysregulated, stochastic, or de-repressed fashion. Indeed, in a separate summary of this model, authors from the same laboratories note that a few studies indicate that TAG accumulation alone may be insufficient for lipid droplet formation[82, 103] and suggest that while they do not believe proteins to be essential for coalescence of a lipid drop, they may act in the facilitation or regulation of these "spontaneous" steps[95].

Indeed, the concept of proteins effecting lipid droplet formation through the manipulation of the physicochemical forces described above is highly attractive. Certain perilipin proteins have been suggested to promote lipid droplet biogenesis by aiding in the generation of curvature of the cytoplasmic ER leaflet[21, 56-59]; in light of the spontaneous emulsification model, these proteins can now be viewed to act in stabilizing the surface tension of a nascent lipid droplet. Our laboratory has found the phosphatidic acid hydrolase that generates DAG from PA (lipin in mammals; Pah1p in yeast) to play a role in lipid droplet formation via the generation of DAG independent of its subsequent conversion into neutral lipid[82]; by this model, DAG produced by lipin could effect lipid droplet biogenesis by acting as a co-surfactant. These potential explanations are yet untested, however. Furthermore, additional proteins found to drive or regulate lipid droplet formation, namely seipin and the FIT proteins[103, 104], do not present such obvious mechanisms for incorporation into a facilitated emulsification model, largely because little is known about their action. This work will focus on the potential role of seipin as a key regulator of lipid droplet

formation and as a potential driver of lipid droplet biogenesis, or at least a catalyst for lipid droplet emulsion.

SEIPIN AND HUMAN DISEASE

Congenital Generalized Lipodystrophy

The seipin gene was first discovered through mutation in the most severe recorded cases of congenital generalized lipodystrophy, a striking and devastating defect of the primary fat storage organ, adipose tissue[104, 105]. Lipodystrophy in general refers to any relative deficit of adipose tissue and ranges from atypical redistribution of adipose tissue, to local lipodystrophy at the site of certain types of injuries, to partial lipodystrophy affecting limited regional adipose depots, and finally to generalized, or whole-body lipodystrophy. affecting all metabolic (and some non-metabolic) adipose depots. Lipodystrophy can further be classified as congenital or acquired: most congenital, also known as familial, forms have been associated with single genetic mutations, while extensive acquired lipodystrophy usually results from either an autoimmune disorder (one of which has now been associated with genetic mutation) or as a side effect from drug therapy (HIV-associated lipodystrophy in patients treated with anti-retroviral therapy; the most common form of lipodystrophy)[106].

The first known recorded case was a partial, likely acquired lipodystrophy reported in 1885, in a female patient presenting with progressive fat wasting of the upper body[107]. In this instance, only a subset of adipose depots were affected, and the patient was otherwise

asymptomatic – many such patients with local or limited partial lipodystrophy retain enough functional adipose tissue to avoid major metabolic complications[106, 107].

More extensive adipose loss, however, can result in a drastic metabolic syndrome. The first thorough report of a generalized lipodsytrophy was published in 1946; this acquired generalized lipodystrophy (AGL) was at the time often termed "lipoatrophic diabetes" after the diabetic complications that developed in such patients, and has also been referred to as Seip-Lawrence syndrome[108]. Due to the lack of functional adipose tissue for storing fat, patients with generalized or severe partial lipodystrophy present with extreme hypertriglyceridemia; subsequent ectopic accumulation of triacylglycerol in non-adipose tissues generates a clinical picture of muscular hypertrophy, organomegaly, eruptive xanthomas, and recurring pancreatitis. Complications resulting from this ectopic lipid storage include fatty liver disease, umbilical hernia, insulin-resistant (type II) diabetes, and polycystic ovarian syndrome in female patients; further metabolic abnormalities include low levels of high density lipoprotein, leptin, and adiponectin, and increased basal metabolic rate, body temperature, and appetite[104, 106].

The first reports of congenital generalized lipodystrophy (CGL) described children who appeared obviously lipodystrophic at birth or within their first two years; these case reports were made by W. Berardinelli in 1954[109] and Martin Seip in 1959[110], and hence the syndrome became known as Berardinelli-Seip congenital lipodystrophy (BSCL). The clinical picture is remarkably worse for children with CGL compared to adults with AGL because the early onset of the manifestations described above leads to additional complications. Many of these are hormonal effects typically seen in other syndromes of

infantile hyperinsulinemia: accelerated growth followed by early growth arrest, mild acromegaloid appearance, and moderate genital enlargement[111].

No treatment has yet been found to reverse lipodystrophy. Agonists of PPARγ (thiazolidinediones; TZDs), the major transcriptional activator of adipocyte differentiation[112], can promote adipogenesis in vitro[113], but they do not seem able to produce this effect to generate new adipose tissue in CGL. Reports disagree as to whether TZDs can promote differentiation of CGL adipocytes in vitro[114-116], and although treatment of both CGL patients and lipodystrophic mouse models can result in improved metabolic parameters[117, 118], and some adipose gains were observed in TZD-treated seipin knockout mice[118], long-term treatment of CGL patients resulted in little to no gain of adipose tissue[117]. Treatment has therefore necessarily focused on treating the metabolic complications of CGL, mainly in terms of ameliorating hypertriglyceridemia and diabetes. TZDs have shown to be particularly effective in this regard[117]; other treatments that have met some success are the triglyceride-lowering fibrates, the anti-diabetic metformin, and the later withdrawn appetite-suppressant fenfluramine[119, 120]. Leptin replacement therapy has also been utilized: since leptin is synthesized and secreted by mature adipocytes, CGL patients are extremely leptin-deficient[121], and leptin treatment was found to be effective in a mouse model of lipodystrophy[122]. Leptin replacement therapy is extremely effective in CGL patients, both suppressing appetite and correcting most metabolic parameters[123-126]; as a result, leptin recently became the first FDA-approved treatment for congenital generalized lipodystrophy[127].

Congenital generalized lipodystrophy is an autosomal recessive disorder observed in at least 250 families, with an estimated global prevalence of 1 in 10 million[128]. Notable clusters of families have been identified in populations in Norway, Brazil, and Lebanon[105, 129-131]. These families were used to identify the first two genetic loci associated with CGL: *AGPAT2* (*BSCL1*)[132, 133], encoding an enzyme responsible for the acylation of lysophosphatidic acid to yield PA (1-acylglycerol-3-phosphate-*O*-acyltransferase) in the neutral and phospholipid synthesis pathway[134, 135], and *BSCL2*[105], encoding a protein of unknown function, later named seipin after the pediatrician who identified, characterized, and performed early treatments on the first Norwegian BSCL2 patients[110, 119]. Much later, sequencing of candidate genes in a few CGL patients lacking *BSCL1/2* mutations identified mutations in *CAV1*, encoding caveolin-1[136], and *PTRF*, encoding cavin-1[137]; these proteins are known to be critical for caveolar function, which in turn is understood to play a role in adipocyte signaling and lipid uptake[138, 139]. *AGPAT2, BSCL2, CAV1,* and *PTRF* are the only genes with mutations linked to CGL thus far, however there are a few known CGL patients lacking mutations in these four genes, indicating at least one CGL-linked locus yet to be identified[106, 140].

Although patients with mutations in any one of these four genes present the classic picture of generalized lipodsytrophy with hypertriglyceridemia and insulin resistance, several phenotypic differences have been noted. One of the most remarkable differences is in the distribution of adipose depots affected: all four sets of patients are deficient in subcutaneous and visceral adipose, but patients bearing mutations in *AGPAT2* retain mechanical adipose depots (retro-orbital, buccal, tongue, palmar, plantar, crista galli, scalp, perineum, vulvar,

periarticular, epidural, and pericalyceal fat pads)[141], while *CAV1* and *PTRF* patients retain bone marrow adipose[136, 137]. *BSCL2* represents the most extreme form of generalized lipodystrophy, as no preserved adipose tissues have been detected in these patients, and they also often present with an earlier onset of diabetes than patients in the other classes[121, 141, 142]. Even *BSCL2* patients are not 100% deficient in adipose, however: subcutaneous biopsies from Seip's patients show rare scattered clusters of small adipocytes[143]. Whether these residual adipocytes retain significant functionality to modulate the CGL phenotype remains unclear[104].

In each class of patients, additional phenotypes have been noted which are not obviously related to the loss of adipose tissue, and are likely due to the roles these genes play in non-adipose cell types. *AGPAT2* patients display lytic bone lesions not found in patients with mutations in the other genes[141, 144], and *CAV1* and *PTRF* patients suffer from a host of additional manifestations relating to renal and muscle dysfunction[136, 137, 145-147]. *BSCL2* patients present with the unique manifestations of mild mental retardation[105], hypertrophic cardiomyopathy[142, 144], and male infertility[148].

Evidence suggests that each of these additional *BSCL2* phenotypes is likely due to cell-autonomous effects of seipin mutation, rather than indirect effects of adipose loss. Seipin is highly and broadly expressed in brain tissue[105, 149, 150], and a neuronal-specific seipin knockout mouse displayed affective disorders that could be related to the mild cognitive deficit in human patients[151] (discussed further in Section 2-4-1). Furthermore, histology of *BSCL2* patient heart tissue indicates no ectopic lipid accumulation in cardiomyocytes or coronary arteries, precluding hypertriglyceridemia as a likely cause of cardiomyopathy[152].

Finally, seipin is also highly expressed in testis[105], and a germ cell-specific seipin knockout mouse presented with teratozoospermia similar to that observed in the systemic knockout mouse and a male *BSCL2* patient[118, 148].

Numerous distinct mutations have been identified in *BSCL2* patients (Table 1). These are mostly comprised of frameshifts, nonsense mutations, or large deletions, all of which result in large regions missing from the seipin gene. Four missense mutations were identified in the conserved core of the gene[105, 153, 154]; of these, one has been suggested to be unstable[155]. Thus most of the mutations identified likely result in an effective lack of seipin; the few notable missense mutants and shorter truncations that provide some insight into seipin function are discussed in Section 2-4.

Several tools have been generated for studying seipin-deficient lipodystrophy in model organisms. Three independently generated seipin knockout mice have been reported in the last three years: all three mouse models display a severe lipodystrophic phenotype (albeit with less severe adipose loss than in human patients) that manifests with fatty liver and insulin resistance[115, 118, 156]. Seemingly paradoxically, transgenic adipose-specific overexpression of seipin resulted in a similar phenotype, suggesting that dosage of seipin may be important[157]. A fruit fly seipin knockout also presented with deficiencies in the fat body, an organ with both adipose and liver features, indicating that this role for seipin in fat tissue function is well conserved[158].

The lack of adipose tissue in the absence of seipin is believed to be due to a defect in adipocyte differentiation. Studies of seipin knockdown in established pre-adipocyte cell lines have indicated no defect in determination to the pre-adipocytic lineage[159], but rather drastic

Truncations	OMIM #	First report
L63fsX75	606158.0001	Ref. [105]
L100fsX111	606158.0002	"
P105fsX112	606158.0003	"
P105fsX111	606158.0005	"
V108fsX113	606158.0006	"
R138X	606158.0007	"
Y213fsX232	606158.0010	"
R224fsX225	606158.0011	"
R224ΔY255-Q271fsX288	606158.0012	"
R275X	606158.0015	Ref. [160]
E189X**	606158.0016	Ref. [161]
I262fsX273**	---	Ref. [162]
Q391X	---	Ref. [153]
Large Deletions		
del/ins exons 5-6	606158.0004	Ref. [105]
del exon 4	606158.0008	"
del exons 4-6	---	"
del exon 5	---	Ref. [153]
Missense mutations		
A212P	606158.0009	Ref. [105]
N88S*	606158.0013	Ref. [163]
S90L*	606158.0014	"
T78A	---	Ref. [153]
L91P	---	"
Y187C	---	Ref. [154]

Table 1. Known disease-causing seipin mutations in humans. Modified from Cartwright and Goodman 2012. * Mutations causing neuronal seipinopathy without lipodystrophy; ** mutations found in one compound heterozygous lipodystrophic patient with dystonia; known homozygotes display no neuronal symptoms

deficits in the differentiation of 3T3-L1 and CBH10T1/2 pre-adipocytes into mature adipocytes[114, 159]. Similar defects in terminal adipocyte differentiation were observed in mouse embryonic fibroblasts (MEFs) obtained from seipin knockout mice[115, 118]. Both of these studies have noted increased basal lipolysis during terminal adipocyte differentiation; while one such study suggested that this lipolysis may be the causal factor preventing adipocyte maturation[115], another found that adipogenesis could be rescued by TZD administration without any suppression of lipolysis[118]. This suggests a potential additional role for seipin in the maintenance or function of mature adipocytes, corroborating with the lipodystrophic phenotype observed in an adipose-specific knockout mouse reported recently[164].

Seipin therefore plays a clear role in the function of the adipose organ; the mechanism of this role, however, remains unclear. Three candidate binding partners for mammalian seipin have been proposed to transduce seipin's effect on adipogenesis. Yang et al. have suggested that the adipogenic block could occur as a result of a defect in actin cytosekelton remodeling necessary for mature adipocyte development and formation of a large unilocular lipid droplet. They further reported evidence that seipin may interact with 14-3-3β to effect actin remodeling, but these experiments were only performed with overexpressed protein, so this interaction could be artefactual. Similarly overexpressed co-immunoprecipitation studies identified a potential interaction between seipin and lipin, a protein found to be mutated in a spontaneous syndrome of lipodystrophy and peripheral neuropathy in mice[165-167], and this putative interaction appeared to be required for adipogenesis[168, 169]. The potential interaction between seipin and lipin will be more thoroughly discussed in Section 5-2-2.

Bi et al. very recently reported immunoprecipitation studies identifying an interaction in *Drosophila* fat body cells between endogenous seipin and SERCA, an ER calcium pump[170]; SERCA specifically[171, 172] and ER calcium homeostasis in general[173, 174] have both been implicated in adipocyte differentiation, although the mechanism of this effect is not clear. These authors also suggested that the seipin-SERCA action could explain the cognitive and cardiac defects in *BSCL2* patients, as intracellular calcium homeostasis is key for the function of both neurons and cardiomyocytes[170]. Given the wide range of potential mechanisms proposed by these putative interactions, these three binding partners will have to be validated, and additional experiments will be required to determine which of these (if any) represents the primary pathogenesis of seipin deficiency.

Neuronal Seipinopathies

Intriguingly, lipodystrophy is not the only disease associated with seipin; two seipin point mutations, N88S and S90L, were identified in patients with apparently normal adipose tissue but displaying symptoms of motor neuropathy[163]. Patients with either of these two seipin mutations can present with either upper or motor neuron disruption, so that most are diagnosed with either spastic paraplegia (muscle weakness and spasticity in lower limbs, also termed Silver syndrome) or distal hereditary motor neuropathy (distal limb weakness), although broader patterns of motor neuron dysfunction not falling within these two syndromes have also been observed[175]. The several motor neuropathy syndromes associated with either of these seipin mutations have been collectively termed "seipinopathies."[176] Two

additional patients have been identified with neuronal dysfunction associated with seipin mutations other than the two classic seipinopathy mutants: one patient with a large seipin deletion was found to suffer from more extreme, early onset, fatal neurodegeneration of the cerebral cortex and basal ganglia[177], while another compound heterozygous patient with mutations known individually to be associated with lipodystrophy[161, 178] presented with both lipodystrophy and motor neuron dysfunction[162]. Whether seipin is indeed the cause of these unusual presentations of neuronal dysfunction, or whether other independent or modifier loci are present in these patients, is not yet known.

While congenital generalized lipodsytrophy is an autosomal recessive disorder[105], neuronal seipinopathies are inherited in an autosomal dominant pattern[163], suggesting that CGL represents a loss of seipin function and seipinopathy may reflect a gain of toxic function[176]. The primary mutations causing neuronal seipinopathy, N88S and S90L, were found to disrupt a site of asparagine-linked glycosylation on the seipin protein, resulting in relative unfolding, ubiquitination, and marked aggregation of seipin, as well as activation of ER stress and apoptotic pathways[163, 179]. Several transgenic models have recapitulated this phenotype in lower organisms: an N88S transgenic mouse developed spastic motor neuron defects and muscular atrophy with seipin aggregates and ER stress[180, 181]; a transgenic mouse expressing an N88S/S90L double mutant developed late-onset locomotor and gait abnormalities with seipin aggregates, ER stress, disrupted Golgi, and increased autophagy[182]; and transgenic N88S zebrafish larvae demonstrated decreased motility[183]. Remarkably, neuronal death does not appear to be required for neuropathy: in the N88S transgenic mouse, clear motor deficits were apparent without observable motor neuron death, although a

decrease in axonal transport was detected[180]. This suggests that glycosylation mutants of seipin may interfere with neuronal function; indeed, cultured neurons overexpressing N88S seipin display defects in both excitatory and inhibitory post-synaptic current, impaired docking of synaptic vesicles, and partial colocalization of seipin with synaptophysin[184]. Since motor neuron death has yet to be reported in patients with seipinopathy, these neuronal defects in these patients could be due to a dysfunction in vesicular transport rather than neuronal cell death, although the etiology of such vesicular defects is not known.

Interestingly, although aggregation of seipin was initially thought to be the pathological cause of motor neuron degeneration in seipinopathy patients, the formation of seipin aggregates may actually be cytoprotective: HeLa cells expressing N88S seipin were actually found to be more likely to exhibit ER stress and cell death if they lacked seipin aggregates. These seipin aggregates, also referred to as inclusion bodies, colocalized with a variant of α-antitrypsin, suggesting their possible accumulation in unique cytoplasmic vesicles known as ER-derived protective organelles (ERPO)[181].

Intriguingly, the ultrastructure of these putative ERPO vesicles (by electron microscopy with immunolabeling for seipin) more closely resembles the appearance of lipid droplets than that of membrane-bound vesicles[181]; although the authors of this study did not note this similarity, the possibility that glycosylation mutants of seipin could be sequestered on lipid droplets is attractive, given the putative role of lipid droplets in protein sequestration (Section 2-1-2) and the intimate functional and physical association between seipin and lipid droplets (Section 2-4). This hypothesis that mutant seipin inclusion bodies may actually represent sequestration on the lipid droplet is supported by recent experiments connecting

seipinopathy and triacylglycerol storage. In cultured motor neurons, expansion of lipid droplet stores via either exogenous oleate treatment or inhibition of lipolysis could alleviate N88S-induced ER stress. Exogenous oleate treatment was also able to improve motility in N88S transgenic zebrafish larvae, and, importantly, redistribute seipin N88S from the ER to lipid droplets[183].

Thus lipid droplets could be the "ER-derived protective organelle" in which unglycosylated seipin accumulates; consistent with this potential role as a protective organelle, lipid droplets are upregulated by multiple ER stressors, although they are not essential for cell viability under ER stress conditions[185]. This putative role for lipid droplets may even be conserved across multiple etiologies of neuropathy: two other proteins associated with spastic paraplegia, atlastin and REEP1, have been implicated in both ER morphology and lipid droplet function[186, 187]. It will be important to determine whether the redistribution of mutant seipin to lipid droplets does indeed account for the aggregation of seipin seen in motor neurons, and whether this redistribution results in any pathological effects on lipid metabolism.

SEIPIN AND LIPID DROPLETS

Dissection of Metazoan and Ancient Functions

Several lines of evidence exist that seipin does not only function in the differentiation of adipocytes, but plays a role in non-adipocytic cells as well. As described in Section 2-3-1,

seipin-deficient patients display multiple apparently cell-autonomous defects in neurons, cardiomyocytes, and sperm cells. The putative role of seipin in the brain was supported by a study of seipin knockdown in cultured cortical neurons, which found that seipin deficiency impaired excitatory post synaptic currents and cell surface localization of AMPA receptors without affecting inhibitory currents[188]. This finding was supported by the report of depression-like behavior in the neuron-specific seipin knockout mouse[151]; it is possible that both affective disorders in mice and mental retardation in humans could be due to relative over-inhibition as a result of cortical excitatory/inhibitory imbalance[151, 188, 189]. However, this depressed phenotype could additionally or alternatively be due to the same etiology that generates the adipogenic defect, since suppression of PPARγ appears to play a role in the phenotype of neuronal seipin knockout mice[151].

The fly seipin knockout provides additional evidence that seipin may function in non-adipose cells in a manner distinct from its role in adipocytes. In addition to the lipodsytrophic phenotype observed in the larval fat body, *dSeipin* flies exhibited ectopic lipid accumulation into large lipid droplets in the midgut and salivary glands; surprisingly, these phenotypes were found to be cell-autonomous, independent of the fat body deficit[158]. Additionally, cultured fibroblasts and lymphoblastoid cells derived from *BSCL2* patients exhibit disorganized lipid droplets, further indicating a potential role for seipin in lipid manipulation in non-adipose tissues[190, 191]. This was underscored by the identification of seipin knockout lipid droplet phenotypes in the unicellular yeast *S. cerevisiae*[190, 192] (thoroughly reviewed in Section 2-4-2).

Finally, experiments in fruit flies and in 3T3-L1 cells have indicated that the roles of seipin in adipose and non-adipose tissue may in fact be dissectible, produced by distinct regions of the seipin protein. Analysis of the gene sequence of seipin homologues in multiple species indicates a conserved core region (essentially equivalent to the full gene in yeast; see Section 2-4-2) flanked by highly variable N- and C-terminal domains[190]. Yang et al. found that seipin overexpression suppressed lipid droplet formation in undifferentiated 3T3-L1 preadipocytes (which generally behave as fibroblasts), and used this phenotype as an output for testing seipin function in non-adipocytes[193]. They identified the conserved core sequence of mouse seipin as sufficient for function in non-adipocytes, while the more divergent C-terminal domain was necessary for function in adipogenesis; this dissection of function was conserved in the fly despite highly divergent sequence of the C-terminal domain[158]. Phylogenetic analysis indicated that only 22 seipin homologues, all within mammalian species, contained a C-terminal domain of high sequence similarity to that of human seipin. These authors therefore concluded that the mammalian seipin C-terminal domain and its function in adipogenesis were acquired very recently in evolution[193], although some conservation of function does appear in fly fat body lipogenesis. In contrast, the core sequence is present across animal, plant, and fungal species, indicating an evolutionarily conserved role for seipin in the fundamental cell biology of non-adipose tissues[190].

Seipin in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, or baker's yeast, has proved a valuable model organism for lipid droplet studies because of the thorough characterization of yeast biosynthetic pathways, the abundance of genetic and biochemical tools and protocols, the ease of microscopic visualization, and the relative homogeneity of yeast LDs[194]. The *S. cerevisiae* homolog of seipin, was identified in two independent screens of a yeast genomic knockout library for genes affecting the appearance of lipid droplets[190, 192]. One of these two screens generally grouped genes into two classes: "few lipid droplets," (fld) and "many lipid droplets" (mld); the first-pass analysis of the seipin knockout strain placed it into the former group, leading to the gene name *FLD1*[192]. Comparison by both sequence homology and predicted secondary structure led to the identification of this gene as homologous to human seipin; both human and mouse seipin were able to successfully complement the yeast *fld1*Δ lipid droplet phenotype[190, 192].

Almost all of the *FLD1* gene consists of the evolutionarily conserved "core" sequence (approximately 210 amino acids in length): only a total of 23 amino acid residues lie outside of the core alignment[190]. Human seipin was found to be an integral ER membrane protein with two transmembrane domains, cytosolic N- and C-termini, and a large luminal domain, often referred to as the "luminal loop"[195]. Yeast Fld1p was also found to localize to the endoplasmic reticulum[192], specifically concentrated into puncta[196] found to be associated with lipid droplets[190, 197, 198]. Fld1p purified from ER membranes was found to self-associate into a stable homooligomeric toroidal-shaped complex of approximately nine subunits[155]; subsequent analysis of human seipin identified a complex of twelve subunits of a circular

shape consistent with the yeast seipin toroid[169]. Seipin therefore assembles into circular complexes at the LD-ER junction.

All of the known seipin missense mutations identified in human patients are located in the luminal domain of the protein. The A212P lipodystrophy mutant of human seipin was unable to rescue lipid droplet appearance in *fld1*Δ yeast[190, 192, 198], nor could analogous mutations of the yeast gene, *fld1*^{S224P} and *fld1*^{G225P} [190]. The Y187L and L91P lipodystrophy mutations were also non-functional in yeast, and along with A212P did not display normal LD-ER puncta localization[198]. The N88S and S90L seipinopathy mutants, however, were fully functional in yeast[192], consistent with the lack of a glycosylation site in yeast seipin (Joel Goodman, unpublished observations).

Yeast lacking seipin produced a remarkable phenotype of disorganized lipid droplet morphology. While wild-type yeast lipid droplets are remarkably homogenous in size (generally 0.3-0.4 μm diameter[192]), lipid droplets in the *fld1*Δ strain were strikingly heterogenous. Two predominant morphological patterns were noted: 1) unusually large lipid droplets (0.5-1.5 μm in diameter; up to 50 times the volume of a wild type droplet), often termed supersized lipid droplets (SLDs) (arbitrarily defined in the literature as >1μm diameter)[99, 190, 192]; 2) clusters or amorphous aggregates of small droplets, often appearing enmeshed in excess ER membrane, in which case they are sometimes termed LD-ER tangles[83, 190, 192].

The prevalence of each of these morphological classes can be shifted based on the type of media in which *fdl1*Δ yeast are cultured. After growth in minimal glucose media, a majority of cells displayed supersized lipid droplets, while in rich media containing glucose

or oleate, the clustered/amorphous aggregate phenotype predominated[99, 192]. Fei et al. therefore speculated that nutrients available in rich yeast media but not in minimal medium were responsible for this phenotypic shift; indeed, inositol was found to fit these criteria, suppressing the appearance of supersized lipid droplets[99] in favor of droplet clusters[198]. While both inositol and choline reversed the SLDs found in yeast mutants of phospholipid synthesis, choline was not capable of suppressing SLDs in *fld1*Δ. Fei et al. further found that ethanolamine produced an effect opposite to that of inositol, increasing the percentage of cells displaying supersized lipid droplets[99], although this effect of ethanolamine was not reproduced in another report[198]. While inositol treatment also produced an increase in the phospholipid to neutral lipid ratio of isolated *fld1*Δ droplets, corresponding with the increased surface area to volume ratio of LD clusters compared to SLDs, no detectable effect was seen after ethanolamine treatment[99].

These phenotypic shifts could potentially be explained by the basic physicochemistry of the phospholipids synthesized from inositol and ethanolamine. Phosphatidylinositol, due to its large head group, generates a positive curvature especially suited for smaller lipid droplets; thus increased PI on the droplet surface could potentially promote a smaller droplet morphology, generating the observed clusters. Phosphatidylethanolamine is quite the opposite, generating a negative membrane curvature, leading Fei et al. to suggest that excess PE generates a lipid droplet with less surface integrity, promoting fusion of LDs to generate supersized droplets. Indeed, while lipid droplet fusion events were never observed for wild-type yeast, a minority of *fld1*Δ cells demonstrated fusion of smaller LDs into an SLD during a course of time-lapse microscopy. Purified *fld1*Δ LDs were also more fusogenic in vitro,

and in vitro fusion was potentiated by addition of the negative-curvature phospholipids PA and PE[99, 192]. However, the Goodman laboratory has not detected any evidence of lipid droplet fusion in our studies of lipid droplet biogenesis in *fld1*Δ (Chris Hilton, Derk Binns, and Bethany Cartwright, unpublished observations), and so it remains to be determined whether fusion is indeed a significant cause of supersized lipid droplets in seipin-deficient yeast.

Several research groups have avidly searched for abnormalities in lipid composition or metabolism in the absence of seipin, and while several shifts in both neutral and phospholipid composition have been observed, these are generally modest or subtle, with no clear pattern for an obvious single-enzyme or single-lipid defect. In terms of neutral lipid, Fei et al. reported a doubling in steady state levels of both triacylglycerols and steryl esters in *fld1*Δ compared to wild type[192], consistent with increased salivary gland lipogenesis in the *dSeipin* fly[158]. Members of the Goodman laboratory were unable to reproduce this increase in neutral lipid in *fld1*Δ yeast, however (Chris Hilton, unpublished data).

A few genes encoding enzymes of phospholipid synthesis were upregulated in fld1Δ cells[99, 198], although the phospholipid composition of lipid droplets isolated from *fld1*Δ yeast was not found to be significantly different from that of wild-type droplets[199]. A slight increase was observed for PA in isolated ER membranes[99], and microscopy of fluorescent probes for PA has indicated an unusual concentration at the LD-ER junction in *fld1*Δ cells (Sungwon Han, unpublished data). Slight shifts were also observed in the fatty acyl composition of phospholipids, from long unsaturated fatty acids to shorter, saturated FAs[192]. A similar modest shift was observed in PLs and TAG in lymphoblastoid cell lines derived

from *BSCL2* pateints, apparently suggesting a defect in fatty acid Δ9 desaturase activity[191]; this activity has not yet been directly assayed in the absence of seipin, however.

These subtle lipid abnormalities could be due to direct functions of seipin, or they could be due to altered functionality of the morphologically abnormal seipin-deficient LDs[104]. Several lines of evidence have suggested that *fld1*Δ lipid droplets are not fully functional. Inheritance of lipid droplets into daughter cells during mitosis is impeded in *fld1*Δ cells, possibly due to the entrapment of many *fld1*Δ LDs in tangles of ER membrane[83]. Furthermore, *fld1*Δ cells display increased sensitivity to terbinafine[198], a fungicidal inhibitor of the sterol synthesis enzyme Erg1p[200], which is known to associate with lipid droplets[71].

No severe defect in protein targeting to the lipid droplet surface has been observed: Erg6p, an abundant and commonly-used lipid droplet marker, was generally able to localize to lipid droplets in the absence of seipin[190], and only modest changes were observed in the proteomics profile of lipid droplets isolated from *fld1*Δ cells[199]. A more subtle defect has been observed, however, in the targeting of the dominant yeast triacylglycerol lipase, Tgl3p, to the lipid droplet surface. Wolinski et al. found that Tgl3p localized to a discrete punctum on each lipid droplet when cells were in stationary phase, then redistributed across the entire lipid droplet surface when cells were switched to fresh media to promote growth. In *fld1*Δ cells, this pattern was disrupted; only a subset of lipid droplets had associated Tgl3p, and those that did often exhibited diffuse surface rather than punctate localization in stationary phase. Presumably due to this lipase mislocalization, *fld1*Δ cells exhibited decreased rates of lipolysis, a further indication of diminished functionality of *fld1*Δ droplets[83].

The primary etiology of *fld1*Δ lipid droplet morphology and associated defects remains to be determined. Data on the potential interaction of seipin with other proteins has been even more limited in yeast than in mammalian cells. Few other proteins have been found to show an LD-ER junction localization pattern. Lro1p, one of the two triacylglycerol synthases in yeast, localizes to LD-ER junctions, but it does not colocalize with Fld1p, indicating that additional LD-ER junctions exist besides those marked by seipin, and that Lro1p is unlikely to physically interact with Fld1p. Nem1p, a component of a phosphatase complex that acts in the activation of yeast lipin (Pah1p), also localizes to LD-ER junctions, although it accumulates in fewer puncta per cell than Fld1p. The possibility of a functional or physical interaction between Fld1p and Pah1p will be discussed in Section 5-2-1.

One physical interaction has been well demonstrated for yeast seipin, however: Wang et al. recently thoroughly demonstrated and characterized an interaction between Fld1p and Ldb16p, a protein of unknown function. Ldb16p also localized to puncta at LD-ER junctions, a significant portion of which colocalized with Fdl1p, and a physical interaction was demonstrated by both co-immunoprecipitation of endogenously-tagged proteins and by a yeast two-hybrid assay. Furthermore, *ldb16*Δ essentially phenocopied *fld1*Δ lipid droplet morphology, presenting with both supersized lipid droplets and droplet clusters/LD-ER tangles as well as manipulation of phenotype by inositol and increased sensitivity to terbinafine. Unfortunately, the discovery of this interaction does little to further an understanding of seipin action, since Ldb16p has no identified function and no apparent homologs in higher eukaryotes. Intriguingly, however, while *FLD1* was unable to rescue the *ldb16*Δ phentoype, expressing human seipin did restore lipid droplet morphology in the

absence of Ldb16p. Since the human seipin gene contains no apparent regions of homologous sequence with *LDB16*, this suggests that the functions of Fld1p and Ldb16p may have converged during evolution[198].

Seipin as a Driver of Lipid Droplet Biogenesis

Although the effects of yeast seipin on lipid droplet morphology have been thoroughly studied by multiple groups, there was no evidence as to whether this morphological defect represented abnormalities in lipid droplet biogenesis or more downstream effects. The localization of seipin at the LD-ER junction, however, seemed to indicate a potential role in lipid droplet emergence from the endoplasmic reticulum. Members of the Gooodman laboratory therefore utilized a new system for direct analysis of de novo lipid droplet formation in *S. cerevisiae* (discussed in Chapter Three and Section 4-3-2) and found that *fld1*Δ cells display a severe impediment in lipid droplet biogenesis. In the absence of seipin during early de novo droplet formation, few droplets are generated, and excess neutral lipid instead accumulates in the endoplasmic reticulum (Chris Hilton and Derk Binns). We have therefore hypothesized that seipin functions in the regulated release of neutral lipid from the ER into lipid droplets, and that in the absence of this function, neutral lipid blebs stochastically from the ER, generating relatively dysfunctional lipid drops of unregulated composition and morphology (Fig. 2).

For my thesis work, I set out to examine this role for seipin at the lipid droplet. In Chapters Three and Four, I will present experiments confirming the aberrant nature of seipin-

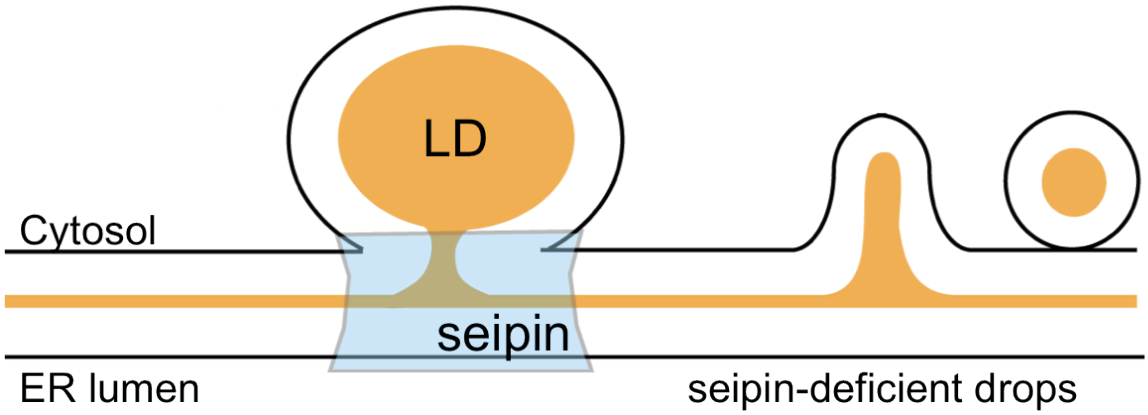

Figure 2. Seipin model of lipid droplet biogenesis. Seipin concentrates at the LD-ER junction and is required for efficient de novo LD formation, likely participating in the packaging or transfer of neutral lipid from its site of synthesis in the ER. In the absence of seipin, neutral lipid accumulates within the ER, until instability is likely to drive the formation of abnormal lipid drops by chaotic "blebbing" outward into the cytosol. Figure from Joel Goodman.

deficient droplets, probing the structure of the yeast seipin complex, and dissecting the function of seipin in lipid droplet morphology and biogenesis. In Chapter Five, I will present impressions and hypotheses for the mechanism of action of seipin in these processes, and suggest further experiments for a better understanding of the role that seipin plays in this fundamental aspect of lipid biology.

CHAPTER THREE
Methodology

MATERIALS

Reagents

All materials were reagent grade. All antibodies were purchased: polyclonal anti-GFP (Millipore), monoclonal anti-myc (National Cell Culture Center), moncolconal anti-Dpm1p (Abcam), and polyclonal anti-dsRed (Clontech). BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentomethyl-4-bora-3a,4a-diaza-s-indacene) was purchased (Invitrogen) and stored at 1mg/mL in DMSO. Zymolyase 100-T was purchased from Zymo Research. Neutral lipid standards for TLC quantitation were purchased from Nu-Chek, phospholipid standards from Avanti Polar Lipids, and TLC plates from Whatman.

Strains

Strains used in this study are listed in Table 2. All genomic integration strains (knock-ins and knockouts) were generated by homologous recombination[201], and PCR (followed by sequencing when necessary) was used to confirm all strains. GFP-tagged strains were obtained from the Yeast-GFP Clone Collection in the BY4741 parental strain background, which utilized *HIS3* as an auxotrophic marker (Invitrogen, developed by Huh et al.[196]). The *FLD1* gene was knocked out in these strains by substitution of the *FLD1* ORF

with the auxotrophic marker *URA3*. The BY4742 *fld1*Δ background strain used for plasmid studies was obtained from the Yeast Knockout Collection (Open Biosystems) and contained the *KanMX* cassette in place of the *FLD1* ORF, conferring resistance to the antibiotic G418. Seipin deletion mutant knock-ins and corresponding wild-type and knockout controls were made by generation of a cassette utilizing the auxotrophic marker *ADE2* (Joel Goodman) and insertion into the parental strain BY4742 (Invitrogen) or the *3.5KO(*^{GAL}*DGA1)* strain (Derk Binns; full genotype in Table 2). Strains transformed with plasmids (see below and Table 3) are indicated in the text or figure legends when appropriate.

Plasmids

Plasmids used in this study are listed in Table 3. Plasmids were transformed into background strains as described in the text and selected with an auxotrophic maker (*LEU2* for pRS315 plasmids; *URA3* for pRS316). The p^{PGK}*fld1*^{Luminal}*-myc*$_{13}$ was constructed by cloning the *PGK1* promoter in place of the *FLD1* promoter in p^{FLD1}*fld1*^{Luminal}*-myc*$_{13}$. The p^{PGK}*fld1-mCherry* deletion series was constructed by inserting Gly$_2$-*mCherry*-STOP in place of the *FLD1* stop codon in the corresponding member of the p^{PGK}*fld1* deletion series, except for p^{PGK}*fld1*^{Luminal}*-mCherry*, where *mCherry* was inserted in place of *myc*$_{13}$ in p^{PGK}*fld1*^{Luminal}*-myc*$_{13}$, and p^{PGK}*fld1*$^{\Delta Luminal}$*-mCherry*, which was generated by PCR fusion of *fld1*^{NTM} and *fld1*^{CTM}*-mCherry* with a Gly$_6$ linker. All other plasmids were obtained from the sources indicated.

Strain Name	Genotype	Source
ERG6-GFP	BY4741 *erg6::ERG6-GFP HIS3*	Invitrogen[196]
FAA4-GFP	BY4741 *faa4::FAA4-GFP HIS3*	Invitrogen[196]
YEH1-GFP	BY4741 *yeh1::YEH1-GFP HIS3*	Invitrogen[196]
TGL1-GFP	BY4741 *tgl1::TGL1-GFP HIS3*	Invitrogen[196]
TGL3-GFP	BY4741 *tgl3::TGL3-GFP HIS3*	Invitrogen[196]
TGL4-GFP	BY4741 *tgl4::TGL4-GFP HIS3*	Invitrogen[196]
*ERG6-GFP fld1*Δ	BY4741 *erg6::ERG6-GFP HIS3 fld1::URA3*	This study
*FAA4-GFP fld1*Δ	BY4741 *faa4::FAA4-GFP HIS3 fld1::URA3*	This study
*YEH1-GFP fld1*Δ	BY4741 *yeh1::YEH1-GFP HIS3 fld1::URA3*	This study
*TGL1-GFP fld1*Δ	BY4741 *tgl1::TGL1-GFP HIS3 fld1::URA3*	This study
*TGL3-GFP fld1*Δ	BY4741 *tgl3::TGL3-GFP HIS3 fld1::URA3*	This study
*TGL4-GFP fld1*Δ	BY4741 *tgl4::TGL4-GFP HIS3 fld1::URA3*	This study
*fld1*Δ (background for plasmids)	BY4742 *fld1::KanMX*	Open Biosystems[202], [202]
$fld1^{\Delta Nterm}$	BY4742 $fld1::fld1^{43\text{-}858}$ *ADE2*	This study
FLD1 (positive control for $fld1^{\Delta Nterm}$)	BY4742 *fld1::FLD1 ADE2*	This study
*fld1*Δ (negative control for $fld1^{\Delta Nterm}$)	BY4742 *fld1::ADE2*	This study
$fld1^{\Delta Nterm}$*-tdtomato*	BY4742 $fld1::fld1^{43\text{-}858}$*-tdtomato ADE2*	This study
FLD1-tdtomato	BY4742 *fld1::FLD1-tdtomato ADE2*	This study
tdtomato	BY4742 *fld1::tdtomato*	This study
3.5KO(^{GAL}*DGA1)* (parental strain)	W303-1A *are1::HIS3 are2::LEU2 lro1::URA3 dga1::TRP1-GAL1-10*(promoter)-*DGA1*	Derk Binns
3.5KOfld1$^{\Delta Nterm}$ *(*^{GAL}*DGA1)*	W303-1A *are1::HIS3 are2::LEU2 lro1::URA3 dga1::TRP1-GAL1-10*(promoter)-*DGA1 fld1::*$fld1^{43\text{-}858}$ *ADE2*	This study
3.5KOFLD1 (^{GAL}*DGA1)* (positive control)	W303-1A *are1::HIS3 are2::LEU2 lro1::URA3 dga1::TRP1-GAL1-10*(promoter)-*DGA1 fld1::FLD1 ADE2*	This study
*3.5KOfld1*Δ *(*^{GAL}*DGA1)* (negative control)	W303-1A *are1::HIS3 are2::LEU2 lro1::URA3 dga1::TRP1-GAL1-10*(promoter)-*DGA1 fld1::ADE2*	This study

Table 2. Strains used in this study

Plasmid Name	Contents	Source
p^{PGK}	pRS315, *PGK1* promoter, *PGK1* terminator (empty vector)	Ref. [203]
p^{PGK}*FLD1*	pRS315, *PGK1* promoter, *FLD1* ORF, *PGK1* terminator	Ref. [190]
p^{FLD1}*fld1*^{Luminal}-*myc*$_{13}$	pRS315, *FLD1* promoter, *KAR2* signal sequence, *fld1*$^{115-738}$, *myc* (13 tandem copies), HDEL, *PGK1* terminator	Derk Binns, Chris Hilton
p^{FLD1}*fld1*$^{Luminal-G225P}$-*myc*$_{13}$	pRS315, *FLD1* promoter, *KAR2* signal sequence, *fld1*$^{115-738, G225P}$, *myc* (13 tandem copies), HDEL, *PGK1* terminator	Derk Binns, Chris Hilton
p^{PGK}*fld1*^{Luminal}-*myc*$_{13}$	pRS315, *PGK1* promoter, *KAR2* signal sequence, *fld1*$^{114-738}$, *myc* (13 tandem copies), HDEL, *PGK1* terminator	This study
p^{PGK}*CFP-HDEL*	pRS316, *PGK1* promoter, *KAR2* signal sequence, *CFP* ORF, HDEL, *PGK1* terminator	Ref. [190]
p^{PGK}*fld1*$^{\Delta Cterm}$	pRS315, *PGK1* promoter, *fld1*$^{1-822}$, *PGK1* terminator	Derk Binns
p^{PGK}*fld1*$^{\Delta CTM}$	pRS315, *PGK1* promoter, *fld1*$^{1-738}$, *PGK1* terminator	Derk Binns
p^{PGK}*fld1*^{NTM}	pRS315, *PGK1* promoter, *fld1*$^{1-114}$, *PGK1* terminator	Derk Binns
p^{PGK}*fld1*$^{\Delta Nterm}$	pRS315, *PGK1* promoter, *fld1*$^{43-858}$, *PGK1* terminator	Derk Binns
p^{PGK}*fld1*$^{\Delta NTM}$	pRS315, *PGK1* promoter, *fld1*$^{115-858}$, *PGK1* terminator	Derk Binns
p^{PGK}*fld1*^{CTM}	pRS315, *PGK1* promoter, *fld1*$^{739-858}$, *PGK1* terminator	Derk Binns
p^{PGK}*fld1*$^{\Delta Cterm}$-*mCherry*	pRS315, *PGK1* promoter, *fld1*$^{1-822}$, *mCherry, PGK1* terminator	This study
p^{PGK}*fld1*$^{\Delta CTM}$-*mCherry*	pRS315, *PGK1* promoter, *fld1*$^{1-738}$, *mCherry, PGK1* terminator	This study
p^{PGK}*fld1*^{NTM}-*mCherry*	pRS315, *PGK1* promoter, *fld1*$^{1-114}$, *mCherry, PGK1* terminator	This study
p^{PGK}*fld1*$^{\Delta Nterm}$-*mCherry*	pRS315, *PGK1* promoter, *fld1*$^{43-858}$, *mCherry, PGK1* terminator	This study
p^{PGK}*fld1*$^{\Delta NTM}$-*mCherry*	pRS315, *PGK1* promoter, *fld1*$^{115-858}$, *mCherry, PGK1* terminator	This study
p^{PGK}*fld1*^{CTM}-*mCherry*	pRS315, *PGK1* promoter, *fld1*$^{739-858}$, *mCherry, PGK1* terminator	This study
p^{PGK}*fld1*$^{\Delta Luminal}$-*mCherry*	pRS315, *PGK* promoter, *fld1*$^{1-114}$, Gly_6, *fld1*$^{739-858}$, *mCherry*, *PGK1* terminator	This study
p^{FLD1}*fld1*^{Luminal}-*mCherry*	pRS315, *PGK1* promoter, *KAR2* signal sequence, *fld1*$^{115-738}$, *mCherry,* HDEL, *PGK1* terminator	This study

Table 3. Plasmids used in this study

Growth Conditions and Media

All cultures were grown in liquid media in a shaking incubator at 30^{o} C and 210 rpm. In each experiment, a colony from a plate was precultured for 18-48 hours in minimal glucose medium before dilution in the indicated experimental media. Minimal medium refers to SC (synthetic complete) medium, consisting of Yeast Nitrogen Base (Bacto), 2% indicated sugar source (glucose, galactose, or raffinose), and amino acid and base supplements appropriate to each strain's auxotrophic markers (complete set includes 40mg/L adenine, 20 mg/L arginine HCl, 100 mg/L aspartic acid, 100 mg/L glutamic acid monosodium salt, 20 mg/L histidine, 60 mg/L leucine, 30 mg/L lysine mono-HCl, 20 mg/L methionine, 50 mg/mL phenylalanine, 375 mg/L serine, 200 mg/L threonine, 40 mg/L tryptophan, 30 mg/L tyrosine, 150 mg/L valine, and 20 mg/L uracil). Rich galactose medium refers to YPGal, consisting of 10 g/L yeast extract (Bacto), 20 g/L peptone (Bacto), and 2% galactose. Rich oleate medium refers to YPO, consisting of 3 g/L yeast extract (Bacto), 16.9 g/L peptone (Bacto), 0.5% potassium phosphate (5% stock buffered to pH 6.0), 0.2% Tween 80, and 0.1% oleate.

Cells grown in minimal glucose media were diluted from the starter culture to a concentration of 0.1 OD_{600}/mL and grown to saturation unless otherwise indicated. For cells treated with oleate, cells were additionally pre-cultured in low glucose minimal media (0.1% glucose) at 0.1 OD_{600}/mL and grown to saturation for 30 hours before inoculation into rich oleate media at a concentration of 1.0 OD_{600}/mL and incubation for 18-20 hr. In galactose induction experiments, cells were diluted from the starter glucose culture to 0.3 OD_{600}/mL in

minimal raffinose media (for derepression), grown for 18 hours, and then diluted to 0.5 OD_{600}/mL in minimal galactose media (for induction). For time-lapse microscopy, the pre-culturing conditions were the same, with induction in rich galactose liquid media for 30 min with BODIPY before processing for fluorescence microscopy (see below).

PROTOCOLS

Fluorescence Microscopy

Cells stained with BODIPY were pre-treated to a final concentration of 0.2 μg/mL and incubated at 30°C and 210 rpm for 20-30 minutes immediately before collection. Cells were collected for fluorescence microscopy by centrifugation of 1-10mLliquid culture (often depending on cell concentration) at 3000 *x* g for 5 min at room temperature. Liquid was decanted and cells were resuspended in residual media; 2 μL was mounted onto a glass slide with an unsealed coverslip for imaging, except in time-lapse microscopy experiments, where BODIPY-stained cells were resuspended in 10 μL liquid YPGal with 0.2 μg/mL BODIPY, and 7.5 μL cell suspension was placed in the well of a 35 mm glass-bottom culture dish (MatTek) and mixed with 100 μL of melted YPGal with 0.2 μg/mL BODIPY and 1% agar at 42°C. Once solidified, cells in agar were overlaid with 5mL YPGal with 0.2 μg/mL BODIPY.

Fluorescent images were captured on a Zeiss Axioplan 2E microscope with a Sensican digital camera (Cooke) and Slidebook software (v. 4.1.0.3 or 5.5.2; Intelligent

Imaging Innovations). Samples for time-lapse microscopy were imaged on a Tempcontrol 37-2 heated stage (Zeiss) set for 37°C; measurements of liquid media indicated cells were heated to approximately 27-29°C. A 100X 1.3 numerical aperature oil objective was used with a FITC filter set for GFP and BODIPY visualization (excitation wavelength range 490±25 nm, emission 528±38, 2-sec exposure for GFP and 500-msec for BODIPY), a CFP filter set for CFP-HDEL visualization (ex 430±25, em 470±30, 2-sec exposure), and a CY3 filter set for mCherry or tdtomato visualization (ex 555±28, em 617±23, 2-sec exposure). Brightfield images were captured at 100-msec exposure. Time-lapse images were manually refocused and captured every 10 min from 2 hr after galactose induction to 6 hr after induction. Z-sections were captured at 0.5 μm steps and deconvoluted via the nearest neighbors method in Slidebook; all images shown are maximum intensity projections. Manual scoring was used to quantify lipid droplets in Fig. 5, 6, 8, and 9F,G. ImageJ (National Institutes of Health) was used to quantify droplets automatically by intensity thresholding in Fig. 9B-D and to manually measure lipid droplet intensity in Fig. 9H.

Electron Microscopy

Fixation and embedding of yeast for electron microscopy was adapted from the method published by Robin Wright[204] with help from Tom Januszewski in the UT Southwestern Electron Microscopy Facility[203]. 20 mL saturated cultures in rich oleate media were mixed with 20 mL 2X prefix solution (0.2 M PIPES pH 6.8, 0.2 M sorbitol, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 4% fresh glutaraldehyde) and incubated 5 min at room temperature.

Cells were centrifuged gently at 1000 *x* g for 5 min at room temperature, resuspended in 10 mL 1X prefix solution, and incubated over a weekend at 4^{o}C. Cells were gently centrifuged at setting 4 on an IEC clinical centrifuge, transferred to a borosilicate glass tube, washed 3 times in sterile water, and overlaid with 5 mL 2% $KMnO_4$ fixative at room temperature for 45 min. Cell pellets were gently washed in sterile water until visibly clear and overlaid with 5 mL 1% uranyl acetate stain for 1hr at room temperature. Pellets were gently washed 3 times in sterile water, and dehydrated in consecutive washes of increasing ethanol concentration (1 wash each of 25%, 50%, 70% and 90% in sterile water, followed by 6 washes in 100% ethanol). Cells were infiltrated in increasing concentrations of Spurr resin (61% NSA, 24% ERL 4221, 14% DER 736, 0.7% DMAE; resin reagents from Electron Microscopy Sciences): pellets were overlaid with 1% resin in ethanol for 2 hr, 2% resin overnight, and then overlaid in new resin twice daily at increasing concentrations for four days (10%, 20%, 50%, 67%, and four changes of fresh 100% resin). Pellets embedded in final 100% resin were baked overnight at 70^{o}. Sections were cut to 70-90 nm thickness, placed on 200 mesh copper Formvar grids, and poststained by Hongwei Wang at the UT Southwestern Electron Microscopy Facility. Thin sections were imaged on an FEI Tecnai G^2 Spirit electron microscope. Manual scoring was used to quantify lipid droplets from images taken at 6000X magnification; images of representative cells shown in Fig. 6D were taken at 25,000X.

Lysis and Subcellular Fractionation

For protein expression of seipin deletion mutants, protein extracts were collected by a rapid and simple boiling method[205]: 2.5 OD_{600} units of cells were collected from liquid culture by centrifugation at 3000 *x* g for 5 min at room temperature, followed by resuspension in 0.1 M NaOH and incubation at 5 min at room temperature. Cells were centrifuged again and resuspended in 50 μL PAGE sample buffer (120 mM Tris/HCl pH 6.8, 10% glycerol, 4% sodium dodecyl sulfate, 8% β-mercaptoethanol, 0.004% bromophenol blue) and boiled for 3 min.

For enrichment of ER membranes, large cultures were spheroplasted and lysed by homogenization according to our lab's modification[155] of a method published by the Scheckman laboratory[206]: cells were grown in 2.5 L minimal glucose media to logarithmic growth and collected by centrifugation in a JA10 rotor at 3000 *x* g for 5 min at room temperature. Pelleted cells were resuspended in Tris/DTT buffer (100 mM Tris-HCl pH 9.4, 10 mM fresh DTT) to a concentration of 100 OD_{600}/mL and centrifuged in a JA17 rotor at 41,000 *x* g for 5 min at room temperature. Cells were then resuspended in Lyticase buffer (7.5 g/L yeast extract, 15 g/L peptone, 0.7 M sorbitol, 0.5% glucose, 10 mM Tris-HCl pH 7.4, 1 mM fresh DTT) to a concentration of 100 OD_{600}/mL and spheroplasted with Zymolyase 100-T (4mg/1000 OD_{600}) at 30°C, 210 rpm for 30 min. Spheroplasts were centrifuged in a JA17 rotor at 4100 *x* g for 5 min at 4°C and resuspended in lysis buffer (0.2 M sorbitol, 50 mM potassium acetate, 20 mM HEPES pH 7.4, 2 mM EDTA, 1 mM fresh DTT) to a concentration of 250 OD_{600}/mL. Spheroplasts were then centrifuged in a JA17 rotor at 12,000 *x* g for 5 min at 4°C and resuspended in lysis buffer with added protease inhibitors (0.4 mM 4-[2-amino-ethyl]benzenesulfonyl fluoride hydrochloride, 10 μg/mL

aprotonin, 0.8 μg/mL pepstatin A, 0.8 μg/mL leupeptin, 8 μg/mL *N*α-(*p*-toluene-sulfonyl)-L-arginine methyl ester, 8 μg/mL *N*α-tosyl-L-lysine chloromethyl ketone, 8 μg/mL benzoylarginine methyl ester, and 8 μg/mL soybean trypsin inhibitor) to a concentration of 500 OD_{600}/mL. Spheroplasts were frozen overnight at -80°C. After thawing on ice, spheroplasts were broken by Dounce homogenization at 4°C, and lysate was centrifuged in a JA20 rotor at 1000 *x* g for 5 min at 4°C to pellet nuclei and unlysed cells. The post-nuclear supernatant was centrifuged in a JA21 rotor at 27,000 *x* g, and the resulting medium-speed pellet was resuspended in 100 μL B88 buffer (250 mM sorbitol, 150 mM potassium acetate, 20 mM HEPES pH6.8, 5 mM magnesium acetate).

This medium-speed pellet fraction, enriched in ER membranes, was used for experimentation in Fig. 4: indicated treatments (100 mM Na_2CO_3, 1 M NaCl, or 2-8 M urea) were added directly to the B88 buffer. Cells were sonicated at the lowest setting for 30s where indicated. All treated samples and mock controls were incubated on ice for 30 min before ultracentrifugation at 200,000 *x* g for 1hr in a TLA100 rotor. High-speed pellets were incubated in 80 μL 0.1M NaOH on ice for 90 min to soften the pellet enough for handling. Fractions were mixed 1:1 with PAGE sample buffer before loading on a 10% acrylamide gel and western blotting with the indicated antibodies.

For collection of lipid droplet fractions, large cultures were converted to spheroplasts and gently lysed by hypo-osmotic shock before isolation of lipid droplets by flotation[32]. Specifically, cells were grown for 18-20 hrs in 500 mL rich oleate medium and collected by centrifugation in a JA10 rotor at 8000 *x* g for 10 min at room temperature. Cells were washed twice in sterile water, resuspended in 75 mL 0.1 M Tris-SO_4 pH 9.3 with 10 mM

fresh DTT, and incubated at 30°C in a rotary shaker at 210 rpm for 15 min. Cells were then centrifuged at 7000 *x* g for 10 min at room temperature, washed with 175 mL 1 M sorbitol, and resuspended in 75 mL 1 M sorbitol with 20mM potassium phosphate buffer at pH 7.5. Cells were then spheroplasted with Zymolyase 100-T (4 mg/1000 OD_{600}) at 30°C, 210 rpm for 1-3 hr. Spheroplasts were centrifuged at 7000 *x* g for 5 min at 4°C and resuspended in 2 mL chilled pre-lysis buffer (1M sorbitol, 5 mM MES-Cl, 0.1 mg/mL 4-[2-amino-ethyl]benzenesulfonyl fluoride hydrochloride, 10 μg/mL aprotonin, 1.2 μg/mL pepstatin, 1.2 μg/mL leupeptin) on ice. Cells were slowly pipetted 40 times in 5mL chilled lysis buffer (1M sorbitol, 5 mM MES-Cl, 0.1 mg/mL 4-[2-amino-ethyl]benzenesulfonyl fluoride hydrochloride, 10 μg/mL aprotonin, 1.2 μg/mL pepstatin, 1.2 μg/mL leupeptin) with 2 mL chilled sterile water. Lysate was centrifuged in a JA17 rotor at 3000 *x* g for 7 min at 4°C to pellet nuclei and unlysed cells. Post-nuclear supernatant was transferred to a chilled SW41 tube, overlaid gently with 750 μL-1.5 mL HEPES buffer (20 mM HEPES pH 7.4, 100 mM KCl, 2 mM $MgCl_2$), and centrifuged at 40,000 *x* g for 45 min at 4°C in an SW41 rotor. The lipid droplet fraction was collected by pipetting and scooping off the top white layer after centrifugation. Lipid extracts were obtained from this fraction as described below.

For whole cell lipid extracts, 50 OD_{600} units of cells grown in rich oleate media were collected by centrifugation at 3000 *x* g for 10 min at 4°C and washed twice in cold sterile water, recording pellet wet weight. Pellets were resuspended in 400 μL IP buffer (150 mM NaCl, 50 mM Tris-Cl pH 8, 10% glycerol, 1 mM EDTA, 0.5 mM fresh DTT) and vortexed with 0.5 g acid-washed glass beads for 30 min. Lipid extracts were obtained from lysate as described below.

Lipid Analysis

Lipid extracts were obtained by the Bligh and Dyer method[207] modified by Kent Chapman at the University of North Texas[203, 208]. Samples were transferred to a glass tube, briefly and gently vortexed with 2mL hot isopropanol (70°C), incubated at 70°C for 30 min, and vortexed with 1 mL chloroform. Samples were centrifuged at 1000 *x* g for 5 min at 4°C and incubated overnight at 4°C. Samples were then warmed to room temperature in a dessicator for 30 min and vortexed with 500 μL room-temperature isopropanol. Samples were centrifuged at 1000 *x* g for 5 min at room temperature, and supernatant was transferred to a fresh tube. Samples were vortexed with 1 mL chloroform and 2 mL 1 M KCl, centrifuged at 1000 *x* g for 5 min at room temperature, and the upper phase was removed by aspiration. This KCl wash was repeated 5 more times. The lower phase was transferred to a glass vial and evaporated under a stream of N_2 (4 LPM) in a 37°C water bath for 30 min to 1 hr.

For analysis by thin layer chromatography (TLC), samples were reconstituted into 50 μL chloroform and loaded onto warmed TLC plates alongside quantitative phospholipid and neutral lipid standards (see Reagents) in chloroform. Spots were dried under a stream of N_2. TLCs were first developed in solvent designed to separate neutral lipids (hexane : diethylether : acetic acid, 80:20:1 by volume)[203], air-dried for at least 1 hr, then further developed (in the same direction) with a solvent for separating phospholipids (chloroform : ethanol : water : triethylamine, 35:45:9:35 by volume)[209], and then air-dried overnight.

Plates were then sprayed with 3% cupric acetate in 8% phosphoric acid and charred at 260^{o}F multiple times. Charred spots were quantified by densitometry in ImageJ.

CHAPTER FOUR
Results

DROPLET FUNCTION WITHOUT SEIPIN: PROTEIN TARGETING

Localization of Lipid Droplet proteins

Given the unusual morphology of seipin-deficient lipid droplets in yeast and their likely unregulated origin, it seems likely that other aspects of the lipid droplet could be affected by seipin. Specifically, I looked to determine if seipin-deficient cells presented any defect in the targeting of proteins that localize to the lipid droplet surface. At the time that I started this work, only the localization of Erg6p, an abundant sterol synthesis enzyme that is commonly used as an LD marker, had been investigated in *fld1Δ* yeast; the ability of Erg6p to associate with LDs was not obviously diminished, although it formed an aberrant distribution pattern reflecting unusual *fld1Δ* LD morphology[190]. A later publication found only modest differences in the proteomic composition of lipid droplets isolated from *fld1Δ,* but did not analyze localization patterns by microscopy[199].

I selected a small panel of lipid droplet proteins to analyze: Erg6p, the LD marker previously investigated; Faa4p, a fatty acid activating enzyme also abundantly localized and used as an LD surface marker; Yeh1p and Tgl1p, the two sterol esterases in yeast known to localize at the droplet surface (additional sterol esterases localize to the plasma membrane)[196]; and Tgl3p, Tgl4p, and Tgl5p, the three known triacylglycerol lipases in yeast[60, 61]. While Erg6p and Faa4p act in lipid synthesis, the sterol esterases and

triacylglycerol lipases function in the lipolysis of neutral lipids, the process by which fatty acids are freed from neutral lipid and made accessible to the rest of the cell during times of need.

To observe lipid droplet localization of the selected LD surface protein panel, I obtained strains in which a green fluorescent protein (GFP) tag was integrated at the C-terminus of each gene at its endogenous genomic locus from the Yeast GFP Fusion Library[196]. I then knocked out the seipin gene in each of these strains (*fld1*Δ) to compare each protein's localization in the presence or absence of seipin. Protein localization was then observed by fluorescence microscopy of cells both in the logarithmic phase of growth in minimal glucose medium, where lipid droplets are small and actively being generated and filled, and in stationary phase in rich oleate media, where cells develop particularly large lipid droplets.

While I did not observe complete defect in the localization of any of the selected proteins to the lipid droplet surface (signal is likely lipid droplet-associated by comparison with the brightfield image, not shown), subtle defects were observed. Generally, in the absence of seipin, the localization of each protein appeared more heterogenous than in otherwise wild type cells, largely reflecting the heterogenous droplet morphology: note that Erg6p and Faa4p can be seen to coat both large and small droplets during growth in minimal glucose media (Fig. 3A) and can appear relatively diffuse or patchy when *fld1*Δ droplets are particularly abundant and chaotic after culturing in rich oleate media (Fig. 3B). Therefore while association with the droplet surface is maintained for these two proteins, their cellular distribution is altered as a result of abnormal lipid droplet morphology; whether this

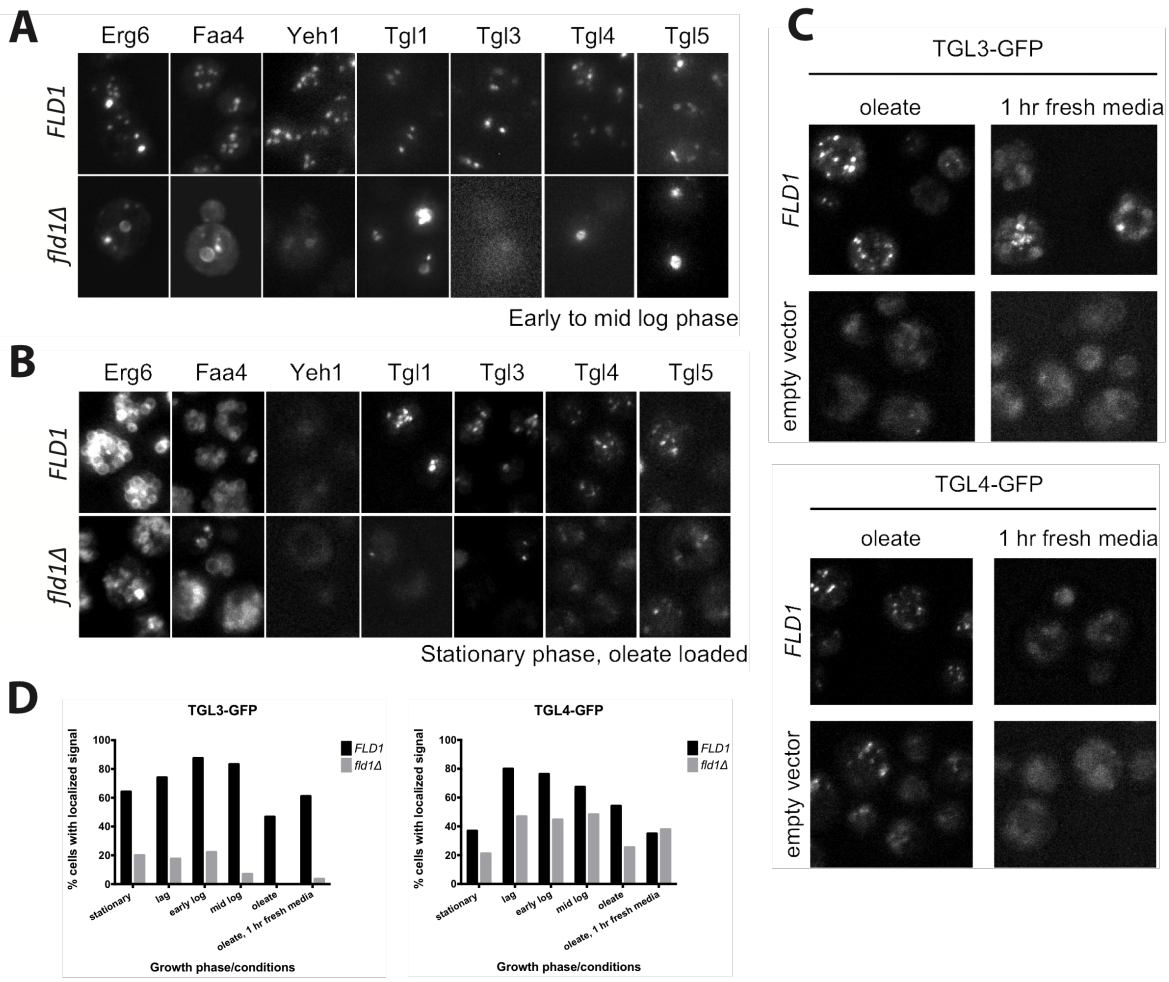

Figure 3. Localization of lipid droplet proteins in the absence of seipin. Strains were selected from the Yeast genomic GFP fusion library[196], in which GFP was integrated at the C-terminus of each indicated protein at its endogenous locus, and *FLD1* was knocked out in the indicated strains (*fld1Δ*). Cells were grown in the growth conditions indicated and imaged by fluorescence microscopy for GFP. (A) Representative projection fluorescence microscope images during early to mid logarithmic growth in minimal glucose media. (B) Representative projection fluorescence microscope images after growth saturation in rich oleate media. (C) Representative projection fluorescence microscope images after growth saturation in rich oleate media, followed by 1 hour of culturing in fresh minimal media. (D) Percent of cells displaying a distinct, localized GFP signal during the growth phases or conditions indicated.

secondary shift in distribution has effects on protein function is not known. More extensive colocalization studies with a neutral lipid dye will be required to confirm LD association of these proteins; such experiments could additionally be used to quantify whether these proteins localize correctly to all lipid droplets or only a subset.

Lipase Targeting and Growth

As was the case for Erg6p and Faa4p, the lipases (this term generally refers to lipid hydrolases: both sterol esterases and triacylglycerol lipases) did not completely lose the ability to associate with lipid droplets in the seipin knockout, although marked losses in signal were seen for both Yeh1p and Tgl3p in minimal glucose media (Fig. 3A), indicating some general defect in the targeting and/or expression of these two proteins. Additionally, the percent of cells that could produce a distinct localized Tgl3p or Tgl4p signal was diminished in the seipin knockout, possibly indicating a more general barrier to lipase localization at the droplet. These effects on Tgl3p and Tgl4p were rescued by introduction of a plasmid overexpressing seipin (Fig. 3C, D)

A more striking defect appeared for all five of the lipases, however, when cells were grown to stationary phase and cultured in rich oleate media (Fig. 3B). The GFP signal generally appeared to decrease in each *fld1Δ* strain compared to *FLD1*, indicating either a dispersion of protein or decreased expression. While this signal decrease occurred even in the wild-type strain for Yeh1p, for the four remaining lipases the effect was specific to the seipin knockout. Strikingly, while Erg6p and Faa4p formed "donuts" surrounding the

surface of large oleate-laden droplets in wild-type cells, Tgl1p, Tgl3p, Tgl4p, and Tgl5p all formed discrete puncta on the under these conditions. These puncta appeared to be LD-associated by association with brightfield images, however specific colocalization studies with an LD marker still need to be performed.

From these observations, I hypothesized that these puncta represent a lipase localization mode unique to cells in the stationary phase of growth. I therefore performed an experiment in which Tgl3p or Tgl4p-labeled cells were first grown to stationary phase and cultured in rich oleate media as before, but were then isolated and diluted in fresh minimal glucose media for 1 hr to induce resumed growth. Remarkably, Tgl3p and Tgl4p puncta in *FLD1* cells largely disspitated after 1 hour in fresh media, forming more diffuse signal around the entire lipid droplet surface (Fig. 3C). Furthermore, signal in *fld1*Δ cells was not as drastically decreased compared to *FLD1* after 1 hour in fresh media, suggesting that seipin may be more specifically affecting the "puncta" mode of localization in stationary cells than the "donut" mode in actively dividing cells. Unfortunately, a GFP antibody was unable to detect endogenous lipase on a western blot, so whether this loss of signal represents mislocalization or decreased enzyme levels remains to be determined.

These preliminary findings were confirmed by Wolinski et al. in a report published while these experiments were being conducted. These authors found that Tgl3p localized diffusely around the LD surface during logarithmic growth of wild-type cells, but localized to discrete LD-associated puncta in stationary phase. In the absence of seipin, Tgl3p localized to only a subset of LDs, and in stationary phase often showed diffuse surface localization rather than puncta. My work has added to these findings by demonstrating both growth- and

seipin-dependent effects on all five of the LD-associated lipases in yeast, indicating that these are modes of regulation conserved across several types of lipolytic enzymes.

The purpose of the lipase localization switch between growth and stationary phase is likely to be in diverting the free fatty acids (FFA) produced in lipolysis to different destinations depending on cellular requirements. Yeast generally enter stationary phase as a result of limited resources in saturated or low-nutrient media; with limited exogenous energy sources, they oxidize free fatty acids released from lipid stores for the energy required to maintain basic cellular processes. When cells divide, however, they must double their phospholipid membrane, and therefore utilize FFAs released from lipid droplets for abundant phospholipid synthesis. The differential localization of lipases between these two conditions could therefore be tailored for either different levels of lipolysis requirements (limited or slow requirements for free fatty acid in stationary cells vs. rapid, abundant free fatty acid requirements in dividing cells) or for different destinations of the fatty acids produced (peroxisomes for fatty acid oxidation in stationary cells vs. endoplasmic reticulum and other membranes for phospholipid synthesis in dividing cells). The function of the puncta remain unclear, however: although we hypothesized that they might represent a junction between the lipid droplet and the peroxisome for free fatty acid transfer, they were not found to colocalize with peroxisomes (Cynthia Torres and Bethany Cartwright, data not shown).

The mechanism by which lipases relocalize is also unknown. Tgl4p is already known to be differentially regulated by phosphorylation during the cell cycle[210], a post-translational modification that could be responsible for the change in localization. This sort of modification has not yet been demonstrated for any of the other lipases, however, and it is

not obvious how phosphorylation would result in redistribution of the protein on the lipid droplet surface. It will be important to determine if these lipase puncta colocalize with any organelles or other factors to determine their nature and putative components/binding partners that may explain their regulation.

The mechanism by which seipin affects the targeting of lipases to the lipid droplet surface also remains undetermined. It is unlikely to be a direct effect of localization via binding seipin, since seipin has never been observed to coat the lipid droplet surface in yeast, and the puncta produced by Tgl1p, Tgl3p, Tgl4p, or Tgl5p did not colocalize with seipin puncta at the LD-ER junction (data not shown). The effect is more likely to be indirect through a role for seipin in the trafficking of proteins to the lipid droplet surface, or even more indirect as a result of aberrant lipid droplet morphology, dysregulated lipid composition, or compensatory feedback on lipase regulatory pathways. It will be necessary to determine the exact mode of seipin's effect (expression, degradation, or mislocalization) to distinguish among these and other possibilities.

DISSECTION OF SEIPIN STRUCTURE: DELETION MUTANTS

Membrane Association of the Luminal Seipin Domain

To better understand the nature and role of seipin at the LD-ER junction, a series of mutants were made in which large regions of the gene were deleted (Derk Binns, Chris Hilton, and Sungwon Han; diagrammed in Figure 5A). I initially focused on a construct in

which both transmembrane domains were deleted, such that only the luminal domain of seipin was expressed. This luminal domain was artificially targeted to the ER by an added KAR2 signal sequence and HDEL retention sequence, so that it could be studied in its usual luminal environment.

This luminal domain of seipin comprises most of the seipin protein. It is often referred to as the "luminal loop domain" and drawn as a disordered, soluble domain in diagrams of the protein.[163] However, this region has a complex predicted secondary structure that contains a significant number of hydrophobic residues, including one short hydrophobic predicted alpha helix (see Fig. 5A). We therefore suspected that, particularly given the protein's likely role in lipid manipulation, this luminal domain could associate with or be embedded in the luminal face of the ER membrane independent of the protein's two transmembrane domains. I therefore experimentally tested the association of this construct with the ER membrane.

Strains were generated in which the seipin luminal construct described above (*fld1*^{Luminal}) was expressed on a low-copy plasmid using a copy of the endogenous seipin promoter. These cells were co-transformed with a plasmid expressing ER-targeted cyan fluorescence protein (CFP-HDEL) to be used as a marker for soluble luminal proteins, and endogenous Dpm1p was used as a marker for integral ER membrane proteins. An ER-enriched membrane fraction was collected and subjugated to one of two treatments known to disrupt the ER membrane and release soluble luminal proteins: sodium carbonate (pH 11), which converts closed membrane vesicles to open membrane sheets[211], and sonication, which physically breaks apart membranes.

After treatment, samples underwent high-speed centrifugation to separate soluble (supernatant) and insoluble (pellet) fractions. While both sodium carbonate and sonication released the soluble luminal marker CFP-HDEL into the supernatant, neither had any effect on Dpm1p or fld1p^{Luminal}, both of which remained with the pellet fraction (Fig. 4A), indicating that the seipin luminal domain can indeed bind membranes in the absence of either transmembrane domain. Furthermore, high salt concentration was unable to release fld1p^{Luminal} from sonicated membranes, indicating that the seipin luminal domain is not peripherally associated with the membrane via electrostatic interactions. Indeed, only strongly denaturing concentrations of urea could diminish either Fld1p^{Luminal} or Dpm1p from the pellet fraction (Fig. 4A and 4C). We do not know the nature of the two faster migrating species derived from Fld1p^{Luminal} that appear in the supernatant with 8 M urea.

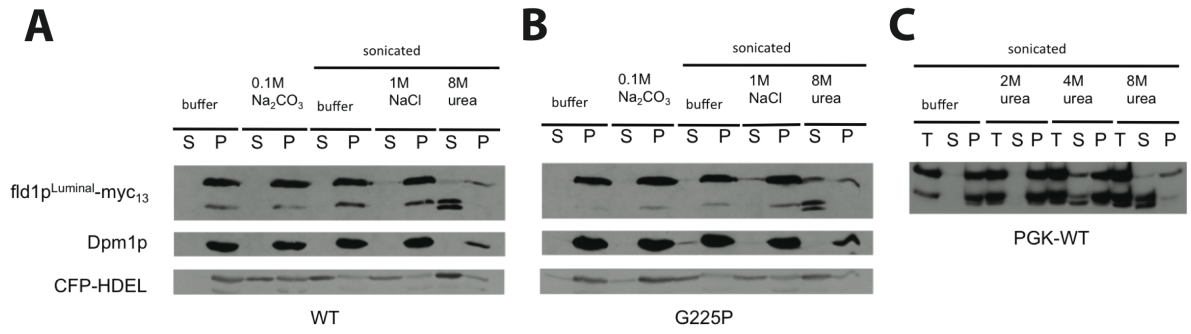

Figure 4. Tight binding of the seipin "luminal loop" to the ER membrane. The seipin luminal loop (*fld1^{Luminal}*, aa 39-246) tagged with myc was expressed on a plasmid in an *fld1Δ* background and targeted to the ER lumen. Cells were co-transformed with overexpressed CFP-HDEL as a luminal ER marker, and endogenous Dpm1p was used as a membrane ER marker. ER membrane fractions were collected from cells expressing (A) *fld1^{Luminal}* from the seipin promoter, (B) *fld1^{Luminal}* with the G225P point mutation from the seipin promoter, and (C) *fld1^{Luminal}* overexpressed from the PGK promoter. Isolated membranes were exposed to the indicated treatments and then pelleted by high speed centrifugation. Western blots shown of equivalent cell units with α-myc (top row), α-Dpm1p (second row), and α-GFP (third row) antibodies. T=total sample, S=supernatant, P=pellet.

I also analyzed the membrane binding capability of the G225P mutant of fld1p^{Luminal} in this system. This mutant, while unstable when expressed in the context of the full length protein[155], was expressed at levels similar to the wild-type version in the context of the luminal domain alone, and displayed equivalent membrane binding (Fig. 4B). This suggests that the instability of the full-length G225P mutant protein requires the remaining domains of seipin and may result from grossly disrupted protein conformation.

Finally, to determine if binding of the luminal domain to membranes involves a saturable binding component, I overexpressed fld1p^{Luminal} from the strong PGK promoter). Overexpressed Fld1p^{Luminal} displayed similar behavior of membrane association as seen with the lower expressing form (Fig. 4C). To confirm that Fld1p^{Luminal} was not simply aggregating independent of membranes, membranes after treatment with chaotropes were subjected to centrifugation in tubes containing a sucrose cushion below buffer. We found that the luminal domain co-fractionated with membranes above the cushion rather than simply forming insoluble aggregates which would pellet under the conditions used (data not shown). Together, these findings suggest that the luminal domain of seipin is tightly embedded in the ER membrane via hydrophobic interactions.

Localization and Stability of Seipin Domains

I then analyzed the larger set of seipin deletion mutants, with the goal of dissecting the components of seipin responsible for its localization, oligomerization, and function. This set was composed of deletions of each of the two terminal cytosolic tails (ΔNterm, aa 15-286;

ΔCterm, aa 1-274), deletions that extended through each of the two transmembrane domains (ΔNTM, aa 39-286; ΔCTM, aa 1-246), constructs which expressed only a transmembrane domain and its associated cytosolic tail (NTM, aa 1-38; CTM, aa 247-286), a construct in which the luminal domain was deleted and replaced with a flexible linker to connect the two transmembrane domains (ΔLuminal, aa 1-38-Gly_6-247-286), and the ER-targeted luminal domain construct described above (Luminal, KAR2-39-246-HDEL) (Fig. 5A). Each mutant was C-terminally tagged with mCherry and overexpressed on a plasmid in an *fld1*Δ background. Each truncated protein migrated appropriate to its predicted size on an SDS-PAGE gel (Fig. 5B). Only one mutant, ΔNTM, showed poor expression by both western blotting and fluorescence microscopy (Fig. 5B,C). Since this mutant is unlikely to be inserted into the ER membrane (see below), it seems likely that this mutant protein is degraded in the cytosol.

I then analyzed the subcellular localization pattern of each of these mutants. While endogenously-expressed yeast seipin localizes specifically to subdomains of the endoplasmic reticulum that associate with lipid droplets, our lab has consistently found that overexpressed seipin displays a diffuse/patchy distribution throughout the ER (unpublished observations and Fig. 5C). Several of the mutants I analyzed preserved a general ability to localize to the ER, although the possibility of more subtle distribution or efficiency defects has not yet been determined; these mutants were fld1p$^{\Delta Cterm}$, fld1p$^{\Delta CTM}$, fld1p$^{\Delta Nterm}$, and, importantly, fld1p^{NTM} (Fig. 5C). Since the Nterm is not necessary for ER localization and NTM is sufficient, our results strongly suggest that the N-terminal transmembrane domain is sufficient for seipin ER targeting.

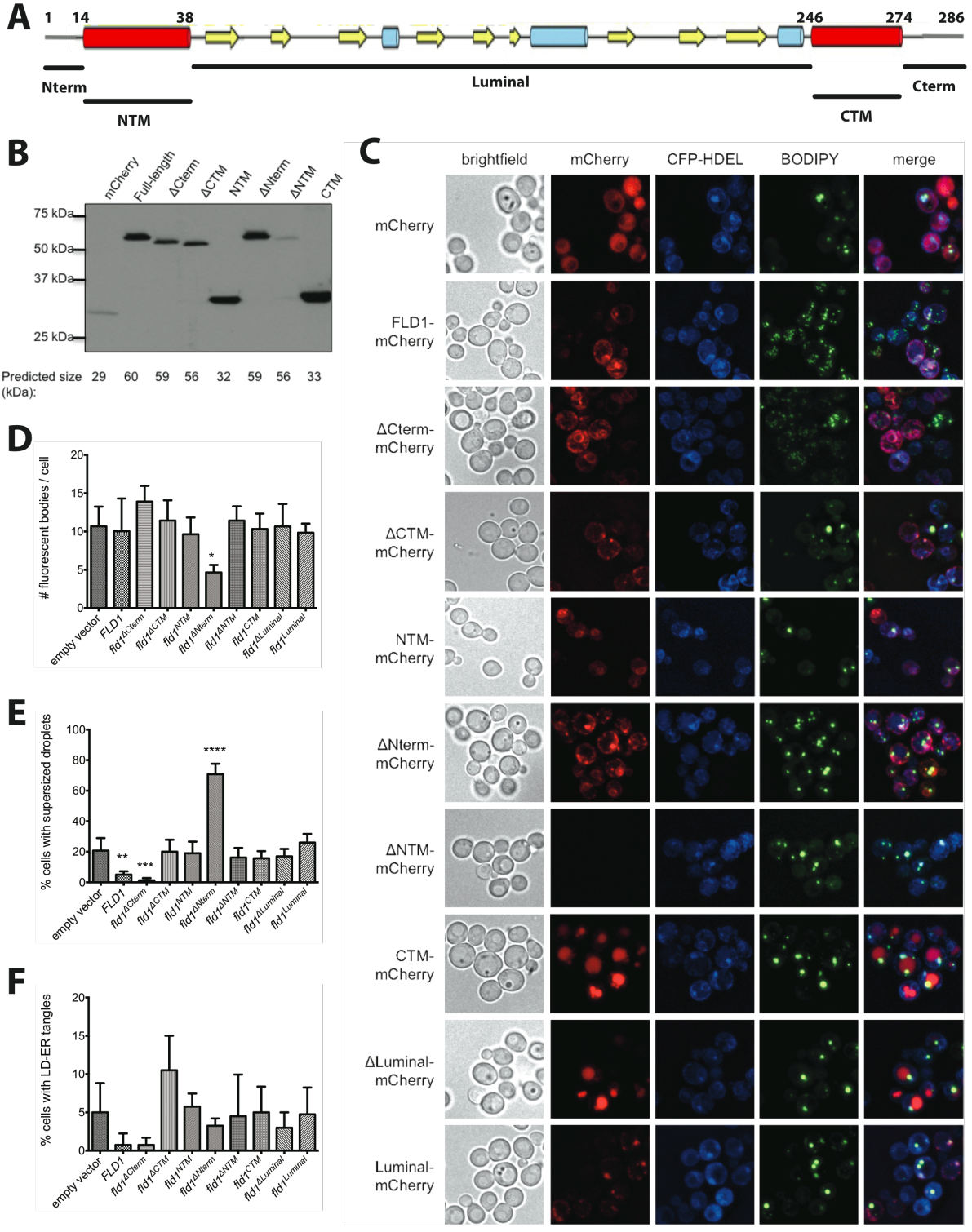

Figure 5. Dissection of seipin. (A) Predicted secondary structure of yeast seipin, modified

from Szymanski *et al.* 2007. Red indicates transmembrane domains, yellow beta sheets, blue alpha helices. (B) Expression of deletion mutants. Indicated mutants were C-terminally tagged with mCherry and overexpressed on a plasmid in an *fld1Δ* background. Western blot shows equivalent cell amounts with an α-dsRed antibody. Predicted sizes are shown based on amino acid number of each mutant. (C) Localization of deletion mutants. Strains from B with coexpression of CFP-HDEL as an ER marker, grown in minimal glucose media and stained with the neutral lipid dye BODIPY. Representative projection fluorescence microscope images shown. (D) Quantitation of strains from C, grown in rich oleate media and stained with BODIPY. Average number of BODIPY-fluorescent bodies per total cells in the indicated strains. (E) Percent of cells displaying one or more supersized lipid droplets (SLDs, defined as >1μm diameter). (F) Percent of cells displaying LD-ER tangles (defined as irregular BODIPY-stained bodies colocalizing with CFP-HDEL densities). Nterm=N-terminal cytosolic tail, NTM=N-terminal transmembrane domain, Luminal=luminal "loop" domain, CTM=C-terminal transmembrane domain, Cterm=C-terminal cytosolic tail. Error bars represent SEM from 4 independent experiments, each N=100 cells from at least 3 fields. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with correction for multiple comparisons.

The localization patterns of the remaining mutants are consistent with this conclusion and demonstrate that the N-terminal transmembrane domain is also necessary for ER insertion. As stated above, fld1p$^{\Delta NTM}$ is present at lower levels than the other mutants, and is not detectable in the ER (Fig. 5B,C). Furthermore, the construct expressing only the C-terminal transmembrane domain, fld1p^{CTM}, was not seen to localize to ER, but rather accumulated within vacuoles, suggesting mislocalization or instability (with possible release of free mCherry). This pattern was also observed for fld1p$^{\Delta Luminal}$; although this mutant protein does contain the likely sufficient N-terminal transmembrane domain, direct linkage to the C-terminal transmembrane domain may destabilize the protein and prevent ER retention. Together, these results strongly implicate the N-terminal transmembrane domain of seipin as necessary and sufficient for insertion in the endoplasmic reticulum. A similar conclusion for mammalian seipin has recently been reached[149].

Experiments were initiated to further refine this observation by identifying the region responsible for LD-ER junction localization once the protein is within the ER. For this purpose a subset of these mutants (*fld1*$^{\Delta Cterm}$, *fld1*$^{\Delta CTM}$, *fld1*$^{\Delta Nterm}$, and *fld1*^{NTM}) were integrated into the genomic seipin locus for endogenous expression with a tandem-dimer tomato (tdtomato) tag for more sensitivity in fluorescence detection compared to mCherry. Of these, only fld1p$^{\Delta Nterm}$ formed LD-adjacent puncta consistently similar to the full-length protein (Fig. 7B) (*fld1*$^{\Delta Cterm}$ did form puncta, but these may be artifactual, see below). The other mutants studied did not produce detectible fluorescence signal. Unfortunately, I was unable to determine whether this lack of signal was caused by dispersion in the cytoplasm or reduced expression, since none of the tdtomato constructs expressed from the endogenous seipin promoter could be detected by immunoblotting with a dsRed antibody, in contrast to overexpressed proteins. More sensitive antibodies or epitope tags could answer this question. However, while there may indeed be a specific, identifiable domain that targets seipin to droplet junctions, seipin could also exist there secondary to its involvement in the early initiation of lipid droplets.

Finally, I analyzed the functionality of each of these deletion mutants by examining the lipid droplet phenotype produced by each upon overexpression. The most obvious effect was caused by *fld1*$^{\Delta Nterm}$, the study of which eventually comprised the main effort of my project and will be more extensively discussed in Section 4-3. Other mutant phenotypes are of note, however: interestingly, the *fld1*^{Luminal} construct was unable to produce a wild-type lipid droplet phenotype despite containing most of the seipin protein with ER targeting, indicating that the transmembrane domains are critical for seipin function. Indeed, *fld1*$^{\Delta Cterm}$

was the only mutant found to produce a phenotype indistinguishable from wild-type seipin, displaying homogenous droplets and little to no incidence of supersized droplets or LD-ER tangles (Fig. 5D-F; the approach used to quantify lipid droplet morphology will be more thoroughly described in the following section); this suggested that the short C-terminal cytosolic tail may be dispensable for seipin function. Furthermore, in *fld1*$^{\Delta CTM}$, I found an increase (although statistically not significant) in LD-ER tangles by fluorescence microscopy (Fig. 5F), with an increase (statistically significant) in the number of droplet clusters observed by electron microscopy (not shown).

I pursued these C-terminal mutants with interest, and analyzed their phenotypes when expressed at endogenous levels from the genomic seipin locus. . There were several issues, however, that made the results difficult to interpret. Under these conditions, fld1p$^{\Delta CTM}$ was not expressed at levels detectable by fluorescence microscopy and produced a lipid droplet phenotype indistinguishable from the seipin knockout. An endogenous tdtomato-tagged version of fld1p$^{\Delta Cterm}$ produced a wild-type lipid droplet phenotype and LD-adjacent seipin puncta, but an endogenous untagged *fld1*$^{\Delta Cterm}$ strain produced a disorganized lipid droplet phenotype, possibly with increased LD-ER tangles like that of the overexpressed *fld1*^{CTM}. Further complicating this picture, a recent publication reported that a 10-amino acid deletion from the Fld1p C-terminus resulted in a phenotype of fewer, larger lipid droplets, a phenotype that was much more similar to that of *fld1*$^{\Delta Nterm}$ than what I have observed for *fld1*$^{\Delta Cterm}$ and *fld1*$^{\Delta CTM}$ [198]. I have therefore concluded that while the Cterm and CTM regions of seipin are functionally important, these deletion mutants are too labile and dependent on context for careful study. Analysis of the C-terminal region of seipin may be an important

avenue to pursue, but it will require more conservative changes, such as shorter deletions or point mutagenesis.

These experiments have only scratched the surface of the potential of these deletion mutants and other avenues to break down the functional components of the seipin protein. More extensive analysis of the localization and oligomerization of these deletions and further refined mutants could provide important insights into the interactions determining seipin complex formation of the LD-ER junction. Furthermore, such mutants could be used in the dissection of seipin function: a particularly fruitful example is presented in the next section.

DISSECTION OF SEIPIN FUNCTION: THE SEIPIN N-TERMINUS

An Extreme Supersized Lipid Droplet Phenotype

The most striking phenotype revealed by the deletion mutant analysis described in the previous section was that of *fld1*$^{\Delta Nterm}$, a deletion of just 14 amino acids from the seipin N-terminal cytosolic tail. Seipin knockout yeast cells (*fld1*Δ) generally have disorganized droplet phenotypes that can vary based on culturing conditions, ranging from the "fewer lipid droplets" phenotype for which the gene was named, seen in minimal glucose media and marked by a small number of supersized droplets and LD-ER tangles (Fig. 8A), to an abundance of heterogeneously sized droplets that appear when cells are cultured in rich oleate media (Fig. 6A). In contrast, overexpression of the N-terminal deletion mutant (*fld1*$^{\Delta Nterm}$) from a plasmid in an *fld1*Δ background resulted in a remarkable prominence of

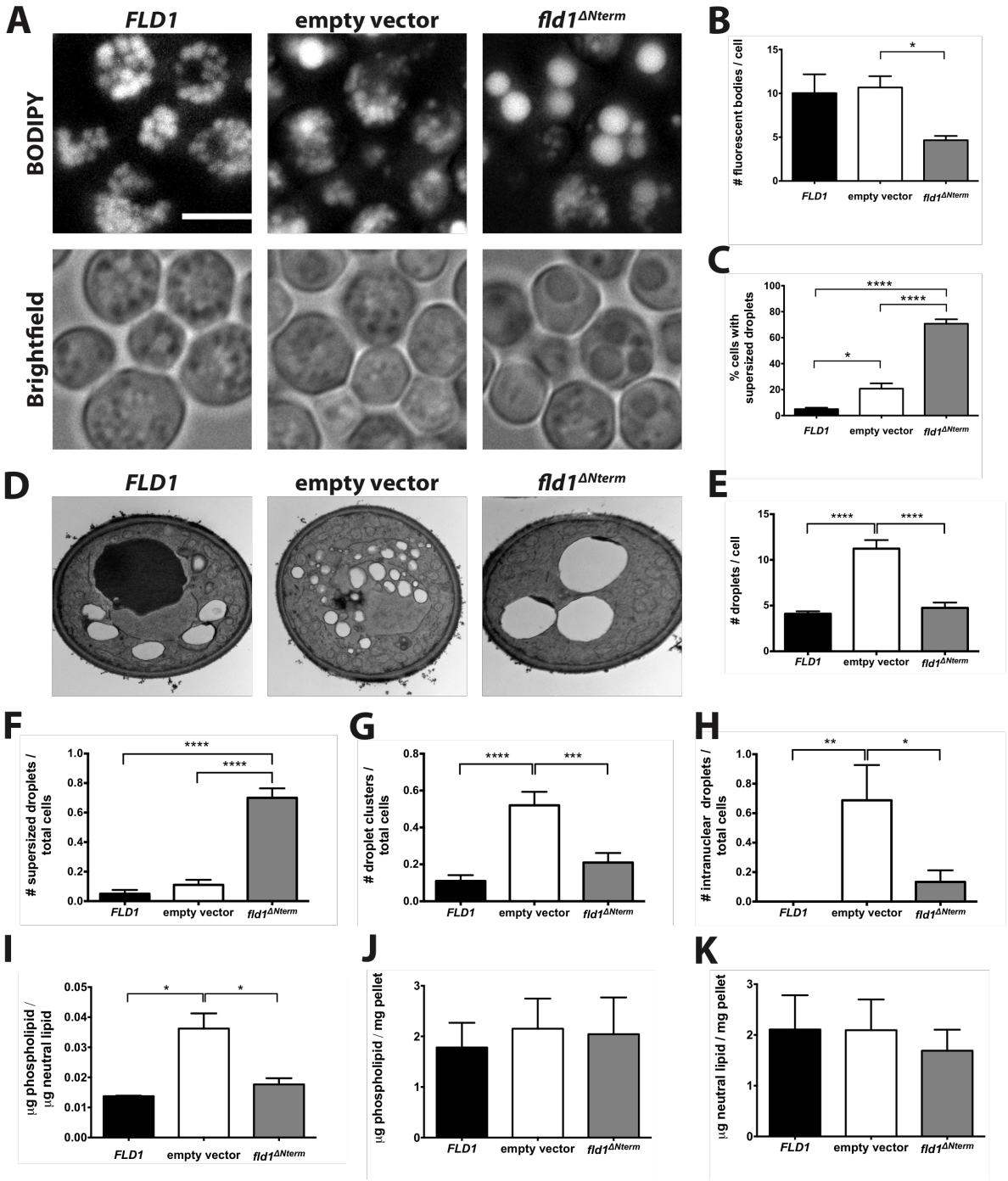

Figure 6. Supersized lipid droplet phenotype of an N-terminal seipin deletion mutant. Seipin knockout cells (*fld1Δ*) were complemented with plasmids overexpressing *FLD1, fld1*$^{\Delta Nterm}$, or empty vector, cultured in rich oleate media. (A) Representative fluorescence microscope projection images after staining with BODIPY to visualize lipid droplets, scale bar 5µm. (B) Number of fluorescent bodies per total number of cells. (C) Percent of cells

displaying one or more supersized lipid droplet (SLD; defined >1μm diameter). Error bars represent SEM from 4 independent experiments, each N=100 cells from at least 3 fields. (D) Representative electron microscope images. (E) Number of lipid droplets per total number of cells. (F) Number of SLDs. (G) Number of lipid droplet clusters (defined >5 adjacent droplets). Error bars represent SEM from 100 cells. (H) Number of intranuclear droplets (defined as within an observable, intact nuclear envelope). Error bars represent SEM from ~30 cells that had visible nuclei. (I) Phospholipid to neutral lipid ratios of isolated lipid droplet fractions, analyzed by TLC. (J) Phospholipid levels of whole cell lysates by TLC, normalized to cell pellet wet weight. (K) Neutral lipid levels as in (J). Error bars represent SEM from 4 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with correction for multiple comparisons.

supersized lipid droplets under both conditions, although the phenotype was most strikingly distinct from that of the seipin knockout in rich oleate media (Fig. 6A). This is consistent with a recently reported phenotype of fewer, larger droplets for a 10-amino acid N-terminal deletion of seipin, but that mutant was not further characterized[198].

I carefully quantified lipid droplet morphology in this strain by staining with the neutral lipid dye BODIPY and manually counting the number of fluorescent bodies per cell (termed “fluorescent bodies” rather than “lipid droplets,” since tiny droplets within clusters or tangles usually cannot be resolved from each other and appear as an irregular blur on fluorescence microscopy). I also manually scored cells that contained supersized lipid droplets (SLDs; arbitrarily defined as spherical fluorescent bodies with greater than 1 μm diameter by Fei et al., determined to be over 30 times the average volume of wild-type lipid droplets[99]). In addition, cells were transformed with a plasmid expressing the ER marker CFP-HDEL to identify LD-ER tangles, defined here as irregularly-shaped (non-spherical) fluorescent bodies colocalizing with an ER signal density of similar shape (example marked by arrowhead in Fig. 8A); a similar such method has previously been reported to be successful in identifying of these structures[83]. While this analysis produced clear results

when cells were cultured in minimal glucose media (Fig. 8E), this method was less useful in rich oleate media, where abundant neutral lipid in the seipin knockout produced large clusters of lipid droplets with relatively limited ER membrane components (Fig. 8E). Therefore, electron microscopy was used to quantify lipid droplet clustering in the rich oleate condition (Fig. 6D, G).

Through these analyses, I found that the *fld1*$^{\Delta Nterm}$ mutant produced fewer droplets per cell (Fig. 6B), more cells with supersized lipid droplets, and more supersized lipid droplets per cells that had them (data not shown) than both WT and seipin knockout cells after growth in rich oleate media. As described in the previous section, this occurred with no apparent defect in localization of fld1p$^{\Delta Nterm}$ to the ER (Fig. 5C), and this mutant additionally appeared to form high molecular weight oligomers (Derk Binns, unpublished data). A similar phenotype was observed for endogenously-expressed *fld1*$^{\Delta Nterm}$ integrated at the genomic seipin locus, although this appeared less extreme than the overexpressed phenotype (Fig. 7A). Furthermore, as described above, the endogenously-expressed mutant protein also localized to LD-adjacent puncta (Fig. 7B). Importantly, although the number of lipid droplets was fewer, the number of fld1p$^{\Delta Nterm}$ puncta formed was not, as most LDs were associated with one or more fdl1p$^{\Delta Nterm}$ puncta, but each cell had several "excess" puncta not associated with droplets. This indicates that the decreased number of lipid droplets in this strain is not caused by a deficiency of the mutant protein to form punctate complexes within the ER.

I further analyzed this supersized droplet phenotype in the overexpressing strain by electron microscopy (Fig. 6D), finding fewer droplets in *fld1*$^{\Delta Nterm}$ than in *fld1Δ*, with an

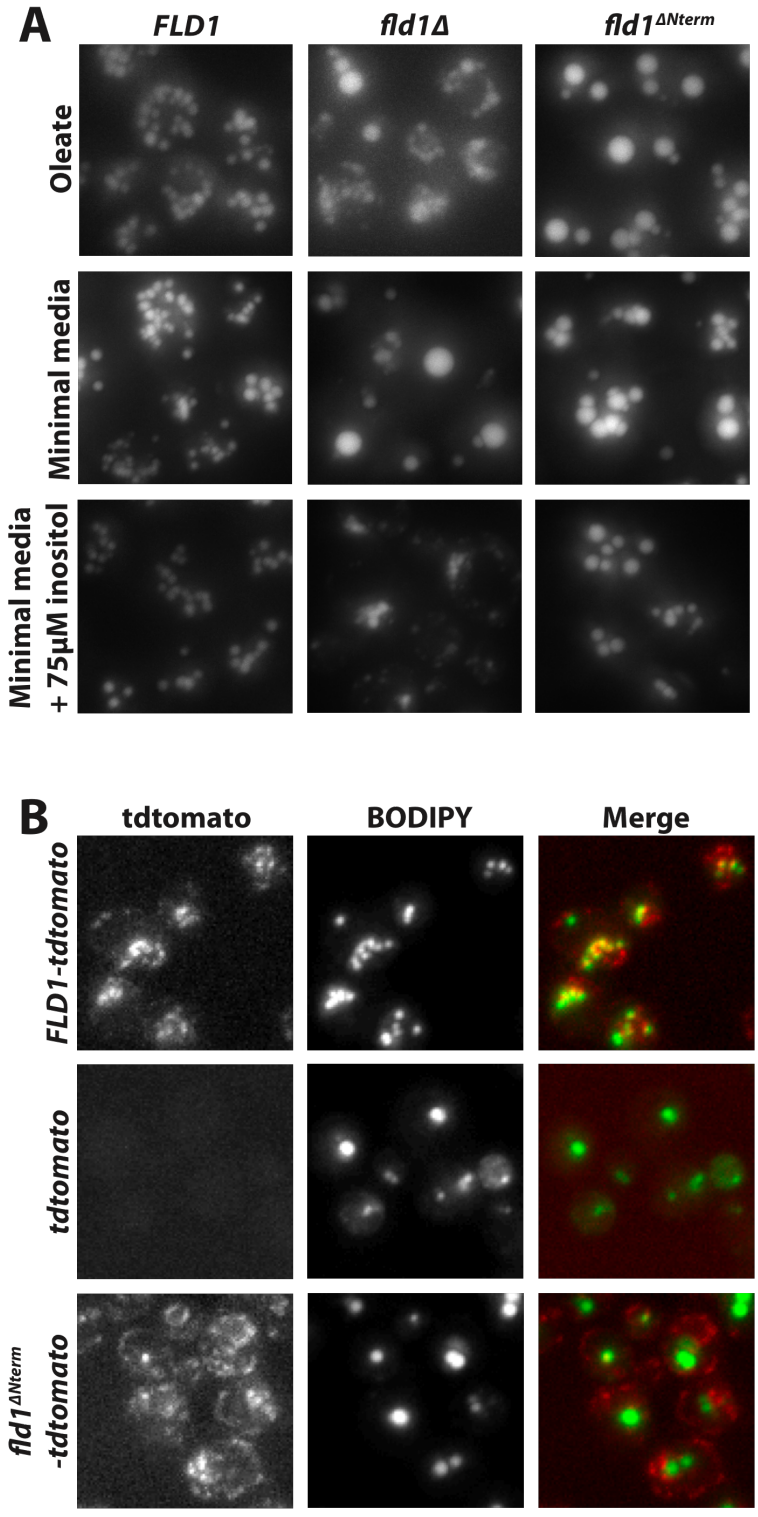

Figure 7. Supersized droplet phenotype and lipid droplet localization of endogenously-expressed *fld1$^{\Delta Nterm}$* (A) *fld1$^{\Delta Nterm}$* and indicated controls were integrated into the genomic

seipin locus. Cells were grown to stationary phase in the indicated media and stained with BODIPY. Representative fluorescence microscope projection images shown. (B) C-terminally tdtomato-tagged *fld1*$^{\Delta Nterm}$ and indicated controls were integrated into the genomic seipin locus. Cells were grown to stationary phase in minimal glucose media and stained with BODIPY. Representative fluorescence microscope projection images from one of three independent experiments shown.

increased number of supersized droplets, consistent with the fluorescence phenotype. Additionally, I detected a concomitant decrease in the prevalence of lipid droplet clusters (arbitrarily defined as at least 5 droplets adjacent to one another) compared to *fld1*Δ cells (Fig. 6G). This was consistent with a decrease in LD-ER tangles compared to *fld1*Δ that I later observed by fluorescence microscopy in minimal glucose media (Fig. 8C,E). The N-terminal deletion therefore appears to display an extreme supersized phenotype, with less heterogenous droplet morphology than the seipin knockout.

Electron microscopy additionally allowed me to detect the unusual incidence of lipid droplets within the nucleus within these mutant strains. While one report has identified potential intranuclear lipid droplets in mammalian hepatocytes[212], we have never observed a single lipid droplet within the nucleus in wild-type yeast. Under conditions with abundant neutral lipid, however, multiple intranuclear lipid droplets can be observed in electron micrographs of *fld1Δ* cells (Fig. 6D,H and Chris Hilton, unpublished data). Intriguingly, although intranuclear droplets were observed in the *fld1*$^{\Delta Nterm}$ strain, they were much more rare than when observed in the knockout (Fig. 6H). It is not yet clear whether this rarity could be explained by the decreased number or increased size of lipid droplets, or whether the bulk of the seipin protein still present in fld1p$^{\Delta Nterm}$ acts to suppress intranuclear lipid droplet formation. There was a trend of a decreased percentage of droplets (as distinguished

from absolute droplet number) appearing within the nucleus in *fld1*$^{\Delta Nterm}$ compared to *fld1*Δ, but this was not determined to be statistically significant (data not shown). Careful quantification of a larger electron micrograph dataset could potentially distinguish among these possibilities. It therefore appears that seipin plays a key role in the direction of lipid droplet formation outward into the cytosol. While further study will be required to determine whether the N-terminus is involved in this function, my hypothesis is that the bulk of the seipin protein acts to direct lipid droplet formation into the cytosol, and that the rare intranuclear droplets in *fld1*$^{\Delta Nterm}$ are caused by rare stochastic lipid droplet formation that occurs independent of the mutant seipin protein (see discussion of Fig. 9 below).

As expected, these alterations in droplet morphology are reflected in general changes in droplet composition. Lipid droplets are generally spherical in shape, with a volume composed of neutral lipid and a surface area composed of phospholipid (and decorated by associated proteins). Geometrically, supersized lipid droplets are expected to have a lower surface area to volume ratio, and therefore a lower phospholipid (PL) to neutral lipid (NL) ratio, than small lipid droplets or especially LD-ER tangles which are marked by excess ER membrane. Indeed, we found that lipid droplets isolated from *fld1*$^{\Delta Nterm}$ cells grown in rich oleate media produced a strikingly lower PL/NL ratio than those isolated from *fld1*Δ cells, consistent with the lower surface area to volume ratio of supersized lipid droplets (Fig. 5I). No difference was detected between *FLD1* and *fld1*$^{\Delta Nterm}$, despite the striking differences in LD size between these two strains.

I calculated predictions of PL/NL ratios for *FLD1* and *fld1*$^{\Delta Nterm}$ using a formula developed by Penno et al. based on the density of triolein (0.95 g/mL) and the surface area

per molecule of phosphatidylcholine (observed to be 1.4 nm^2 on a triolein monolayer[213])[49]. Estimating a typical LD diameter in rich oleate media of 0.5μm for wild-type lipid droplets and 1μm for *fld1*$^{\Delta Nterm}$ SLDs, the resulting predicted PL/NL ratios are 1.2 mass% and 0.56 mass%, respectively. Although this is a two-fold difference, the difficulty in detecting the very small absolute amounts of phospholipid in lipid droplet fractions makes it likely that this small difference is not discernable by our current assay. Importantly, droplet PL/NL composition differences occurred with no change in whole cell phospholipid or neutral lipid levels (Fig. 6J,K), indicating that the abnormalities in lipid droplet composition and morphology are likely to be intrinsic to the lipid droplet rather than reflecting overall cellular abnormalities in lipid synthesis.

I then tested the response of this extreme supersized phenotype to exogenous phospholipid precursors. Fei et al. demonstrated that while choline supplementation in the growth media had no effect on the *fld1*Δ phenotype, inositol can suppress and ethanolamane can promote the appearance of supersized lipid droplets in *fld1*Δ. After growing cells in minimal glucose media with the indicated supplements, I reproduced this suppressive effect of inositol (Fig 8A,B) and additionally found that the suppression of supersized lipid droplets occurred concomitantly with an increase in the percent of cells displaying LD-ER tangles (Fig. 8A,C); this effect was confirmed in a recent publication[198]. While I was not able to reproduce the ethanolamine-induced increase in supersized lipid droplets reported by Fei at al. (Fig. 8B), I did detect a significant suppression of LD-ER tangles in ethanolamine-treated cells (Fig. 8C), suggesting that ethanolamine may indeed shift *fld1*Δ lipid droplet

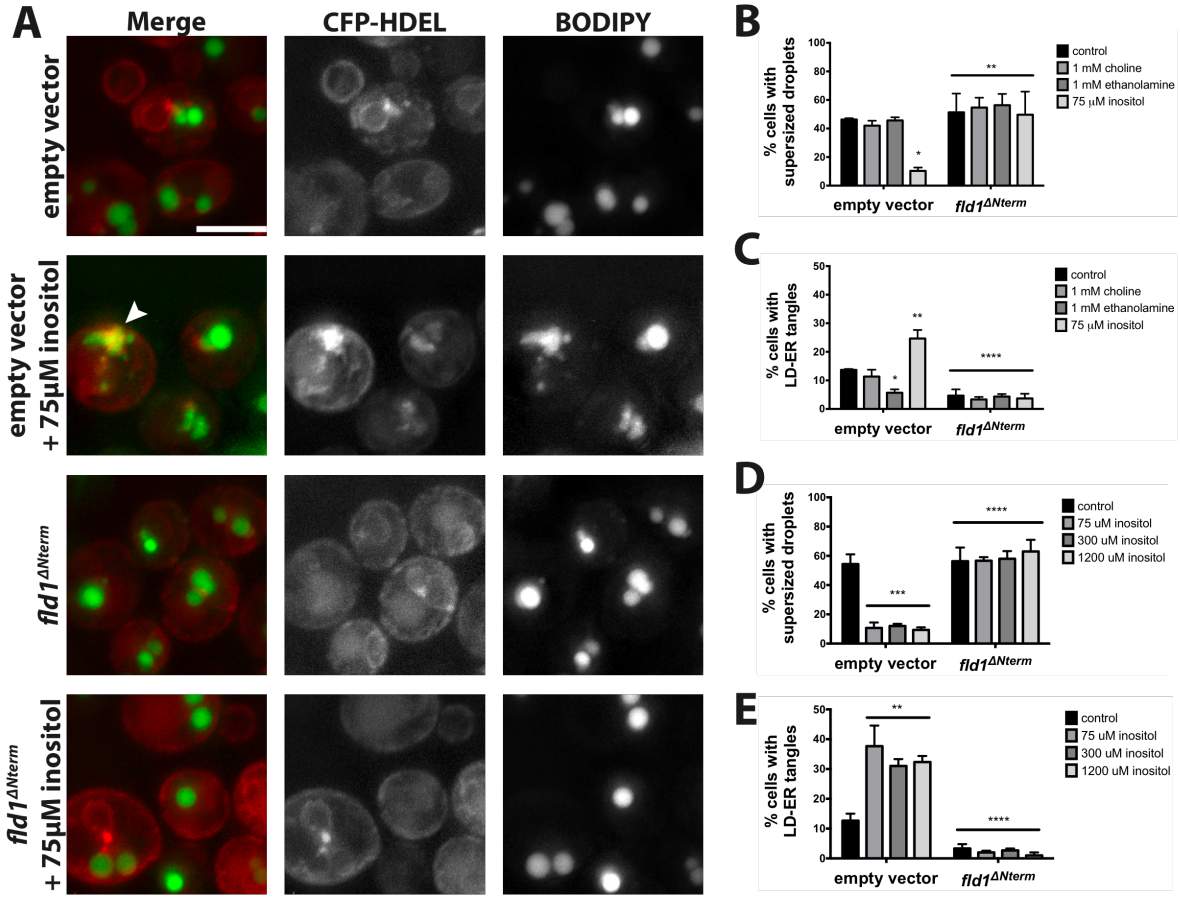

Figure 8. Resistance of the N-terminal supersized droplet phenotype to suppression by inositol. Seipin knockout cells (*fld1Δ*) complemented with plasmids overexpressing *FLD1* and the ER marker CFP-HDEL were grown in minimal glucose media with or without the indicated phospholipid precursor supplements. (A) Representative fluorescence microscope projection images after staining with BODIPY, scale bar 5µm. Arrowhead indicates an LD-ER tangle. (B,D) Percent of cells displaying SLDs. (C,E) Percent of cells displaying LD-ER tangles. Error bars represent SEM from 3 independent experiments , each N=100 cells from at least 3 fields. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by two-way ANOVA with correction for multiple comparisons.

morphology in a direction opposite to that promoted by inositol. Neither effect of ethanolamine was detected in an additional report by Wang et al., however[198].

Both inositol and ethanolamine are believed to exert these effects through upregulation of PI or PE, respectively. The shift in lipid droplet morphology from supersized lipid droplets to LD-ER tangles induced by inositol is likely to be caused by increased available phospholipid surface area and/or the predilection of inositol toward smaller LDs due to its positive curvature. The mechanism of ethanolamine action in the opposite direction has been suggested to be due to the negative curvature of ethanolamine disrupting LD surface integrity and thereby making LDs more fusogenic[99]. The effect of either molecule in producing LD phenotypic shifts seems to rely on changing the phospholipid amount and/or composition on the lipid droplet surface.

Strikingly, unlike those in *fld1*Δ cells, *fld1*$^{\Delta Nterm}$ lipid droplets displayed no detectable response to supplementation with any of the phospholipid precursors tested, in either the appearance of supersized lipid droplets or of LD-ER tangles (Fig. 8A-C). The supersized lipid droplets of *fld1*$^{\Delta Nterm}$ remained resistant even when inositol was applied at several-fold higher concentrations than that typically used for supplementation (Fig. 8D,E), and this relative resistance was reproduced in cells expressing *fld1*$^{\Delta Nterm}$ at endogenous levels (Fig. 7A). Since lipid droplets are similarly unaffected by inositol in wild type cells (Fig. 7A), it appears that excess phospholipids upregulated by inositol may have limited access to the droplet surface when the bulk of the seipin protein is present, thus limiting the effect of exogenous inositol on lipid droplet morphology. I hypothesize that this effect of restricting or regulating phospholipid access to the lipid droplet surface is the main mechanism by

which seipin regulates lipid droplet morphology and is the cause of the relatively homogenous lipid droplet appearance produced by the N-terminal deletion compared to the full seipin knockout; this possibility will be discussed further in Section 5-2-2.

Lipid Droplet Initiation During Biogenesis

After characterizing the effects of the seipin N-terminal deletion on lipid droplet morphology, I then sought to determine whether this morphological phenotype reflected any abnormality in lipid droplet biogenesis. Members of our lab had previously developed a system for studying de novo lipid droplet biogenesis in yeast (Chris Hilton and Derk Binns, unpublished data), and a similar system was independently developed and published by Jacquier et al.[72] For this system, a strain was generated deleting three of the four genes encoding the acyltransferases that synthesize neutral lipid in yeast, while the fourth was placed under a galactose-inducible promoter. Under repressing conditions (culturing in minimal glucose media), no acyltransferase is expressed, no neutral lipid is synthesized, and the cells lack lipid droplets. Upon induction with galactose media (either minimal as in Fig. 9A-D or rich as in Fig. 9E-H), the induced acyltransferase synthesizes neutral lipid and lipid droplets from de novo. Our lab has generated versions this system with the induced acyltransferase being *ARE1* (one of the two steryl ester synthesis enzymes), *LRO1,* or *DGA1* (the two triacylglycerol synthesis enzymes). All of the work described below was conducted in an *are1*Δ *are2*Δ *lro1*Δ strain with *DGA1* expressed in the genome under a galactose promoter (termed *3.5KO*^{GAL}*DGA1*).

Chris Hilton and Derk Binns in the lab previously used this system to characterize the effect of seipin knockout on de novo lipid droplet formation. They found that deletion of seipin resulted in a drastic impediment in lipid droplet formation, as a lower percentage of *fld1*Δ cells (in each of the galactose-inducible backgrounds described above) produced lipid droplets than *FLD1* cells (reproduced in Fig. 9A,C). Importantly, this defect in lipid droplet formation occurs with no defect in neutral lipid levels or synthesis rates, indicating that seipin acts downstream of neutral lipid synthesis. Indeed, multiple lines of evidence suggested that neutral lipid accumulates in the endoplasmic reticulum at early time points of galactose induction in *fld1*Δ cells (Chris Hilton and Derk Binns, unpublished data).

We have therefore hypothesized that seipin acts in the regulated transfer or release of neutral lipid from its site of synthesis in the endoplasmic reticulum to its site of storage in the lipid droplet. This model would explain the chaotic, disorganized nature of lipid droplet morphology in *fld1*Δ cells: without seipin to promote orderly neutral lipid packaging into lipid droplets, neutral lipid accumulates in the ER until it reaches a saturation point beyond which the ER membrane becomes unstable and "oils out"[86,87], upon which droplets bleb stochastically from the ER membrane, with unregulated composition and directionality.

Because of the key role for seipin in lipid droplet biogenesis suggested by these findings, it seemed likely that the N-terminal deletion might effect supersized lipid droplets by acting during early points of lipid droplet biogenesis. I therefore integrated $fld1^{\Delta Nterm}$ into the genomic seipin locus of the *3.5KO(*^{GAL}*DGA1)* strain (using the same integration strategy to generate *FLD1* and *fld1*Δ controls), and observed lipid droplet formation after galactose induction. After 9 hours in galactose, the "supersized" morphology of $fld1^{\Delta Nterm}$ was

generally recapitulated (Fig. 9A), with the *fld1*$^{\Delta Nterm}$ droplet population displaying significantly higher average size than *FLD1* or *fld1*Δ (average area per fluorescent body in a fluorescence microscope maximum projection image: *FLD1* 55.02 pixels^2, *fld1*Δ 80.43 pixels^2, *fld1*$^{\Delta Nterm}$ 116.6 pixels^2), but significantly less skewness than *fld1*Δ (average skewness of area distributions from 3 independent trials: *FLD1* 1.094, *fld1*Δ 2.189, *fld1*$^{\Delta Nterm}$ 1.406), indicating a more normally distributed droplet population than the knockout (Fig. 9B).

Notably, after 2 hours of galactose induction, *fld1*$^{\Delta Nterm}$ produced a significantly lower percentage of cells with at least one lipid droplet than *FLD1* (Fig. 9C), suggesting a partial defect in the ability of *fld1*$^{\Delta Nterm}$ cells to initiate the first droplet at early time points This defect was similar to that of the *fld1*Δ strain, if not as extreme. In cells that did develop lipid droplets, both *fld1*Δ and *fld1*$^{\Delta Nterm}$ produced fewer lipid droplets per cell (Fig. 9D).

I decided to look more closely at the earliest stages of droplet formation by performing time-lapse microscopy on cells induced in rich galactose media embedded in agar, a condition in which we have found lipid droplet formation to be generally slower, and early formation can be directly observed (Fig. 9E). While approximately 40% of *3.5KO(*^{GAL}*DGA1) FLD1* cells produced at least one droplet over the course of the time lapse, only around 10% of *fld1*Δ or *fld1*$^{\Delta Nterm}$ cells could produce a visible droplet (Fig. 9F), confirming the defect observed in Fig. 9C.

Furthermore, I scored the time at which each cell developed its first lipid droplet, and found that while the majority of *FLD1* droplets were produced within the first 2 hours of galactose induction, both *fld1*Δ and *fld1*$^{\Delta Nterm}$ had a severe deficit in the ability to produce a

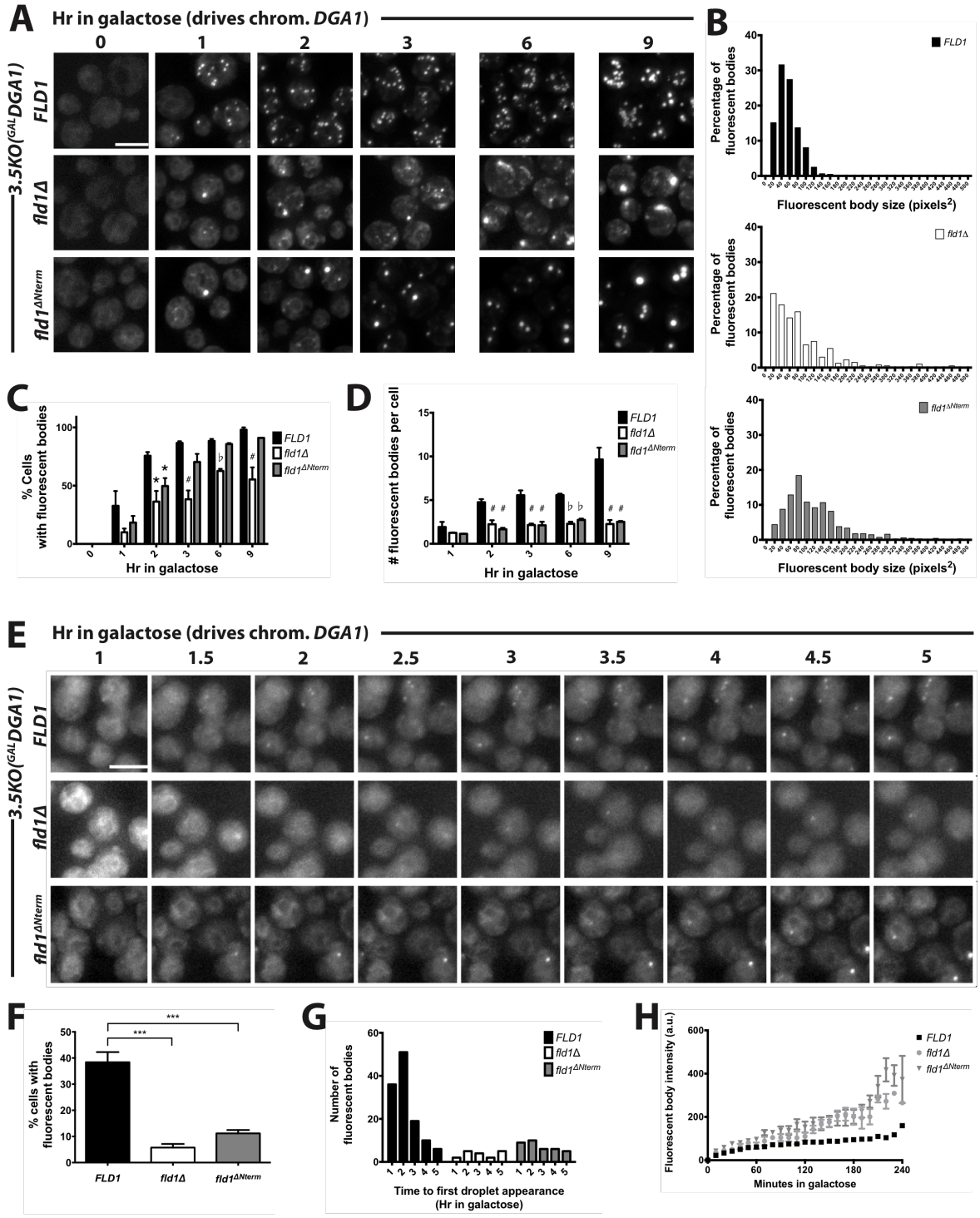

Figure 9. Initiation defect of *fld1*$^{\Delta Nterm}$ during de novo droplet formation. Genomic knock-ins were generated at the seipin locus in the *3.5KO(*^{GAL}*DGA1)* background for *FLD1,*

fld1Δ, and fld1$^{\Delta Nterm}$. Cells were introduced to galactose media at t=0 to induce droplet formation and stained with BODIPY. (A) Representative fluorescence microscope projection images at indicated time points after galactose induction. (B) Histograms of fluorescent body size, given as area in pixels on a maximum intensity projection image. (C) Percent of cells containing at least one fluorescent body. (D) Number of distinct fluorescent bodies per cells that have at least one. Error bars represent SEM from 3 independent experiments, each N=100 cells from at least 3 fields. *$p<0.05$, #$p<0.01$, þ$p<0.0001$ by one-way ANOVA with correction for multiple comparisons. (E) Time lapse fluorescence microscopy of cells embedded into agar after 1 hour of galactose induction in liquid culture. Images taken in 10 min increments (see Supplementary Movies), representative projections of 30 min increment montages shown. (F) Percent of cells that displayed at least one fluorescent body over the course of the time lapse. Error bars represent SEM from 3 independent experiments. ***$p<0.001$ by one-way ANOVA with correction for multiple comparisons. (G) Histogram of time to first appearance for each droplet. (H) Average intensity curves for fluorescent bodies during the time lapse. Time 0 defined as the frame before first appearance of the droplet. Error bars represent SEM from average droplet intensity values per time point over 3 independent experiments.

droplet at these early times (Fig. 9G). Indeed, the lack of a peak at any time period suggests that the time to first droplet appearance is relatively stochastic in both mutant strains.

I also analyzed the rate of droplet growth in these strains by measuring the change in each droplet intensity over time, normalized to background cellular fluorescence (Fig. 9H: Unlike in previous graphs, 0 time was set not at time of galactose induction but rather at 10 minutes before the first appearance of a distinct fluorescent body, while 0 intensity was set at the fluorescence above cell background at 0 time). Although rate of droplet appearance was comparatively sluggish in each mutant (Fig. 9G), once initiated, droplet growth was actually faster in both *fld1*Δ and *fld1*$^{\Delta Nterm}$-cells than in *FLD1* (Fig. 9H). This increased rate is consistent with an increased "pressure" buildup as neutral lipid accumulates within the ER according to our model (described more thoroughly in Section 5-1-2).

It was surprising to me that lipid droplet initiation was so severely affected in *fld1*$^{\Delta Nterm}$. During most of this work, I had viewed the *fld1*$^{\Delta Nterm}$ phenotype as a defect in

lipid droplet size or morphology, however these data suggest that the supersized droplet phenotype actually occurs secondary to a primary defect in lipid droplet number, which is limited by the poor rate of lipid droplet initiation. Once initiated, however, *fld1*$^{\Delta Nterm}$ lipid droplets appear morphologically more normal than *fld1*Δ droplets, with size being their only distinction from wild-type lipid droplets. It therefore appears that the N-terminal mutant uncovers two dissectible roles for seipin: first in promoting the efficient initiation of lipid droplets, for which the N-terminus is required; and second in regulating lipid droplet morphology during subsequent lipid droplet growth or maintenance. These roles are described further in Section 5-1, and hypotheses for the mechanism of action of each role are discussed in Section 5-2.

CHAPTER FIVE
Conclusions and Recommendations

ROLE OF SEIPIN IN NORMAL DROPLET FORMATION

Aberrance of Seipin-Deficient Droplets

I have presented results confirming the role of seipin in the regulated targeting of the major yeast triacylglycerol lipase, Tgl3p, to the lipid droplet surface, as published by Wolinski et al.[83] I further found that in addition to Tgl3p, all of the other known LD-associated triacylglycerols and sterol esters also exhibit a regulated switch between localization modes depending on whether the cell is in a growth or stationary phase, suggesting that this is likely to be a general mode of lipolytic enzyme regulation. Moreover, I found partial to severe defects in the localization of each known LD-associated lipolytic enzyme to the lipid droplet surface, suggesting a broad effect of seipin on lipase targeting (Fig. 3).

This lipase-targeting defect highlights the aberrant nature of seipin-deficient lipid droplets. As described in Section 2-4-2, several aspects of lipid droplet function are impeded in the absence of seipin, including lipolysis, lipid droplet inheritance during cell division[83], and lipid metabolism[99, 158, 191, 192, 198, 199]. This is consistent with our model in which seipin is required for the regulated biogenesis of a lipid droplet, both in promoting drop formation and in organizing the morphology and contents of the organelle. Without these controls, stochastically budding lipid drops are expected to have a relatively random composition

depending on the contents of the local section of the ER membrane from which they happen to bud. An individual lipid droplet that buds in this manner may not happen to obtain the full complement of lipid and protein components required for every lipid droplet function, and the proportion of such components may be out of balance. Because of the stochastic nature of this process, each lipid droplet would be different, accounting for the extreme heterogeneity observed in *fld1*Δ lipid droplet morphology[99, 190, 192], lipase targeting[83], and even in partitioning of LDs between the cytosol and nucleus (Fig. 6). Far from being the regulated organelle that the lipid droplet is now viewed as, seipin-deficient droplets are highly aberrant, heterogenous, and partially dysfunctional.

The implications of these defects in *fld1*Δ lipid droplets for cellular function or disease pathogenesis remain to be determined. Several of the phenotypes associated with seipin deficiency in yeast could be downstream consequences of lipid droplet dysfunction; likely examples include lipase mistargeting[83], subtle defects in lipid metabolism[99, 158, 191, 192, 198, 199], terbinafine sensitivity[198], and lipid droplet fusion[99, 192]. It will be important to consider this possibility when addressing any phenotype associated with seipin, so as not to confuse direct functions of the protein with indirect effects[104].

It is possible that lipid droplet dysfunction could play a role in cellular pathogenesis in either adipocytes or non-adipocytes of *BSCL2* patients. Although dissection of the human seipin protein has suggested that the evolutionarily divergent C-terminal domain is responsible for the adipogenic function of seipin[169, 193], the conserved core sequence of the protein could still potentiate or modulate adipogensis and/or mediate one or more of the multiple non-adipose phenotypes unique to seipin-deficient patients. There are several ways

in which lipid droplet dysfunction could cause these cellular defects. The findings on abnormal lipolysis due to aberrant lipase targeting in *fld1*Δ could explain the observations that the block in adipogenesis in seipin knockout mice is associated with deregulated lipolysis in the later stages of adipocyte differentiation[115, 118]. Although *fld1*Δ cells were found to have decreased lipolysis[83], while seipin-deficient differentiating adipocytes displayed increased lipolysis[115, 118], this difference in output could be explained by the more complex and specialized nature of lipolytic control at the adipocyte lipid droplet surface (a system which includes regulation by perilipins, proteins not found in yeast)[214-216]; lipase mistargeting or misregulation could therefore produce different effects in these widely different systems. A wealth of detailed knowledge is available from intensive study of lipolytic control in adipocytes; use of such tools to study the regulation of ATGL, HSL, and other mammalian lipases in the absence of seipin would be a worthy pursuit.

Although no drastic effects on lipid metabolism are seen in the absence of seipin, the subtle effects observed could also affect cellular processes via the alteration of low-abundance lipid mediators. One particular theory that has been put forth is the suggestion that slightly altered lipid metabolism in seipin-deficient cells could affect the generation of a lipid ligand for PPARγ, the ligand-gated nuclear receptor often referred to as the master transcriptional regulator of adipogenesis. Although the endogenous ligand has not yet been conclusively identified, certain fatty acids have been found to bind and activate PPARγ, and some evidence indicates that an unknown endogenous activating ligand may be produced during adipogenesis[113]. PPARγ function during adipogenesis is clearly at least indirectly disrupted in seipin-deficient pre-adipocytes[114, 116, 159], and recent findings on a potential role

for PPARγ in the pathogenesis of affective behavioral disorders in seipin-deficient mice suggest that it may also play a role in non-adipose seipin phenotypes as well[151]. It has therefore been proposed that seipin-deficient lipid droplets could disrupt the metabolism of an endogenous activating or inhibiting PPARγ ligand[104, 217]. Thorough analysis of minor lipid species in seipin-deficient cells could address this hypothesis.

Finally, disruption of more recently hypothesized functions of lipid droplets could effect a variety of downstream consequences. Given increasing evidence for the role of lipid droplets in the regulation, sequestration, and degradation of a wide variety of proteins[73], disruption of the lipid droplet surface could have broad effects through the misregulation of such proteins. Additionally, it has been hypothesized that proteins at the lipid droplet surface could act in the sensing of lipid storage status[218]; alteration of the morphology or content of LDs could easily alter the information transduced in such a process. However, since these potential lipid droplet functions are still highly uncharacterized, no specific hypotheses have been generated for how general protein regulation or lipid droplet sensing could mediate seipin effects.

Effects of Seipin on Early Droplet Assembly

Members of the Goodman laboratory identified a defect in de novo droplet formation in *fld1*Δ yeast (Chris Hilton and Derk Binns, unpublished data); I found that deletion of the first 14 N-terminal amino acids of yeast seipin reproduced most of this effect, suggesting a role for the seipin N-terminus in lipid droplet biogenesis. Importantly, this defect was

apparent in the earliest stages of lipid droplet formation, impairing the ability of *fld1*Δ and *fld1*$^{\Delta Nterm}$ cells to generate even a single, first LD, suggesting that the N-terminus of yeast seipin promotes the earliest initiation events of the lipid droplet. The scattered time points in which LDs appeared in the mutant strains, compared to the early peak for *FLD1* cells, suggest that lipid droplet formation occurs stochastically in *fld1*Δ and *fld1*$^{\Delta Nterm}$. Once a lipid droplet did appear, filling of the droplet with neutral lipid proceeded at a surprisingly more rapid pace in *fld1*Δ and *fld1*$^{\Delta Nterm}$ than in *FLD1* droplets (Fig. 9).

These data are consistent with our hypothesis that seipin promotes the exit of neutral lipid from the ER via lipid droplet formation; in the absence of seipin, neutral lipid accumulates within the ER membrane until the bilayer becomes unstable and lipid drops bud stochastically, resulting in delayed and diminished lipid droplet formation. Once the barrier to this stochastic blebbing has been overcome and a lipid drop has been initiated, neutral lipid then fills the drop more rapidly as a result of the increased concentration gradient generated by extensive neutral lipid accumulation within the ER. To use an analogy, seipin can be thought of as a "vent" for releasing neutral lipid buildup in the ER: without this vent, neutral lipid accumulates until enough pressure has built up to spring a leak; once this leak has formed, the pressure buildup results in increased flow.

The recently described spontaneous emulsion model of lipid droplet formation posits that the physicochemical forces underlying hydrophobic and hydrophilic interactions serve as the primary driver for lipid droplet biogenesis[41, 95]. I propose that the aberrant LDs generated in the absence of seipin represent these spontaneously formed drops because of their stochastic appearance and aberrant composition. I suggest that while lipids can indeed

coalesce into a drop in the absence of cellular machinery simply as a result of their hydrophobicity, this is not the same process as the biogenesis of a functional, regulated lipid droplet organelle. I therefore propose a careful distinction of nomenclature between an accumulation of lipid within the cell and the actual, constructed organelle: here I use the term "lipid drop," used by the authors of the spontaneous emulsion model to describe the early coalesced lipid structure[41], to refer to neutral lipid aggregates likely originating by spontaneous emulsion, and the term "lipid droplet" to refer to the homogenous, regulated organelle.

Researchers within the field have noted that no single gene appears to be essential for lipid droplet formation[41, 95]; upon deletion of seipin and other proteins suspected to be involved in lipid droplet biogenesis, accumulations of neutral lipid are still seen on microscopy[82, 190, 192, 198]. This is not surprising, however, since unless such mutation completely abrogates triacylglycerol and steryl ester synthesis, neutral lipid still accumulates within the cell, and once it reaches a saturation point within the ER, it must go somewhere. If, however, we adopt a distinction between spontaneously coalesced lipid drops and regulated, constructed lipid droplets, then it becomes apparent that the N-terminus of seipin is indeed required for the efficient and regulated biogenesis of the lipid droplet organelle.

HYPOTHESES FOR SEIPIN MECHANISM OF ACTION

Lipid Droplet Initiation and Lipin

I have presented evidence that the N-terminus of yeast seipin acts to promote lipid droplet biogenesis. Since lipid drops can form stochastically in the absence of seipin, I suggest that during LD initiation, seipin acts as a catalyst in the facilitated emulsion of the lipid droplet. There are many mechanisms by which seipin could carry out this role: direct channeling of neutral lipid, manipulation of ER membrane shape, and recruitment of enzymes for localized/directed lipid synthesis are only a few possibilities. Since the structure and physical interactions of seipin have not been largely characterized, it is not yet possible to narrow down the many potential mechanisms.

However, several parallels between seipin and another lipodystrophy protein, lipin, strongly suggest that these two proteins carry out similar, potentially coordinating or interacting functions in lipid droplet biogenesis. Lipin is a phosphatidic acid hydrolase (Pah1p in yeast) that catalyzes the dephosphorylation of phosphatidic acid to generate diacylglycerol. It is not the only PA hydrolase: upon lipin deletion, DAG can still be generated from PA via alternative pathways, although a dysregulated imbalance leads to relative PA accumulation[219]. Localization to the ER membrane is required for lipin activity, and this localization is regulated by a phosphorylation switch. Phosphorylated lipin is soluble in the cytosol and nucleus; dephosphorylation by an ER phosphatase complex (Nem1p and Spo7p in yeast; CTDNEP1 and NEP1-R1 in mammals) results in binding to the ER membrane via an amphipathic helix[220-223]. Importantly, Nem1p has been found to localize to a few discrete puncta within the ER which associate with lipid droplets, suggesting that much like seipin, lipin may act at LD-ER junctions[82].

Lipin and seipin deletion result in strikingly similar phenotypic patterns in both mammals and yeast. Lipin was first discovered in a spontaneous mutant mouse line presenting with lipodystrophy and peripheral neuropathy[165-167], indicating that, much like seipin, it is likely to play a role in adipocyte and neuronal function. Furthermore, although the *pah1*Δ strain was absent from the yeast knockout library used for both of the screens for lipid droplet morphology that first identified yeast seipin, each of these screens picked up both Nem1p and Spo7p as putative lipid droplet determinants[82, 190, 192].

The *pah1*Δ lipid droplet phenotype was later found to be similar to those of *nem1*Δ and *spo7*Δ[82]; this phenotype also bears several striking similarities to that of *fld1*Δ yeast. Lipid droplets appear heterogenous in *pah1*Δ[82], even including the presence of supersized lipid droplets, albeit at a lower frequency than in *fld1*Δ[99]. The *pah1*Δ strain additionally exhibits decreased triacylglcyerol[219] (with a compensatory increase in sterol ester maintaining total neutral lipid[82]) and extreme proliferation of ER membrane into extensive perinuclear ER stacks. These effects, not observed in *fld1*Δ, likely occur as a result of a relative shift in cellular metabolic flux away from DAG (which primarily acts as a precursor for triacylglycerol synthesis) toward PA (which, when its conversion to DAG is diminished, primarily acts as a precursor for phospholipid synthesis, and additionally results in derepression of phospholipid synthetic enzymes)[224]. Within these ER stacks, abundant neutral lipid inclusions accumulate[82], suggesting that, much like Fld1p, Pah1p acts to facilitate the release of neutral lipid from the ER into LDs.

The minor elevation in PA detected in membranes isolated from *fld1*Δ cells[99] indicates that the similarity between these two strains may extend to effects on PA

metabolism. Pah1p clearly has a stronger effect on PA; since it acts directly as a PA degradation enzyme, *pah1*Δ generates whole-cell increases in PA and phospholipids that result in massive changes to ER morphology[224]. Fld1p is more likely to act in subtle, localized manipulation of PA at the LD-ER junction, and so produces only a modest increase in local PA and does not generate obvious ER proliferation. This difference is likely to explain why neutral lipid accumulation within the ER can only be observed during early lipid droplet biogenesis in *fld1*Δ (Chris Hilton and Derk Binns, unpublished data), while clear NL inclusions can be seen in the ER bilayer in steady-state *pah1Δ* cells: the extensive proliferation of ER membranes in *pah1Δ* provides a greatly increased capacity for absorbing neutral lipid before saturation[82]. Notably, another mutation associated with congenital generalized lipodystrophy, in the *BSCL1/AGPAT2* gene[131, 132], also results in elevated PA levels[225, 226]. This similar effect of three different lipodystrophy proteins on PA suggests that manipulation of phosphatidic acid may represent a common/convergent mechanism for promoting lipid droplet biogenesis or lipid droplet initiation[217].

In Chapter Four, I presented evidence that seipin performs at least two dissectible functions in initiating and maintaining the lipid droplet. Members of our laboratory have recently found evidence that PA accumulation co-segregates with the lipid droplet initiation function carried out by the seipin N-terminus. In *fld1Δ* cells, localized PA accumulation at the LD-ER junction can be detected by use of multiple fluorescent probes for PA; these probes display a weak perinuclear membrane distribution in *FLD1* cells, but accumulate into LD-associated puncta in *fld1Δ*. Strikingly, these PA puncta are also observed in *fld1*$^{\Delta Nterm}$ (Sungwon Han, unpublished data), suggesting that accumulation of PA is associated with the

lipid droplet biogenesis defect of *fld1Δ*, rather than with dysregulated LD maintenance. This provides further evidence that seipin and lipin act through similar mechanisms to promote lipid droplet formation.

Together, these patterns present a strong case that seipin and lipin carry out at least highly similar, parallel roles in lipid droplet biogenesis, making lipin a prime candidate for transducing the effect of the seipin N-terminus on LD formation. This possibility is made even more attractive by the finding that human seipin and lipin can co-immunoprecipitate[168, 169], although these studies need to be more carefully validated, and a physical interaction has not yet been observed for yeast Fld1p and Pah1p. An interaction between Fld1p and Pah1p, whether a direct physical interaction or an indirect functional coordination, would be likely to result in the localized conversion of PA to DAG at the LD-ER junction. Diacylglycerol has been found to promote lipid droplet formation independent of its role as a precursor for TAG[82]; a localized DAG accumulation generated by seipin/lipin could therefore act as a co-surfactant in lipid droplet emulsion[41].

Manipulation of localized diacylglycerol levels via lipin action therefore seems an intriguing possible mechanism for the action of seipin in the facilitated emulsion of lipid droplets. Efforts are currently underway in the Goodman laboratory to quantify Pah1p stability and activity in the absence of seipin and to identify potential protein binding partners for seipin in yeast, including a candidate approach to specifically address the possibility of an interaction between Fld1p and Nem1p at the LD-ER junction. Studies of de novo lipid droplet formation in *pah1Δ* may also be called for, to confirm a lipid droplet biogenesis defect similar to that of *fld1Δ*. Although a functional interaction with lipin is only one

possibility for the mechanism of seipin action in lipid droplet biogenesis, it is my opinion that the body of evidence paralleling lipin and seipin makes this an important avenue to pursue.

Lipid Droplet Regulation: Phospholipid Trafficking

I found that although *fld1*$^{\Delta Nterm}$ at least partially recapitulated the lipid droplet biogenesis defect of *fld1Δ* (Fig. 9), there were significant differences between the two strains in terms of lipid droplet morphology. Lipid droplets in *fld1*$^{\Delta Nterm}$ are much more homogenous in size and shape than those in *fld1Δ*; indeed, their only apparent distinction from *FLD1* droplets is increased size and decreased number, likely owing to limited LD number as a result of sluggish lipid droplet biogenesis (Fig. 9). I have therefore concluded that the N-terminal seipin deletion dissects two roles for seipin: one in lipid droplet biogenesis, and one in regulation of lipid droplet morphology.

The response of both of these mutants to inositol provides a valuable clue to the mechanism of seipin action in promoting lipid droplet homogeneity. While inositol supplementation increases the phospholipid to neutral lipid ratio of *fld1Δ* lipid droplets and shifts their morphology from supersized droplets to LD-ER tangles, inositol produces no effect on lipid droplet morphology in *FLD1* or *fld1*$^{\Delta Nterm}$ (Fig. 8 and data not shown). Inositol action in *fld1Δ* is likely due to upregulation of phospholipid synthesis; increased available phospholipids can then access the droplet surface to alter morphology by increasing the surface area to volume ratio. Since these effects do not occur in *FLD1* or *fld1*$^{\Delta Nterm}$, I hypothesize that the bulk of the seipin protein (the entire sequence except for the short N-

terminus) participates in the regulation or restriction of phospholipid access to the LD surface. Importantly, once a lipid droplet is generated, multiple phosphatidylcholine synthesis enzymes have been found to associate with the lipid droplet surface[48, 227]. Therefore, seipin could act only in regulating or limiting the free flow of phospholipid access from the ER to the LD surface, or it could block it entirely, allowing phospholipid for LD growth to be generated by surface enzymes.

Experiments analyzing phospholipid flux between the ER membrane and the LD monolayer would be extremely valuable in testing this hypothesis, however the tools for such experiments are not yet available. Unfortunately, fluorescent probes for phospholipids are severely lacking. Most such probes attach the fluorescent label in place of an acyl chain (tail-labeled) rather than to the head group (head-labeled)[228]. As such, the tail-labeled fluorophore can be cleaved and incorporated into different lipid classes by lipid metabolic enzymes, often ultimately ending up labeling neutral lipid within the LD core[229]. Head-group labels would be much more specific for phospholipids and could theoretically be used to differentiate different PL classes, however due to the ease of chemical conjugation, only head-labeled phosphatidylethanolamine is currently available[230]; even this labeled PE may have limited utility in yeast. With the technology currently available, phospholipid flux could be analyzed by incorporation of radiolabeled phosphate into a lipid droplet fraction during de novo biogenesis, but such experiments are likely to be laborious. The development of better fluorescent phospholipid probes would be ideal.

Unfortunately, the lack of structural and functional information on seipin makes it difficult to construct an informed hypothesis on how seipin might carry out this potential

regulation of phospholipid flux. Seipin could act as a physical barrier to phospholipid diffusion, an active transporter of certain phospholipids to the LD surface, a coordinator of new phospholipid synthesis, or a determinant of local ER membrane shape, among other possibilities. Because interactions with the phospholipid bilayer are likely to be crucial for this function, it will be important to determine whether seipin generates membrane curvature or unusual phospholipid structures; this could be addressed by studies of purified seipin in giant unilamellar vesicles.

Conclusion

During my thesis work, I have probed the function of seipin by analyzing the effects of seipin mutations on lipid droplet protein targeting, morphology, and biogenesis. I have found evidence supporting the hypothesis that seipin is required for the regulated assembly of the lipid droplet organelle, and I have implicated dissectible regions of the protein in the facilitation of lipid droplet biogenesis and the maintenance of regular lipid droplet morphology. These studies have provided valuable insight into the role of seipin at the lipid droplet in yeast, and further investigation to determine if these findings are conserved in mammalian cells would provide additional insight into disease pathogenesis in seipin-deficient patients. Moreover, these experiments have supported a view of lipid droplets as regulated organelles constructed and maintained by cellular machinery, rather than spontaneously coalescing lipid inclusions, providing insight into one of the most fundamental processes of the eukaryotic cell.

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