Control of endoplasmic reticulum homeostasis by Doa10-dependent protein degradation

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“Ho un’intelligenza mediocre, il mio merito è l’impegno”

Rita Levi Montalcini

This has been the best harsh time I have ever gone through.
I have lived my PhD years very intensely, in many respects, and it is now that I can clearly see how all those feelings have shaped me into the scientist and person I have become. While this change was going on I was never alone, neither as a scientist nor as a person. There are many people I want to acknowledge for making it happen.

The biggest acknowledgement goes to my supervisor Pedro. I thank him for having been motivating (sometimes in his own way), pushy (in his very own way), brilliant and hopeful, and for always having a “plan B”.

The past and present members of the lab: I learned from their knowledge, gained strength from their support and laughed at their jokes.

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Abstract

The function, shape and identity of cellular organelles are too a large extent determined by their lipid and protein composition. In order to maintain cellular homeostasis, the rate of synthesis and degradation of proteins and lipids must be accurately controlled. Proteolysis by the ubiquitin-proteasome system plays a major role in regulating the half-lives of a range of proteins. A multitude of cellular processes depends on timely controlled and selective protein degradation; just to mention a few, these include intracellular trafficking and secretion, elimination of damaged polypeptides and DNA repair. Remarkably, anomalies in the ubiquitin-proteasome system have been linked to several human pathologies.

Misfolded proteins in the membrane and lumen of the endoplasmic reticulum (ER) are constitutively generated during protein biosynthesis. These species are potentially toxic and are eliminated by the ubiquitin-proteasome system through a quality control pathway called ER-associated protein degradation (ERAD). Beyond this well-studied role, ERAD controls the levels of some folded, functional but short-lived ER proteins by eliminating them under a specific physiological condition, thereby in a regulated fashion. Of note, sterol production is adjusted to cell needs through feedback control of the HMGR enzyme stability.

Despite its importance in ER homeostasis, regulated degradation through ERAD still accounts for only few examples.

Yeast Doa10 is one of three ER ubiquitin ligase enzymes implicated in the degradation of misfolded proteins. To seek for regulated Doa10 clients, we pursued a proteomics screening. We identified potential targets involved in diverse cellular functions and further characterized some of them. We demonstrate that Doa10-dependent degradation critically impacts lipid homeostasis through regulated disposal of the sterol pathway enzyme Erg1. Moreover, we show that Doa10 mediates degradation of proteins belonging to lipid droplets, an ER-derived organelle; this finding highlights a role for ERAD in protein spatial control and maintenance of ER identity.

Resumen

La función, forma e identidad de los orgánulos celulares es determinada, en gran medida, por su composición lipídica y proteica. Para mantener el equilibrio celular, las tasas de síntesis y degradación tanto de proteínas como de lípidos deben controlarse con exactitud. La proteólisis mediante el sistema ubiquitino-proteosómico cumple un papel importante en la regulación del tiempo de vida media de una variedad de proteínas. El normal funcionamiento de numerosos procesos celulares requiere degradación selectiva de proteínas en forma precisa y oportuna; entre estos procesos algunos ejemplos prominentes son: tráfico intracelular y secreción, eliminación de polipéptidos dañados y reparación de ADN. Valga resaltar que anomalías en el sistema ubiquitino-proteosómico han sido asociadas a varias patologías humanas.

Durante la proteosíntesis algunas proteínas mal plegadas se generan, de forma constitutiva, en la membrana y en el lumen del retículo endoplasmático (RE). Estas especies, potencialmente tóxicas, son eliminadas mediante el sistema ubiquitino-proteosómico por una ruta de control de calidad denominada degradación asociada al retículo endoplasmático (DARE). Más allá de esta bien conocida y estudiada función, DARE controla también la abundancia de algunas proteínas del RE correctamente plegadas y funcionales, pero de vida media corta. En este caso la selección y degradación de substratos responde a condiciones fisiológicas específicas y constituye un proceso regulado. De particular relevancia, la síntesis de esteroles se ajusta a los requerimientos celulares a través del control de la estabilidad de la enzima HMGR mediante un mecanismo de retroalimentación.

A pesar de su importancia en la homeostasis del RE, hasta el momento sólo se conocen algunos pocos ejemplos de degradación regulada mediada por DARE.

En el RE de *S.cerevisiae* tres enzimas ligasas de ubiquitina, entre ellas Doa10, participan en la degradación de proteínas mal plegadas. Con el propósito de encontrar sustratos regulados de Doa10 llevamos a cabo un examen proteómico. Encontramos varios candidatos, involucrados en diversas funciones celulares, y caracterizamos algunos de ellos en mayor profundidad. Demostramos que la

degradación dependiente de Doa10 tiene un impacto crucial en la homeostasis de lípidos por medio de la eliminación regulada de Erg1, una enzima del anabolismo de esteroles. Más aún, encontramos que Doa10 lleva a la degradación de proteínas pertenecientes a los cuerpos lipídicos, un orgánulo derivado del RE; este descubrimiento resalta el rol que DARE juega en el control espacial de proteínas y el mantenimiento de la identidad del RE.

Preface

The work described in this thesis has been entirely conducted in the Cell and Developmental Biology program at Center for Genomic Regulation (CRG) under the supervision of Dr. Pedro Carvalho.

The results presented here illustrate a previously unreported role for the ubiquitin ligase Doa10 in control of endoplasmic reticulum homeostasis through selective and regulated protein degradation.

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1. INTRODUCTION

1.1 The endoplasmic reticulum

In eukaryotic cells compartmentalization allows distinct cellular functions to be carried out in dedicated organelles. Remarkable for its structural organization and functional diversity, the endoplasmic reticulum (ER) has attracted the interest of cell biologists since its first description in 1953. It is composed by a dynamic network of sheets and branching tubules, which in higher eukaryotes extends from the nuclear envelope to the cell periphery, throughout the entire cytoplasm; in yeast, the nuclear ER, in continuity with the inner nuclear membrane (INM), connects to the cortical (or peripheral) ER by few tubules (Shibata et al., 2006).

These morphologically different ER subdomains are also functionally distinct. Biosynthesis of membrane and secretory proteins is confined to sheets, or rough ER; here, translating ribosomes are targeted to the SRP receptor on the ER by the signal recognition particle (SRP) bound to the N-terminal signal peptide in the emerging polypeptide. The polypeptide chain extends and is simultaneously translocated into the ER through a translocation channel. Membrane insertion of hydrophobic sequences in transmembrane proteins occurs co-translationally, whereas polypeptides lacking transmembrane domains are fully released in the ER lumen (Shao and Hegde, 2011). Notably, certain protein groups use alternative, SRP-independent pathways (reviewed in (Aviram and Schuldiner, 2014)). Among these are tail-anchored (TA) proteins, bearing a single transmembrane span at their C-terminus. The C-terminal hydrophobic sequence is not exposed until translation is complete (Borgese et al., 2003); after release from the ribosome, the TA protein is inserted post-translationally in the ER membrane by the TRC40/GET pathway, which is completely different from the co-translational pathway (Borgese and Fasana, 2011).

In all cases, the polypeptide is aided in folding by a plethora of chaperones and other modifying enzymes, which, *e.g.*, attach glycans and form and oligomerize disulfide bonds (Braakman and Hebert, 2013).

Lipid synthesis in the ER is believed to occur primarily in the tubules, which are devoid of active ribosomes (thereby the definition of smooth ER). ER tubules establish direct contacts with other organelles (*e.g.* mitochondria and endosomes), thereby facilitating lipid exchange [reviewed in (Rowland and Voeltz, 2012)] and controlling organelle division (Friedman et al., 2011; Rowland et al., 2014).

Importantly, the ER also initiates the biogenesis of other organelles, namely peroxisomes [reviewed in (Hettema et al., 2014)] and lipid droplets [reviewed in (Pol et al., 2014)] and is the major calcium store in the cell.

1.2 Protein quality control

Protein synthesis is a delicate process: protein function relies on the acquisition of the correct folding and adequate post-translational modifications. In an organelle with intense protein synthesis such as the ER, newly synthesized polypeptides are subjected to accurate scrutiny by numerous chaperones, which ensure acquisition of the native structure before proteins can reach their final locations. Moreover, in many cases polypeptide folding is further facilitated by covalent modifications; these include N-linked glycosylation and disulfide bond formation. At this stage, subunits of multiprotein complexes need to assemble with specific stoichiometries.

Several circumstances can compromise protein folding efficiency. Mutations in the primary sequence, unbalanced subunit synthesis or stochastic failure to fold into a native structure lead to the production of defective molecules, which account for one third of newly synthesized polypeptides (Schubert et al., 2000). Recognition and elimination of such species by quality control mechanisms is required to avoid their accumulation and possible aggregation.

When folding fails aberrant proteins are retained in the ER. They are recognized by chaperones, returned to the cytoplasm (in a step called retrotranslocation), marked by a polyubiquitin chain and degraded by the proteasome. Polyubiquitination requires the sequential and repeated activity of E1, E2 and E3 enzymes (Figure 1).

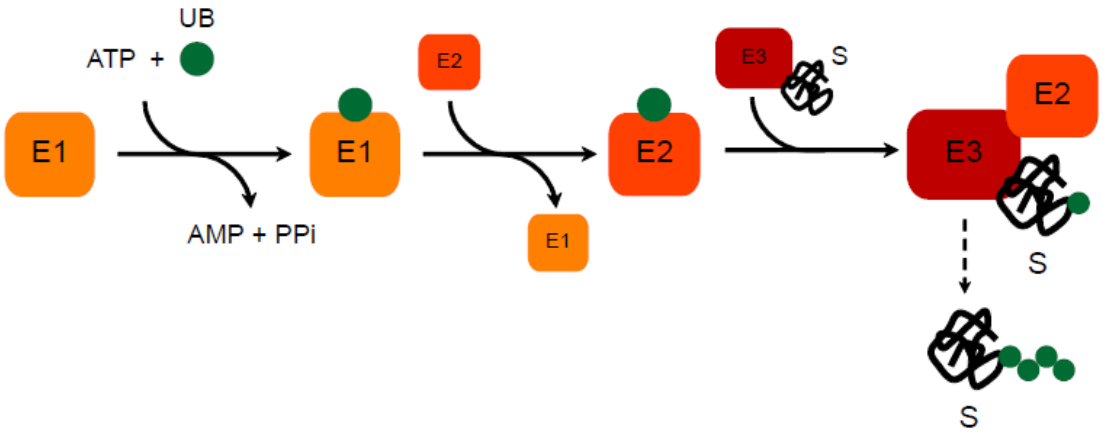

Figure 1: Schematic of the ubiquitination process. Ubiquitin (UB) is activated in an ATP-dependent manner by the ubiquitin-activating enzyme (E1), forming a thioester bond between a cysteine residue and the C-terminus of ubiquitin. Subsequently, ubiquitin is transferred to the catalytic cysteine of an ubiquitin-conjugating enzyme (E2). An ubiquitin ligase (E3) facilitates transfer of ubiquitin from the E2 to a lysine residue in the substrate (S). The E3 is responsible for substrate specificity. The series of reactions is repeated to build a polyubiquitin chain on the substrate.

Collectively, the series of events for disposal of ER misfolded proteins is referred to as ER-associated protein degradation (ERAD) (Figure 2).

Although protein quality control is constitutively operative, conditions like increased protein synthesis, exposure to high temperature or pharmacological agents, can raise the amount of aberrant proteins and challenge the folding capacity of the organelle. Cells possess adaptive responses, namely the unfolded protein response (UPR), which manage misfolded protein-induced stress by enhancing biosynthesis of membrane lipids, chaperones and ERAD factors (Gardner et al., 2013). Prolonged or irreversible ER stress can ultimately lead to apoptotic cell death (Tabas and Ron, 2011). Importantly, the pathogenesis or exacerbation of several human diseases (*e.g.* neurodegeneration) is a consequence of ER stress and ER-stress-triggered apoptosis (Fonseca et al., 2011; Hetz and Mollereau, 2014; Lin and Lavail, 2010). These studies highlight the importance of quality control pathways in maintaining cell and organism homeostasis.

1.3 ERAD

The early evidence for an ER quality control was the lysosome-independent degradation of unassembled T cell receptor subunits in a pre-Golgi compartment (Lippincott-Schwartz et al., 1988). Since the ubiquitin-proteasome system (UPS) was first implicated in ER quality control in yeast (Sommer and Jentsch, 1993), numerous studies have collected evidences for ubiquitin- and proteasomal-dependent degradation of ER membrane (*i.e.* mutant cystic fibrosis transmembrane conductance regulator, ΔF508 CFTR) and luminal proteins (*i.e* mutant CPY), both *in vivo* (Hiller et al., 1996; Jensen et al., 1995; Ward et al., 1995) and *in vitro* (Werner et al., 1996).

In early nineties, most yeast *UBC* genes encoding E2 ubiquitin-conjugating enzymes had been cloned and characterized (Jungmann et al., 1993; Seufert and Jentsch, 1990; Seufert et al., 1990) and several were later showed to be required for ERAD (Chen et al., 1993; Sommer and Jentsch, 1993). Several other ERAD components were discovered through genetic analysis. Screenings for identifying the genes responsible for "Hmg-CoA reductase degradation" and "degradation of alpha2" (the yeast transcriptional repressor Matα2) uncovered the *HRD* and *DOA* genes, respectively (Hampton et al., 1996; Swanson et al., 2001). Hrd1 and Doa10 were shown to be ER membrane-bound RING E3 ubiquitin ligases (Bays et al., 2001a; Swanson et al., 2001), which act at the core of the ERAD complexes (Figure 3).

As more topologically diverse ERAD clients were identified, the degradative route was shown to depend on the location of the misfolded lesion with respect to the ER membrane (Huyer et al., 2004; Vashist and Ng, 2004). Systematic biochemical analysis helped deciphering how substrates are partitioned between the two yeast ERAD complexes (Carvalho et al., 2006). In general terms, lumenal or membrane polypeptides with a lesion in the lumen (ERAD-L) or in the membrane (ERAD-M) are disposed by the Hrd1 complex; Doa10 complex clients carry a cytosolic misfolded defect (ERAD-C) (Figure 3) (Carvalho et al., 2006). Notably, certain Doa10 substrates are cytosolic or nuclear proteins (Ravid et al., 2006). Additionally, cytosolic ubiquitin ligases can participate in ERAD (Stolz et al., 2013).

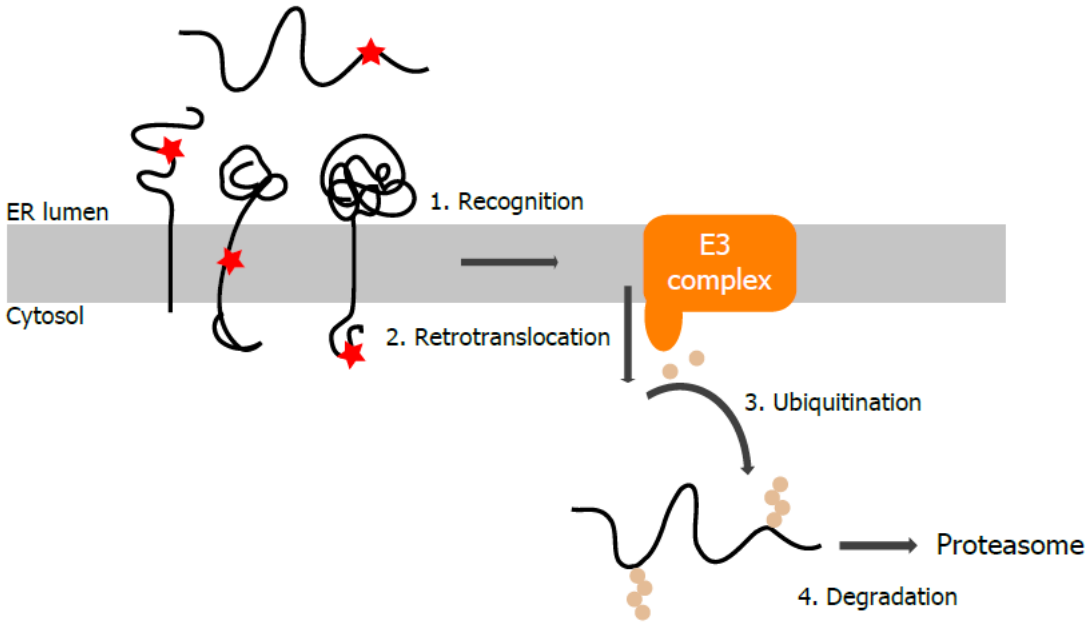

Figure2: Schematic of the ERAD pathway. The canonical ERAD steps are depicted. Red star indicates a misfolded lesion.

More recently, the ASI complex in the INM has been shown to contribute to disposal of misfolded or native proteins that escape the ER through the nuclear pore complex; thereby, it prevents accumulation of ERAD substrates that might not be accessible to the other E3 ubiquitin ligases (Foresti et al., 2014).

Mammalian homologues for the components of the Doa10 and Hrd1 complexes have been identified; in addition, metazoans have many other ubiquitin ligases, some of which only poorly characterized (Christianson and Ye, 2014; Ruggiano et al., 2014). As in yeast, they presumably serve a unique set of misfolded substrates; however, data in this direction are still scarce. A functional homologue for the yeast ASI complex is at present not known.

Irrespective of the E3 complex involved, in a common series of late events ubiquitinated substrates are handled by several ubiquitin-conjugate binding proteins before proteasomal degradation. The ATPase p97/Cdc48 complex is required for membrane extraction (Ye et al., 2001, 2003); in some cases, the ubiquitin-chain assembly factor (or E4), like yeast Ufd2, supports optimal ubiquitination by elongating pre-existing ubiquitin chains, particularly under stress conditions (Koegl et al., 1999). Finally, adaptor proteins, like Rad23 and

Dsk2, escort the substrate to the proteasome for degradation (Richly et al., 2005).

a) ERAD substrates

Misfolded polypeptides expose hydrophobic patches and thus are potentially harmful for their propensity to aggregate (Fink, 1998). Not surprisingly, these molecules are the primary clients of the ERAD pathway. Similarly, unassembled subunits of multiprotein complexes unmask hydrophobic interaction surfaces and, therefore, are also amenable to ERAD.

In more recent years, several reports collectively showed that the mevalonate pathway enzyme 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR) undergoes lipid-induced degradation by ERAD (DeBose-Boyd, 2008). This represented for many years the only example of a potentially functional protein degraded for regulatory purposes.

Substantially different mechanisms define how misfolded or unassembled polypeptides and regulated substrates are engaged in ERAD. These differences will be discussed below.

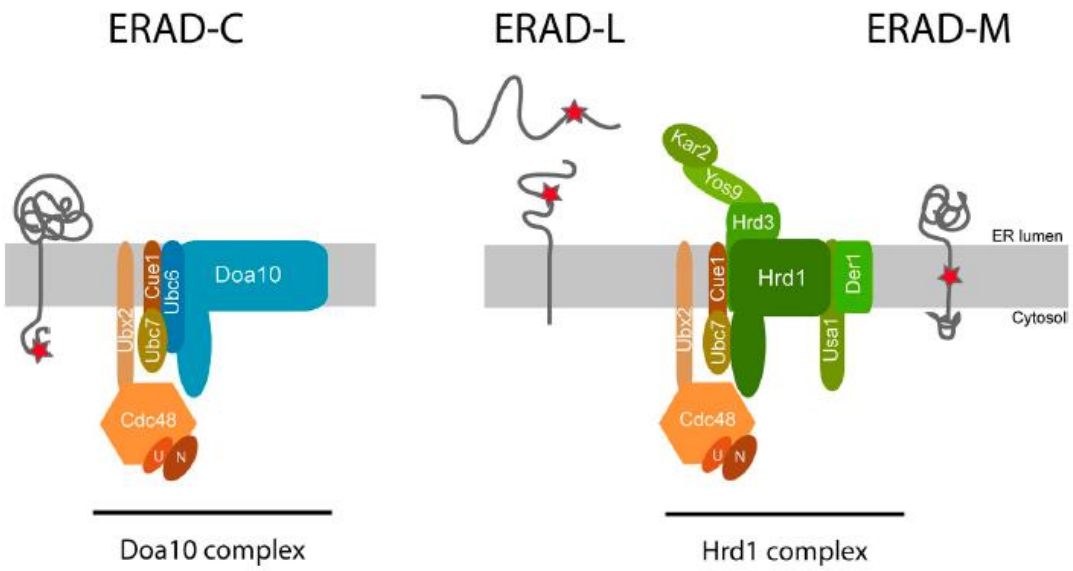

Figure 3: Substrate specificities of the ERAD complexes in yeast. Adapted from (Ruggiano et al., 2014).

- Misfolded proteins

Recognition of these species poses a major challenge to cells, as it relies on the ability to discriminate terminally misfolded polypeptides from folding intermediates. In certain cases, eliminating potentially misfolded molecules is safer than having to deal with the consequences of misfolding. A prominent example of ERAD stringency is degradation of CFTR. CFTR has a complex folding path and a conspicuous fraction of the wild-type polypeptide is disposed by ERAD (Ward and Kopito, 1994). On the other hand, ER must prevent escape of toxic species. Thus, client disposal is carefully controlled at the recognition step by balancing stringency and accuracy. A key role is played by molecular chaperones (*e.g.* Hsp70s and lectins), which aid folding and can target substrates for degradation if this process fails; however, it is not entirely known how these seemingly opposing functions coexist (Brodsky, 2007).

Misfolded protein recognition has been best characterized for misfolded glycoproteins in the ER lumen (*e.g.* misfolded carboxypeptidase, CPY*) (Benyair et al., 2014). The branched N-linked glycan moiety is attached to proteins soon after they have been translocated. It is built from two N-acetylglucosamine, nine mannose and three glucose residues and it can be processed by glycan-trimming enzymes (Herscovics, 1999). Early-acting enzymes (glucosidases) remove the glucoses, allowing binding of the lectins (*e.g.* calnexin and calreticulin) which initiate protein folding. Several cycles of re-glucosylation by UDP-glucose:glycoprotein glucosyltransferase (UGGT) and calnexin/calreticulin re-association might lead to successful folding. Late-acting enzymes (*e.g.* Htm1 in yeast, EDEMs in mammals) trim a terminal mannose residue, thereby acting as timers and releasing the polypeptide from further folding attempts (Figure 4); the terminally misfolded glycoprotein is then diverted to an E3 ubiquitin ligase complex (Benyair et al., 2014; Tannous et al., 2014).

A canonical calnexin/calreticulin cycle does not exist in yeast due to the absence of UGGT. Nevertheless, the framework of glycan-dependent ERAD is conserved, as Htm1 is required for substrate degradation in yeast (Jakob et al., 2001).

Unglycosylated luminal polypeptides are aided by luminal Hsp70s (Kar2 in yeast, BiP in mammals), which also permit disposal of the misfolded client by the Hrd1 complex if folding fails (Okuda-Shimizu and Hendershot, 2007; Plemper et al., 1997).

Folding of the cytosolic domains in membrane proteins is assisted by cytosolic Hsp70s (*e.g.* yeast Ssa1-4). These same facilitate substrate binding to the ERAD E3 ligase if the native structure is not acquired (Metzger et al., 2008; Nakatsukasa et al., 2008).

Core components of the E3 complexes also participate in client recognition. The yeast lectin Yos9, a member of the Hrd1 complex, binds to a hallmark (α1,6-linked mannose) in the misfolded glycoprotein (Bhamidipati et al., 2005; Kim et al., 2005; Quan et al., 2008; Szathmary et al., 2005); in parallel Hrd3, another component, selects the substrates before tethering them to the ubiquitination machinery (Denic et al., 2006; Gardner et al., 2000). Finally, some ERAD-M substrates can be recognized through the transmembrane domain of the Hrd1 ubiquitin ligase itself (Sato et al., 2009).

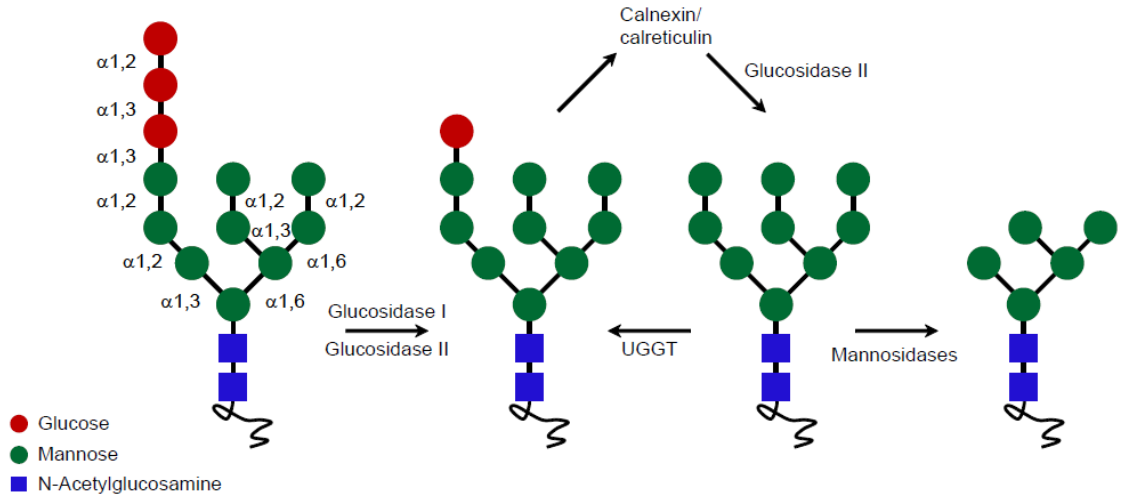

Figure 4: Glycan-trimming enzymes and glycan-dependent folding. The first and second glucose residues of the glycan are trimmed by glucosidase I and II, respectively. The mono-glucosylated peptide is bound by calnexin (CNX) and calreticulin (CRT) for folding. After release from CNX/CRT, the last glucose is removed by glucosidase II. Re-glucosylation by UGGT dictates re-entry in the CNX/CRT cycle. Extensive mannose trimming by mannosidases prevents further folding attempts and diverts the substrate to ERAD.

- Orphaned subunits of protein complexes

Plasma membrane protein complexes form in the ER and then traverse the secretory pathway. However, assembly efficiency is inherently low, thereby reducing cell surface expression of mature complexes to 10-40% of the overall synthesized pool. Unassembled subunits are retained in the ER and disposed by ERAD. Notable examples are the TCRα and CD3δ subunit of the octameric T cell receptor (TCR) (Yang et al., 1998); subunits of the pentameric nicotinic receptor (Christianson and Green, 2004); β2 microglobulin-orphaned major histocompatibility complex (MHC)-I heavy chain (Hughes et al., 1997).
While degradation ultimately relies on the canonical ERAD components, it is difficult to depict a general mechanism for recognition of the unassembled subunits. However, as anticipated, exposed hydrophobic stretches are a hallmark of incompletely assembled complexes. An example is provided by TCRα, a single-pass transmembrane subunit of the TCR, whose biogenesis is well-characterized (Kearse et al., 1995). When stoichiometric amounts of its binding partners (CD3δ and CD3ε) are not available, the unassembled TCRα is not retained in the membrane and transiently enters the lumen. It is consequently engaged by the luminal chaperone Bip, which commits TCRα to ERAD (Feige and Hendershot, 2013). It is unclear whether a similar mechanism applies to other orphaned subunits.

- Regulated substrates

The ER membrane enzyme 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR) is rate-limiting in the biosynthesis of sterols and other isoprenoids (*i.e.* the mevalonate pathway, Figure 5). Early studies demonstrated that the end product cholesterol turns off HMGR gene transcription, thus blocking further sterol synthesis (Brown and Goldstein, 2009).
Sterols also trigger HMGR rapid proteasome-dependent degradation (Ravid et al., 2000), thereby adding another crucial feedback mechanism to control overall sterol production. Similarly, yeast Hmg2, one of the two HMGR

homologs, is degraded by the proteasome in response to metabolite flux through the mevalonate pathway (Hampton et al., 1996; Hampton and Rine, 1994). Studies in mammalian cells and yeast demonstrated that HMGR/Hmg2 is ubiquitinated by an ERAD ubiquitin ligase prior to proteasomal degradation (Bordallo et al., 1998; Song et al., 2005b). It became clear that disposal of misfolded proteins was not the only function of the ERAD pathway, and the concept of regulated degradation of functional proteins in response to certain stimuli emerged.

As anticipated, engagement of HMGR/Hmg2 in ERAD depends on a metabolite signal, which reflects the pathway activity. In mammalian cells, buildup of lanosterol and its derivative 24,25-dihydrolanosterol favors substrate binding to the adaptor protein Insig-1, which bridges the interaction with the ubiquitin ligase gp78 (Song et al., 2005a; Song et al., 2005b). However, HMGR degradation can also depend on different ubiquitin ligases (Jo et al., 2011; Tsai et al., 2012). Conditional interaction between HMGR and Insig-1 occurs by virtue of a transmembrane region known as sterol-sensing domain (SSD) in the substrate (Sever et al., 2003b). Intriguingly, an early non-sterol metabolite (geranylgeraniol, a geranylgeranyl pyrophosphate derivative, Figure 5) also promotes HMGR degradation at a post-ubiquitination step (Elsabrouty et al., 2013; Sever et al., 2003a). HMGR also interacts with the membrane protein UBIAD1, a prenyltransferase, when its degradation is stimulated by sterols; geranylgeraniol disrupts this interaction (Schumacher et al., 2015). It has been postulated that the interaction HMGR-UBIAD1 protects HMGR and delays its degradation at the retrotranslocation step (Morris et al., 2014; Schumacher et al., 2015).

Stability of yeast Hmg2 is mainly regulated by an early isoprenoid (geranylgeranyl pyrophosphate, GGPP), which builds up in anaerobiosis (Garza et al., 2009). Under this condition, the oxygen-consuming sterol synthesis is blocked and the flux through the pathway sensibly declines. When sterol biosynthesis is active, lanosterol favors Hmg2 association with the Insig homolog Nsg1, a condition that protects Hmg2 from degradation (Flury et al., 2005); conversely, low lanosterol causes dissociation. The combinatorial effect of low lanosterol and high GGPP stimulates Hmg2 degradation (Theesfeld and

Hampton, 2013). Ubiquitination is executed by the gp78 homolog Hrd1, which was initially identified in the "Hmg-CoA reductase degradation" (HRD) screening (Hampton et al., 1996). As HMGR, Hmg2 possesses a SSD that is required for regulated degradation (Theesfeld et al., 2011). It has been proposed that a signal-mediated structural change in the SSD might impart sufficient conformational instability for recognition by the Hrd1 complex (Shearer and Hampton, 2005).

ERAD contribution to feedback inhibition of sterol synthesis illustrates how regulated degradation by ERAD ubiquitin ligases can impact cellular homeostasis. While HMGR/Hmg2 remains so far the model regulated substrate, regulated degradation through ERAD is likely to have a more prominent role. More effort is being put towards the identification of endogenous (*i.e.* non-misfolded) substrates.

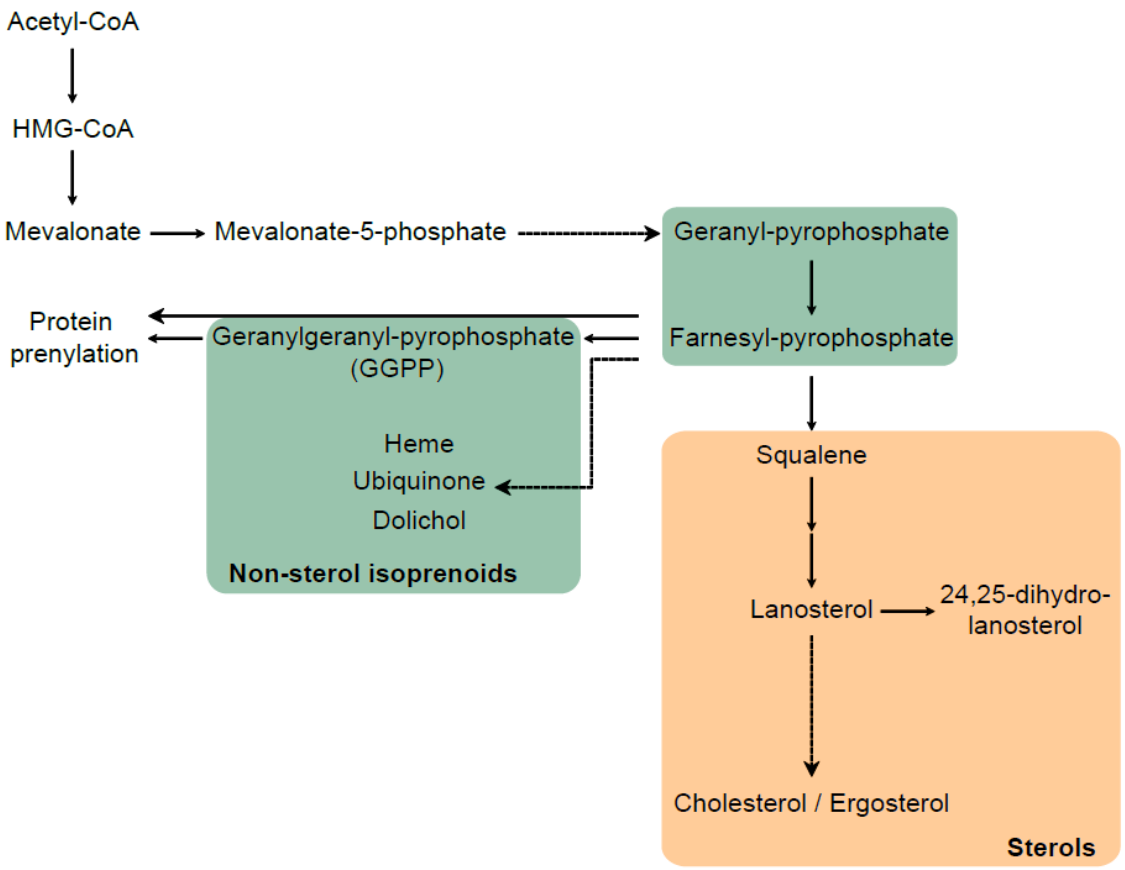

Figure 5: The mevalonate pathway. Discontinuous arrows indicate multiple reactions.

- Viral hijacking

Certain viruses hijack the ERAD system as a mean to evade immune surveillance. In many instances, a viral protein facilitates disposal of an immune system protein, thereby preventing it from reaching the cell surface and be functional. A notable example is clearance of MHC-I, which is induced by the cytomegalovirus membrane glycoproteins US2 and US11. Despite similarities in the mode of action, US2 and US11 exploit different ERAD components to ultimately trigger MHC-I degradation. Studies on these viral proteins have been instrumental in defining the mammalian ERAD pathway. US2 engages MHC-I is a complex comprising the signal peptide peptidase, SPP, and the ER ubiquitin ligase Trc8 (Loureiro et al., 2006; Stagg et al., 2009). Conversely, Us11-mediated degradation requires the ERAD components Derlin-1 and Sel1, the p97 ATPase complex and its membrane adaptor VIMP (Lilley and Ploegh, 2004; Mueller et al., 2006; Ye et al., 2005; Ye et al., 2004), and the recently identified ubiquitin ligase TMEM129 (van de Weijer et al., 2014; van den Boomen et al., 2014).

Similarly to US2 and US11, the HIV1 protein Vpu depletes the CD4 receptor by promoting its ubiquitination by an ubiquitin ligase of the SCF family, its p97-dependent membrane extraction and proteasomal degradation (Fujita et al., 1997; Magadan et al., 2010; Schubert et al., 1998).

Overall, manipulation of immune system protein levels appears to be a common mechanism operated by viruses to avoid an effective immune response and establish chronic infections.

On a different level, polyomaviruses (*e.g.* SV40) use the ERAD machinery to facilitate their replication strategies. After entering the cell, they travel in endocytic vesicles to the ER, where the reducing environment allows remodeling of the capsid proteins; the partially uncoated particle co-opts the retrotranslocation machinery to reach the cytosol; here the capsid proteins undergo further rearrangements before the particle associates to the nuclear pore and releases the viral DNA. Interestingly, different polyomaviruses use different Derlins for retrotranslocation (Lilley et al., 2006; Schelhaas et al., 2007); however, it remains unclear how they avoid proteasomal degradation.

b) Substrate retrotranslocation and degradation

ERAD clients must exit the ER in order to be accessible to the proteasome. This event, commonly referred to as retrotranslocation, has been postulated to occur through a protein conducting channel (in analogy to the translocation of nascent chains through the Sec61 complex). However, the identity of the retrotranslocation channel has been matter of debate for many years.

Pioneering studies in this direction suggested the Sec61 translocon as the export route for misfolded proteins. This notion was mainly substantiated by Sec61 interaction with MHC-I in US2-expressing mammalian cells (Wiertz et al., 1996) and with the yeast proteasome (Kalies et al., 2005). In addition, functional studies employing yeast *sec61* mutants showed hampered degradation of model ERAD substrates in conditions where protein import was not impaired (Pilon et al., 1997; Plemper et al., 1997). However, evidences for a role of the Sec61 complex in retrograde transport are not conclusive.

Multispanning membrane components of the ubiquitin ligase complexes have also emerged as good candidates for forming or being part of a retrotranslocation channel. These include the Derlins (Der1 in yeast) and the ubiquitin ligases themselves. Site-specific crosslinking experiments in yeast demonstrated the interaction between a model ERAD substrate (CPY*) and Der1 (Carvalho et al., 2010; Mehnert et al., 2014), even with its transmembrane domain. Thus, Der1 has been proposed to initiate the insertion of the substrate into the membrane, before handling it to Hrd1 for ubiquitination (Mehnert et al., 2014). The conduit for the ultimate passage might consist of Der1 in combination with other proteins. Hrd1 itself is a strong candidate, as substrate crosslinking to the ubiquitin ligase has also been reported to likely happen in the membrane bilayer (Carvalho et al., 2010). Remarkably, substrate ubiquitination and release could be recapitulated in an *in vitro* system where Hrd1 was the solely membrane component (Stein et al., 2014).

The multispanning ubiquitin ligase Doa10 has been speculated to contribute to the formation of a conducting channel for its membrane clients (Kreft et al., 2006), but this hypothesis has remained untested so far.

While much still remains to be understood regarding retrotranslocation, the sequence of later events at the cytoplasmic side is clearer. Luminal substrates are ubiquitinated as they emerge in the cytoplasm, whereas ubiquitination of membrane substrates can happen before retrotranslocation. After ubiquitination, all substrates require the homoexameric ATPase p97 (Cdc48 in yeast) in complex with two cofactors in order to finalize their membrane extraction (Bays et al., 2001b; Ye et al., 2001, 2003). Recruitment of the complex to the ER requires the UBX domain-containing protein VIMP, or UBXD8 (Ubx2 in yeast) (Neuber et al., 2005; Schuberth and Buchberger, 2005; Ye et al., 2004). Beyond the p97/Cdc48 complex, the ATPase subunits of the 19S proteasome can provide the driving force for releasing the substrate from the membrane (Lipson et al., 2008). The Cdc48 complex interacts with other ubiquitin-binding proteins, *e.g*. de-ubiquitinating enzymes (DUBs) (Ernst et al., 2009; Rumpf and Jentsch, 2006). Removal of ubiquitin chains by DUBs (*e.g*. proteasomal DUBs) is essential to allow channeling of the substrate into the proteolytic chamber of the proteasome (Verma et al., 2002; Yao and Cohen, 2002). However, DUBs are likely to have also a more direct role in extraction; this is illustrated by the dominant-negative effect on retrotranslocation of inactive DUB variants (Ernst et al., 2009; Wang et al., 2006). Interestingly, DUBs also antagonize retrotranslocation of non-canonical, non-ubiquitinated substrates, suggesting that they could act on ubiquitinated ERAD components rather than on substrates (Bernardi et al., 2013). In Vpu-mediated degradation of CD4, DUBs were shown to counteract the E3 activity and influence polyubiquitination kinetics; this provides a mechanism to enhance substrate selection (Zhang et al., 2013).
The Cdc48 complex also scaffolds the interaction with adaptor proteins that ultimately shuttle the substrate to the proteasome for degradation (Richly et al., 2005).

1.4 ER-derived organelles

The ER participates in the biogenesis of other organelles. Among these are peroxisomes, ubiquitous organelles whose main function is related to lipid

oxidation. They mostly form through growth and division of pre-existing peroxisomes (Menendez-Benito et al., 2013; Motley and Hettema, 2007); vesicular and non-vesicular transport from the ER provides membrane constituents for their growth (Agrawal et al., 2011; Lam et al., 2010; Raychaudhuri and Prinz, 2008). However, a *de novo* biogenesis route exists, where peroxisome precursors originate from the ER (Kim et al., 2006; Tam et al., 2005; van der Zand et al., 2010; van der Zand et al., 2012). However, peroxisome biogenesis is still controvertial.
Conversely, numerous evidences support the origin of lipid droplets from the ER.

a) Lipid droplets

Lipids (*e.g.* fatty acids) represent a major energy source for the cell. Excess is potentially toxic and it is converted to inert neutral lipids (NL), mainly triacylglycerol (TAG) and sterol ester (SE) species. These are stored in organelles called lipid droplets (LD), which exist in nearly all cells. NLs can be retrieved from LDs for new membrane synthesis and energy production.
In contrast to the other organelles, LD surface is delimited by a single phospholipid layer.
Several observations support the notion that LDs emerge directly from the ER. ER hosts neutral lipid synthesizing enzymes (Buhman et al., 2001; Sorger and Daum, 2003); thus, it has a primary role in LD biogenesis. Moreover, LDs are found in intimate association and sometimes connected to the ER (Blanchette-Mackie et al., 1995; Robenek et al., 2006). Nevertheless, the precise mechanism for their formation is still elusive. In the prevailing model, neutral lipids are deposited in between the two ER membrane leaflets, accumulate over a certain threshold and drive budding of a droplet from the cytosolic side of the ER (Walther and Farese, 2009). In yeast LDs stay connected to the ER, thus the LD monolayer is continuous with the ER membrane outer leaflet (Jacquier et al., 2011). This connection could facilitate exchange of lipids and proteins.
LD monolayer is decorated with proteins, which influence LD growth or shrinkage. Among these, structural proteins shield LDs and confer stability by

preventing coalescence or lipolysis (*e.g.* oleosin in plants and perilipin in animals) (Brasaemle et al., 2000; Greenberg et al., 1991; Tzen and Huang, 1992). Several lipid metabolizing enzymes also localize to the LDs; they dynamically move onto LDs depending on the metabolic state of the cell to catalyze local lipid synthesis (Krahmer et al., 2011; Kuerschner et al., 2008; Sorger and Daum, 2002; Wilfling et al., 2013).

- Sorting of proteins to lipid droplets

Proteomic analysis in yeast (Currie et al., 2014; Grillitsch et al., 2011) and in different cell lines (Bartz et al., 2007; Brasaemle et al., 2004; Fujimoto et al., 2004; Krahmer et al., 2013) provided a comprehensive inventory of LD proteins.

The unique structure of LDs only favors certain protein topologies for localization on their surface. Based on several studies, at least two common targeting signals have emerged: amphipathic α-helix and hydrophobic hairpin of two α-helices which dips in and out of the phospholipid monolayer (Walther and Farese, 2012) (Figure 6). In the latter case, positively charged aminoacids in the flanking region are also necessary for targeting in one instance (Ingelmo-Torres et al., 2009). In contrast, proteins with a membrane-spanning helix and hydrophilic domains on both sides are excluded from LDs.

Amphipathic helix-containing proteins (*e.g.* Tip47, a member of the perilipin family) presumably associate to the LD surface through their hydrophobic side, and are recruited post-translationally from the cytosol (Wolins et al., 2006). An exception is the hepatitis C virus non-structural (NS4B) protein, which binds the ER bilayer before segregating onto the LD monolayer (Tanaka et al., 2013).

It is not entirely clear how amphipathic helix-containing proteins distinguish LDs from other organelles. Specificity might depend on specific surface lipids. For instance, CTP:phospho-choline cytidylyltransferase (CCT), a rate-limiting enzyme in phosphatidylcholine (PC) synthesis, is recruited to LD membranes with low PC content (high surface tension) for local PC production (Krahmer et al., 2011).

Hydrophobic hairpin-containing proteins (*e.g.* plant oleosin) were shown in yeast and higher eukaryotes to accumulate in the ER when cells are devoid of LDs (Jacquier et al., 2011; Sorger et al., 2004; Wilfling et al., 2013; Zehmer et al., 2009). These studies strongly suggest that LD membrane proteins insert into the ER membrane and subsequently diffuse onto LDs through contact sites between the two organelles. Reciprocally, during LD regression (lipolysis), proteins (*e.g.* those containing hydrophobic hairpins) can localize back to the ER (Jacquier et al., 2011; Zehmer et al., 2009).

Some hairpin-containing proteins have been shown to have a dual localization to ER and LDs and to dynamically exchange between the two organelles (*e.g.* some TG synthesizing enzymes) (Czabany et al., 2007; Wang and Lee, 2012; Wilfling et al., 2013; Zehmer et al., 2009). This poses the question of how protein sorting between ER and LDs is regulated. Studies in yeast indicate that targeting to LDs is independent from temperature and energy and does not require vesicular trafficking (Jacquier et al., 2011). In *Drosophila*, formation of membrane bridges between ER and LDs has been shown to depend on the Arf1/COPI machinery, which in turn allows targeting of specific LD proteins, including the lipase ATGL/*brummer* and specific TG synthesizing enzymes (Wilfling et al., 2014).

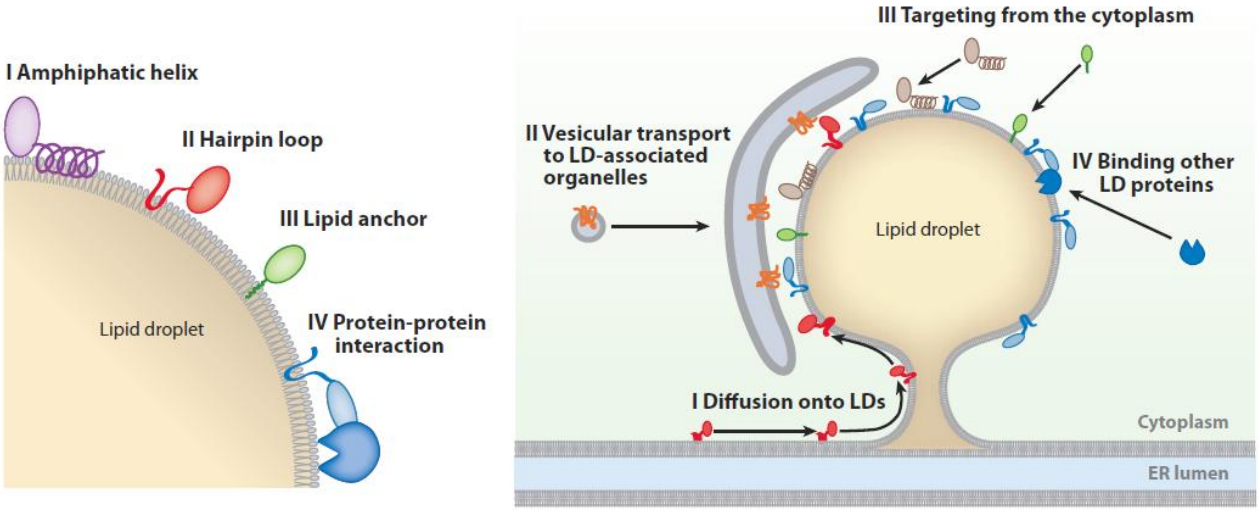

Figure 6: Protein sorting onto lipid droplets. Left, different LD targeting signals are shown; right, relative LD targeting mechanisms. Adapted from (Walther and Farese, 2012)

Despite the recent advances, overall the targeting mechanisms and how these are integrated with metabolic stimuli still need investigation.

- Degradation of lipid droplet proteins

Starvation induces engulfment of LDs in a lysosome for lipolysis (Singh et al., 2009; van Zutphen et al., 2014); during this process, named lipophagy, LD proteins undergo lysosomal degradation.

Several studies reported ubiquitination and proteasomal degradation of some LD proteins. (*e.g.* some perilipins) (Nian et al., 2010; Xu et al., 2006; Xu et al., 2005). Proteasome-dependent protein turnover at the LDs has been shown to indirectly regulate LD physiology by targeting specific modulators of neutral lipid metabolism. For example, degradation of the perilipin family protein ADRP facilitates TAG consumption and consequent LDs regression (Hooper et al., 2010).

In addition, components of the ERAD system have been localized to LDs, but their function here is independent from proteolysis and rather regulatory. One such protein is UBXD8, the p97 membrane adaptor (Ye et al., 2004). UBXD8 localizes to ER and LDs, but it is predominantly at LDs in fatty acid-loaded cells; here, UBXD8 recruits the segregase p97, which uncouples the lipase ATGL from its activator CGI-58 and in turn inhibits lipolysis (Olzmann et al., 2013).

At present no evidence is available for a direct role of ERAD in degradation of LD proteins.

2. RESULTS

Feedback regulation of Hmg2 stability through ERAD is important to control sterol synthesis and, in turn, ER homeostasis. For many years Hmg2 has been the only known ERAD regulated substrate.

In order to gain a deeper understanding on how regulated degradation through ERAD impacts ER homeostasis, a systematic screen for physiological (*i.e.* non-misfolded) substrates was needed. For this purpose we designed a proteomics screening. We took advantage of SILAC (Stable Isotope Labeling by Aminoacids in Culture) followed by quantitative mass spectrometry (de Godoy et al., 2006). We used yeast wild-type strain and strains mutated in one of the ERAD components (*doa10*Δ; *hrd1*Δ; *ubc7*Δ; *ubc6*Δ). Wild-type and mutant were differentially labeled in culture with a heavy or a light lysine isotope, respectively, and mixed before protein extraction. For nearly every protein identified by mass spectrometry a heavy/light ratio (H/L) is calculated; this ratio indicates protein abundance in mutant relative to wild-type. Based on heavy/light value (H/L<0.8), we scored a list of potential substrates for the Hrd1 and the Doa10 complexes.

We focused on the Doa10 complex and validated some of the substrates as *bona fide* clients. These proteins have diverse biological functions. It emerged that their degradation follows substantially different conditions or stimuli. The data have been collected in two independent manuscripts and are presented below.

Ombretta Foresti, Annamaria Ruggiano, Hans K Hannibal-Bach, Christer S Ejsing, Pedro Carvalho. Sterol homeostasis requires regulated degradation of squalene monooxygenase by the ubiquitin ligase Doa10/Teb4.

Elife. 2013 Jul 23;2:e00953. doi:10.7554/eLife.00953.

2.1 Erg1 engagement in ERAD

The regulated substrate Hmg2 is disposed by the ubiquitin ligase Hrd1 in response to metabolite levels in the mevalonate pathway. When lanosterol is present, Hmg2 is protected from ERAD by virtue of its interaction with the Ngs1 chaperone; when high GGPP and low lanosterol conditions display, Hmg2 uncouples from Nsg1 and is engaged in ERAD; consistently, in the absence of Nsg1 and its homolog Nsg2, Hmg2 degradation is accelerated (Theesfeld and Hampton, 2013). It has been proposed that Hmg2 sterol sensing domain (SSD) acquires features of a misfolded protein that trigger its degradation (Shearer and Hampton, 2005); Hmg2 is directly recognized through specific residues in the Hrd1 membrane domain (Sato et al., 2009).

Given that Hmg2 and Erg1 work in a common pathway and undergo a signal-dependent turnover (Foresti et al., 2013), we tested whether the Nsgs chaperones would also participate in Erg1 degradation.

We assessed Erg1 turnover in strains deleted of *NSG1*, *NSG2* or both upon blockage of protein synthesis by cycloheximide. As shown in Figure 7, Erg1 is a short-lived protein and its turnover is strongly hampered in *doa10*Δ cells, as previously shown (Foresti et al., 2013). However, we could not detect any delay or acceleration in Erg1 turnover in *nsg1*Δ, *nsg2*Δ and *nsg1,2*Δ mutants. This excluded any role for Nsg1 and Nsg2 in Erg1 degradation.

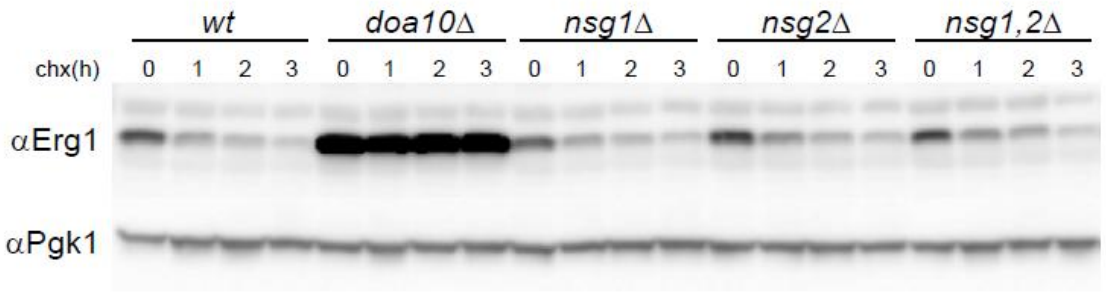

Figure 7: Degradation of Erg1 in the indicated mutant backgrounds after inhibition of protein synthesis by cycloheximide. Pgk1 is a loading control.

A similar protective role for Erg1 might be played by an unknown chaperone different from Nsg. Alternatively an adaptor might exist that mediates interaction with the ubiquitin ligase Doa10 in presence of lanosterol. In the latter case, the adaptor would have a function similar to Insig in the regulated degradation of mammalian HMGR (Song et al., 2005b). These possibilities will be explored in future studies.

Article in preparation

Spatial control of lipid droplet proteins by the ERAD ubiquitin ligase Doa10

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Introduction

The endoplasmic reticulum (ER) plays a central role in the biogenesis of membrane and secretory proteins, facilitating the folding and the posttranslational modifications necessary for their proper function. Protein folding in the ER is under the surveillance of a stringent quality control and polypeptides failing to acquire a native structure are eliminated by ER-associated degradation (or ERAD). This process involves the recognition of a substrate, its translocation from the lumen or membrane of the ER into the cytoplasm, ubiquitination and delivery to the proteasome for degradation (Ruggiano et al., 2014). These events are carried out by ER membrane-embedded protein complexes that have at their core an ubiquitin ligase. While highly conserved across eukaryotes, the mechanisms of ERAD are better characterized in the yeast *S.cerevisiae*. In yeast, genetic and biochemical studies identified three ubiquitin ligase complexes involved in ERAD, the Hrd1, Doa10 and Asi complexes, showing different specificity for misfolded proteins (Bays et al., 2001a; Foresti et al., 2014; Swanson et al., 2001). Besides misfolded proteins, both Hrd1 and Doa10 complexes were shown to degrade some folded, fully functional proteins but in a regulated manner, only upon a specific signal (Foresti et al., 2013; Hampton et al., 1996; Hampton and Rine, 1994). This mode of regulated degradation is important to control certain ER functions, such as sterol biosynthesis. The Asi complex localizes specifically to the inner nuclear membrane (INM), preventing the accumulation of misfolded proteins in this highly specialized ER subdomain. Moreover, the Asi complex degrades ER proteins mistargeted to the INM, suggesting that ERAD also integrates spatial cues (Foresti et al., 2014).

The ER also has a direct role in the biogenesis of other organelles, such as lipid droplets (LDs). These storage organelles consist of a core of neutral lipids, mainly triacylglycerides (TAG) and sterol esters (SE), enclosed by a phospholipid monolayer and a set of LD-specific proteins. These proteins are primarily enzymes promoting the synthesis, remodeling and consumption of lipids in LDs (Walther and Farese, 2012). Therefore the metabolic status of individual LDs is largely determined by the proteins at their surface. Typical

membrane proteins with hydrophilic domains on both sides are not favorably accommodated in the monolayer of LDs; thus, the association of proteins with the surface of these organelles is primarily mediated either by amphipathic helices or hydrophobic hairpins. Proteins with the former motif are recruited to LDs directly from the cytoplasm (Wolins et al., 2006). In contrast, proteins of the latter type are initially targeted and membrane-inserted in the ER; they subsequently diffuse on the monolayer of LDs, which in yeast and in a large fraction of mammalian LDs is continuous with the outer leaflet of the ER membrane (Jacquier et al., 2011; Zehmer et al., 2009). How, among all the ER membrane-anchored proteins, some concentrate specifically at the LD monolayer is not entirely clear. In a few cases, positively charged amino acids in flanking the membrane anchor favor their retention in LDs (Ingelmo-Torres et al., 2009); however a consensus signal or sequence has not been identified. Moreover, it is unclear why some proteins concentrate in LDs soon after their integration at the ER while others accumulate much slower. In some cases, the relative distribution of proteins between ER and LDs varies with the metabolic state of the cells. For example, in quiescent yeast cells the enzyme Dga1 localizes to LDs where it synthesizes TAG, while in actively dividing cells a prominent fraction of Dga1 localizes to the ER in an inactive state (Sorger and Daum, 2002).

In a quantitative proteomics screening for novel endogenous ERAD substrates, we identified a subset of LD proteins as specific targets of the ubiquitin ligase Doa10. The common feature of the LD proteins eliminated by ERAD is a hydrophobic membrane anchor, which is needed for their LD targeting. We show that Doa10 disposes specifically the ER pool of these proteins, and the membrane anchor in the LD protein is required for this. An implication of our results is that the signals for ERAD and LD sorting overlap. Our data reveal a role for ERAD in protein spatial control, where the Doa10 complex limits the accumulation of LD specific proteins in the ER, thereby reinforcing organelle identity.

Results and discussion

ERAD degrades LD proteins

Quantitative proteomic screenings recently performed in our lab generated a long list of potential endogenous ERAD substrates (Foresti et al., 2013). Among these, the LD-specific proteins Pgc1, Dga1 and Yeh1 were overrepresented in *doa10Δ* cells, suggesting that their levels might be controlled by ERAD. Curiously, *doa10Δ* mutants also have defects in LD morphology (Fei et al., 2009), strengthening a potential connection between ERAD and LD regulation. The levels of other LD proteins, such as Erg6, Pet10 or Hfd1, were unaffected in *doa10Δ* cells, as detected by SILAC and cycloheximide chase experiments (data not shown). Here, we characterize the Doa10-mediated degradation of Pgc1. To directly assess the role of Doa10 in controlling the levels of Pgc1, we performed cycloheximide chase experiments. In *wild-type* cells, Pgc1 was short-lived with a half-life of ~45 minutes (Figure 1A). In agreement with the proteomics data, its degradation was significantly delayed in cells lacking the ubiquitin ligase Doa10 or its binding partners Ubc6 and Ubc7 but was not affected in *hrd1Δ* cells, lacking another ERAD ubiquitin ligase (Figure 1A). In fact, deletion of all three ERAD ubiquitin ligases in *doa10Δhrd1Δasi1Δ* cells did not lead to further stabilization of Pgc1 (Figure S2A, C), indicating that it is a specific substrate of the Doa10 complex. Similar results were obtained for the LD proteins Dga1 and Yeh1, also identified in the SILAC dataset (Figure S1A-C and S2B, C). Mutants with impaired proteasomal function, such as *pre2* cells, showed delayed elimination of Pgc1, indicating that the degradation is proteasome-dependent (Figure 1B). In contrast, Pgc1 turnover was unaffected in cells lacking the vacuolar protease Pep4 (Figure S2A, C). Altogether, these data show that Pgc1, Dga1 and Yeh1 are *bona fide* ERAD substrates of the Doa10 complex.

ERAD of membrane-bound substrates requires the cytoplasmic Cdc48 ATPase complex, which drives the final release of substrates into the cytoplasm for proteasomal degradation (Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). Surprisingly, temperature-sensitive mutations in

Cdc48 or in its binding partner Npl4 did not detectably affect the kinetics of degradation of Pgc1 (Figure 1C), attached to the ER by a membrane anchor (see below). The Doa10 substrate Erg1 was stabilized in the same cells, confirming efficient inactivation of c*dc48* and *npl4* alleles (Foresti et al., 2013). Similar results were obtained with Dga1 (Figure S1E), attached to the membrane by a hydrophobic hairpin (Jacquier et al., 2011). These results suggest that Pgc1 and Dga1 are released from the membrane in a Cdc48-independent manner, perhaps with the aid of the proteasomal ATPases of the 19S regulatory particle, shown to facilitate the membrane extraction of some substrates (Lipson et al., 2008).

Pgc1 localizes to LDs and behaves as an integral membrane protein

Pgc1 has been predicted to associate with membranes through a C-terminal hydrophobic anchor and in recent proteomic analysis was identified as a high confidence LD protein (Currie et al., 2014). Indeed, when endogenously expressed as an N-terminal GFP fusion, GFP-Pgc1 localized to LDs both in *wt* and *doa10Δ* cells (Figure 2A). Next, we analyzed the role of the C-terminal hydrophobic region in Pgc1 membrane association. Upon subcellular fractionation, endogenously expressed Pgc1 bearing an N-terminal HA epitope was found in the microsomal fraction (Figure 2B, mock). Importantly, the microsomal association of Pgc1 was maintained after alkaline treatment, which removes peripherally associated proteins such as Kar2, and the protein was only released upon detergent solubilisation of membranes. A similar behaviour is displayed by a truncated version encoding the last 47 amino acids of Pgc1 (3HA-GFP-Pgc1$^{275\text{-}321}$) encompassing the predicted hydrophobic region (Figure 2C). Thus, Pgc1 stably associates with membranes through its hydrophobic C-terminal region. Interestingly, this C-terminal region alone localized to LDs, albeit at lower efficiency than the full length Pgc1 (Figure 2D). In fact, in a fraction of cells, besides the LD staining, 3HA-GFP-Pgc1$^{275\text{-}321}$ also co-localized with the ER marker Sec63-Cherry. These data suggest that, like Dga1 and many

other LD proteins, Pgc1 is first targeted to the ER and subsequently concentrates on LDs. Moreover, the localization to both organelles depends on the C-terminal region of Pgc1.

Pgc1 is degraded by Doa10 at the ER

Next, we analyzed the localization of Pgc1 in cells lacking LDs, such as the *are1Δare2Δlro1Δdga1Δ* mutant deficient in neutral lipid synthesis (Oelkers et al., 2002; Sandager et al., 2002). In this mutant ("no LDs") Pgc1 perfectly overlapped with the ER marker Sec63-Cherry (Figure 3A), in agreement with the idea that Pgc1 is targeted to the ER before concentrating on LDs. Given that the Doa10 complex localizes exclusively to the ER, it would be expected that restricting Pgc1 to this organelle, as in the *are1Δare2Δlro1Δdga1Δ* mutant, would result in its faster degradation. Indeed, the kinetics of Pgc1 degradation was significantly accelerated in this mutant (Figure 3B). In the absence of LDs, Pgc1 degradation was still dependent on Doa10, as the protein was stabilized by additional mutation of this ubiquitin ligase, while deletion of *HRD1* had no effect (Figure 3B). These experiments show that Doa10 promotes the degradation of the ER pool of Pgc1, either *en route* to LDs or traveling back to the ER. Moreover, they imply that LD-localized Pgc1 is spared from degradation. In agreement with the data in Figure 3, GFP-Pgc1, under constitutive expression, accumulated in the ER only in *doa10*Δ cells (Figure S3). These data reveal a function for the ERAD pathway that is distinct from its role in protein quality control or in signal-dependent degradation. We denominate this novel function as protein spatial control, since it is important to restrict the localization of Pgc1 and likely other proteins to the LD surface. Doa10-mediated spatial control of LD proteins resembles the Asi-mediated degradation of ER proteins mistargeted to the INM, hinting that ERAD contributes to the organization and the properties of different ER subdomains.

Membrane anchor is necessary and sufficient for Doa10-dependent degradation

Next we analyzed which regions of Pgc1 were involved in its spatial control by the Doa10 complex. Since the membrane anchor of Pgc1 is necessary and sufficient for the ER targeting and LD localization, we tested whether it was also important for Pgc1 degradation by ERAD. Derivatives of Pgc1 in which its membrane anchor (residues 275-321) was replaced by the one of the ER proteins Scs2 ($Pgc1^{Scs2MA}$) and Bos1 ($Pgc1^{Bos1MA}$) were generated and their localization analysed by fluorescence microscopy. Both in *wt* and *doa10Δ* cells, the two chimeric proteins labeled the ER, as determined by co-localization with Sec63-Cherry, and were completely excluded from LDs (Figure 4A). Despite their ER localization, the chimeric constructs $Pgc1^{Scs2MA}$ and $Pgc1^{Bos1MA}$ were stable, indicating that the Doa10-mediated ERAD of Pgc1 requires its membrane anchor (Figure 4B). On the other hand, 3HA-GFP-$Pgc1^{275-321}$ was extremely short lived in *wt* cells, while its turnover was strongly delayed in *doa10Δ* mutants (Figure 4C). This indicates that the membrane anchor of Pgc1, responsible for the LD localization, is necessary and sufficient for its Doa10-dependent degradation. The overlap of the signals mediating LD localization and ERAD offers the potential for regulating these competing events, for example by the metabolic status of the cells.

The hairpin is the degradation signal for Doa10

We envisioned that the aforementioned dual signal was a structural feature in Doa10 clients. A hydrophobic hairpin is a common signature in LD membrane proteins, as it was previously shown for Dga1 (Jacquier et al., 2011). This raised the possibility that the ER-localized hairpin is a Doa10 degron.

In order to investigate Pgc1 C-terminal anchor topology, we needed to characterize the orientation of its C-terminus with respect to the membrane. Thus, we appended an opsin tag containing a glycosylation site at the C-terminus of the protein (Pgc1-NKT) and checked its modification status. A

higher MW, PNGase-sensitive band was detected, indicating that some molecules did become glycosylated (Figure S4A). This might reflect two physiologically existing populations of molecules with different topologies or be the consequence of protein manipulation. On the other end, a higher percentage of Pgc1-Bos1TM chimera was glycosylated. Importantly, Pgc1-NKT was competent for LD targeting (Figure S4B). Similarly, a C-terminally flag-tagged Pgc1 was still detected at the LDs; however, the extent of Doa10-dependent degradation was dramatically reduced (Figure S4C and data not shown). While this observation is consistent with the membrane anchor being important for recognition, we could not be absolutely confident that the manipulation of the C-terminus would not interfere with the native topology as well. We bypassed this issue by replacing Pgc1 membrane anchor (aa275-321) with the hairpin from the *D.melanogaster* LD protein GPAT4 (aa160-216) (Wilfling et al., 2013). We reasoned that this chimera would have retained Doa10-dependent degradation if the degron was indeed a hairpin. The chimera localized to the LDs (Figure 5A), indicating that the targeting mechanism to LDs is conserved, as previously observed for heterologous expression of other LD proteins (Ting et al., 1997; Zehmer et al., 2008). Strikingly, it underwent fast turnover in *wild-type* cells, whereas *DOA10*, but not *HRD1*, deletion significantly hampered its degradation (Figure 5C). In the absence of LDs, PGC1-GPAT4$^{160\text{-}216}$ had an even shorter half-life, which was significantly increased upon deletion of *DOA10* but not *HRD1* (Figure 5D). Consistently with our and other previous observations, this chimera localized to the ER in the absence of LDs (Figure 5B). These data strongly suggest that the hydrophobic hairpin in LD-specific proteins becomes a Doa10 degradation signal in the ER. Moreover, they imply that degradation is independent from primary sequence and rather requires a structural feature in the mislocalized protein.

A possible explanation for these results is that hairpin-containing proteins, while being capable of adapting to the LD monolayer, do not satisfy the topological requirements for spanning the ER membrane double layer; this condition would eventually trigger Doa10-dependent degradation.

Finally, based on our data on *Dm*GPAT4 hairpin, it is tempting to speculate that Doa10-dependent degradation of LD proteins is a conserved phenomenon among species.

Recognition of LD proteins does not require cytosolic chaperones

Engagement of misfolded Doa10 clients has been shown to require the Ssa family of cytosolic chaperones (Ssa1-4) (Metzger et al., 2008; Nakatsukasa et al., 2008). We took advantage of an Ssa1 temperature sensitive mutant with *SSA2-4* deletions in order to assay the role of cytosolic chaperones in Pgc1 and Dga1 degradation.

As shown in Figure 6, either Pgc1 (A) or Dga1 (B) were degraded with nearly wild-type kinetics in the *ssa1-45* mutant at the non-permissive temperature of 37 degrees; conversely, the turnover of the membrane protein Vma12-Ndc10C', a canonical ERAD-C substrate (Furth et al., 2011), was strongly delayed.

This indicates that the SSA chaperones are dispensable for Pgc1 and Dga1 degradation. In agreement with our finding that Pgc1 membrane anchor is necessary and sufficient for degradation, it is possible that recognition of this class of clients takes place in the membrane bilayer rather than in the cytoplasm. An interesting hypothesis to test would be the direct involvement of Doa10 transmembrane domain in the recognition step.

Experimental procedures

Reagents

Rat monoclonal anti-hemagglutinin (HA) antibody (clone 3F10) was purchased from Roche and used at 1:2000 dilution; rabbit polyclonal anti-GFP and anti-Kar2 antibodies were purchased from SantaCruz Biotechnologies and used at 1:1000 dilution; anti-Pgk1 antibody was purchased from Invitrogen and used at 1:10000 dilution; anti-Flag antibody was purchased from Sigma and used at 1:2000; Rabbit polyclonal anti-Erg1 antibody was raised against the full-length protein as described in (Foresti et al., 2014). Cycloheximide (Sigma-Aldrich) was used at 250 μg/ml. PNGase F was purchased from NEB and used accordingly to the supplier specifications. Monodansyl pentane (MDH) was purchased from Abgent and used at 0.1 mM. All other reagents and chemicals were purchased from Sigma-Aldrich.

Yeast strains and growth

Yeast strains were isogenic to wild-type BY4741 (*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0*), BY4742 (*Mata ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0*) or FY251 (*Mata ura3-52 his3Δ200 leu2Δ1 trp1Δ63)*. Single or multiple deletion mutants were obtained by transformation using PCR-based homologous recombination (Longtine, 1998) or by crossing haploid cells of opposite mating types. The list of strains is available in Table1. Cells were grown in YNB medium supplemented with the appropriate aminoacids for plasmid selection.

Plasmids

A complete list of the plasmids used in this study is available in Table2. *PGC1* plasmids are derived from pPC1040 and pPC1051 *via* sub-cloning, fusion PCR or site-directed mutagenesis techniques. To generate pPC1040, *PGC1* promoter (550 bp) was amplified from BY4741 genomic DNA with primers 1515-1516; *3HA-PGC1* was amplified with its own terminator from yPC6800 genomic DNA

with primers 1517-1518. The fusion PCR product obtained with primers 1515-1518 (introducing SacI and PstI restriction site, respectively) was cloned into pRS315 between SacI and PstI sites. To generate bPC1051, *PGC1* promoter was amplified from BY4741 genomic DNA with primers 1515-2091; *GFP-PGC1* was amplified from yPC6834 genomic DNA with primers 2092-1518. The fusion PCR product obtained with primers 1515-1518 was cloned into pRS315 between SacI and PstI sites.

To generate pPC1196, *ADHpr-DGA1-GFP* was amplified from yPC7249 genomic DNA with primers 1779-185. PCR was cloned into pRS415 between SacI and XhoI sites.

To generate pPC1299, Yeh1-3HA was amplified with its own promoter from yPC9214 genomic DNA with primers 185-2148. The PCR product was cloned into pRS316 between XhoI and NotI sites.

Cycloheximide shut-off experiments

Cycloheximide shut-off experiments in exponentially growing cells ($0.8 \leq OD_{600} \leq 1$) were performed at 30 degrees, unless differently specified. Whole-cell extracts for each time-point were prepared from cell pellets in Laemmli buffer and analyzed by western blot.

Microsomes preparation

Microsomes were prepared from exponentially growing cells ($OD_{600}=1$) essentially as described in (Liu et al., 2011) and resuspended in 10mM Hepes pH7.4. For extraction of membrane proteins, equal amounts of microsomes were treated with 10mM Hepes pH7.4, or 0.2M Na_2CO_3 pH11 in water for 1h at 4 degrees or 1%SDS in 10mM Hepes for 1h at room temperature. After incubation, samples were separated into pellet and supernatant by centrifugation at 100000g. Supernatant fractions were TCA-precipitated. Pellets were resuspended in Laemmli buffer and analyzed by SDS-PAGE.

PNGase treatment

The basic protocol provided by the supplier was adapted as follows. Whole cell lysate was prepared in 50 µl from 1 OD of exponentially growing cells (OD_{600}=1) using 4x glycoprotein denaturation buffer and incubating at 65C for 10 minutes. PNGase reaction was carried out in 40 µl with 10 µl of the lysate and 2 µl of the enzyme, in presence of 1%NP40 and 1x G7 reaction buffer. The reaction mix was incubated at 37C for 1 hour. Samples were denatured with Laemmli buffer and analyzed by SDS-PAGE.

Microscopy

Fluorescence microscopy was performed at room temperature in a Zeiss Cell Observer HS with a Hamamatsu CMOS camera ORCA-Flash4.0 controlled by 3i Slidebook6.0 software. A 100x 1.40 oil immersion objective was used. GFP, mCHERRY, and MDH signals were detected using GFP, RFP and DAPI filters, respectively, with standard settings.
Cells were imaged in logarithmic growth phase.

Table 1

Strain	Genotype
yPC6800	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 NAT-TEF-3HA-Pgc1*
yPC6803	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB NAT-ADH-GFP-Pgc1*
yPC6834	*Mat? NAT-ADH-GFP-Pgc1*
yPC7014	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <3HA-PGC1, CEN, LEU>*
yPC7015	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <3HA-PGC1, CEN, LEU>*
yPC7016	*Mata ura3Δ0 his3Δ1 leu2Δ0 ubc6::KANR <3HA-PGC1, CEN, LEU>*
yPC7017	*Mata ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 ubc7::KANR <3HA-PGC1, CEN, LEU>*
yPC7018	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 hrd1::HygB <3HA-PGC1, CEN, LEU>*
yPC7019	*Mata are1::KANR are2::HYGB lro1::KANR dga1::NAT <3HA-PGC1, CEN, LEU>*
yPC7074	*Mat? are1::KANR are2::HYGB lro1::KANR dga1::NAT doa10::HIS <3HA-PGC1, CEN, LEU>*
yPC7249	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 NAT-ADHp-DGA1-GFP-HIS2*
yPC7496	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <GFP-PGC1, CEN, LEU>*
yPC7497	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <GFP-PGC1, CEN, LEU>*
yPC7589	*Mata are1::KANR are2::HYGB lro1::KANR dga1::NAT <GFP-PGC1, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC7590	*Mat? are1::KANR are2::HYGB lro1::KANR dga1::NAT doa10::HIS <GFP-PGC1, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC8115	*Mat? are1::KANR are2::HYGB lro1::KANR dga1::NAT hrd1::HIS <3HA-PGC1, CEN, LEU>*
yPC8150	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <3HA-PGC1$_{1-274}$-BOS1TM, CEN, LEU>*
yPC8151	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <3HA-PGC1$_{1-274}$-BOS1TM, CEN, LEU>*
yPC8152	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <3HA-PGC1$_{1-274}$-SCS2TM, CEN, LEU>*
yPC8153	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <3HA-PGC1$_{1-274}$-SCS2TM, CEN, LEU>*
yPC8336	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <3HA-GFP-PGC1$_{275-321}$, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC8407	*SSA1 ssa3::HIS3 ssa2::LEU2 ssa4::LYS2 his3-11,15 leu2-3,112 lys2 ura3-52 trp1-Δ1 <PRC1pr-VMA12-NDC10C'-3HA, CEN, URA><3HA-GFP-PGC1, CEN, TRP>*
yPC8408	*ssa1-45 ssa3::HIS3 ssa2::LEU2 ssa4::LYS2 his3-11,15 leu2-3,112 lys2 ura3-52 trp1-Δ1 <PRC1pr-VMA12-NDC10C'-3HA, CEN, URA><3HA-GFP-PGC1, CEN, TRP>*
yPC8412	*Mata ura3-52 his3Δ200 leu2Δ1 trp1Δ63 cdc48-3 <3HA-PGC1, CEN, LEU>*
yPC8413	*Mata ura3-52 leu2Δ1 trp1Δ63 npl4-1 <3HA-PGC1, CEN, LEU>*
yPC8620	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8621	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <ADH1pr-DGA1-GFP, CEN, LEU>*

yPC8622	*SSA1 ssa3::HIS3 ssa2::LEU2 ssa4::LYS2 his3-11,15 leu2-3,112 lys2 ura3-52 trp1-Δ1 <PRC1pr-VMA12-NDC10C'-3HA, CEN, URA><ADH1pr-DGA1-GFP, CEN, TRP>*
yPC8623	*ssa1-45 ssa3::HIS3 ssa2::LEU2 ssa4::LYS2 his3-11,15 leu2-3,112 lys2 ura3-52 trp1-Δ1 <PRC1pr-VMA12-NDC10C'-3HA, CEN, URA><ADH1pr-DGA1-GFP, CEN, TRP>*
yPC8934	*Mata ura3Δ0 his3Δ1 leu2Δ0 ubc6::KANR <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8935	*Mata ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 ubc7::KANR <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8936	*Mata hrd1::HIS <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8937	*Mata ura3-52 his3Δ200 leu2Δ1 trp1Δ63 cdc48-3 <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8938	*Mata ura3-52 leu2Δ1 trp1Δ63 npl4-1 <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8941	*Mata doa3-1 (pre2) <3HA-PGC1, CEN, LEU>*
yPC8942	*Mat? PRE2 <3HA-PGC1, CEN, LEU>*
yPC8943	*Mata doa3-1 (pre2) <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8944	*Mat? PRE2 <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8973	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <GFP- $PGC1_{1-274}$-Scs2TM, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC8974	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <GFP- $PGC1_{1-274}$-Scs2TM, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC8975	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <GFP-$PGC1_{1-274}$-Bos1TM, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC8976	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <GFP-$PGC1_{1-274}$-Bos1TM, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC8977	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <3HA-GFP-$PGC1_{275-321}$, CEN, LEU>*
yPC8978	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <3HA-GFP-$PGC1_{275-321}$, CEN, LEU>*
yPC8979	*Mata doa10::HIS hrd1::KANR asi1::NATR <3HA-GFP-$PGC1_{275-321}$, CEN, LEU>*
yPC8980	*Mat? pep4::URA3 <3HA-PGC1, CEN, LEU>*
yPC8981	*Mat? pep4::URA3 doa10::KANR <3HA-PGC1, CEN, LEU>*
yPC8982	*Mat? pep4::URA3 <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8983	*Mat? pep4::URA3 doa10::KANR <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC8984	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <3HA-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*
yPC8985	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <3HA-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*
yPC8986	*Mata hrd1::HIS <3HA-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*
yPC8987	*Mata are1::KANR are2::HYGB lro1::KANR dga1::NAT <3HA-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*
yPC8988	*Mat? are1::KANR are2::HYGB lro1::KANR dga1::NAT doa10::HIS <3HA-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*
yPC8989	*Mat? are1::KANR are2::HYGB lro1::KANR dga1::NAT hrd1::HIS <3HA-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*
yPC8990	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 <GFP-$PGC1_{1-274}$-$GPAT4_{160-216}$, CEN, LEU>*

yPC8991	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 doa10::HygB <GFP-PGC1$_{1-274}$-GPAT4$_{160-216}$, CEN, LEU>*
yPC8992	*Mat? are1::KANR are2::HYGB lro1::KANR dga1::NAT doa10::HIS <GFP-PGC1$_{1-274}$-GPAT4$_{160-216}$, CEN, LEU> <SEC63-mCherry, CEN, URA>*
yPC9005	*Mat? his3Δ1 hrd1::KANR asi1::NATR <3HA-PGC1, CEN, LEU>*
yPC9006	*Mat? his3Δ1 hrd1::KANR asi1::NATR <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC9007	*Mata doa10::HIS hrd1::KANR asi1::NATR <3HA-PGC1, CEN, LEU>*
yPC9008	*Mata doa10::HIS hrd1::KANR asi1::NATR <ADH1pr-DGA1-GFP, CEN, LEU>*
yPC9214	*Mata ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 Yeh1-3HA*

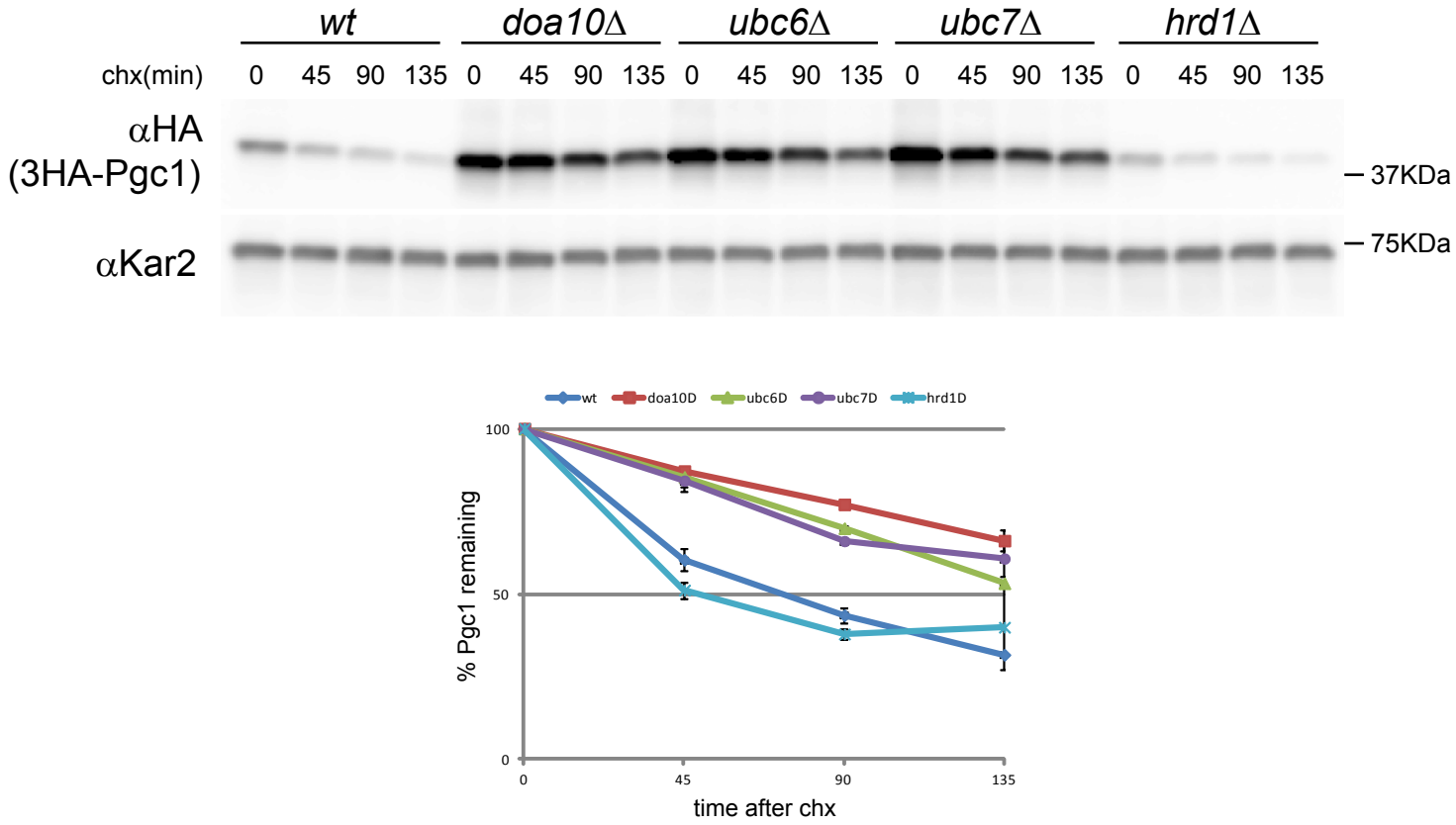
Table 2

Name	Insert/gene	vector	Reference
pPC882	Sec63-mCherry	pRS416	This study
pPC926	Vma12-Ndc10C'	pRS316	Furth *et al*, 2011
pPC1040	3HA-PGC1	pRS315	This study
pPC1051	GFP-PGC1	pRS315	This study
pPC1084	3HA-GFP-PGC1	pRS315	This study
pPC1168	3HA-PGC1$_{1-274}$-Scs2TM	pRS315	This study
pPC1169	3HA-PGC1$_{1-274}$-Bos1TM	pRS315	This study
pPC1170	3HA-GFP-PGC1$_{275-321}$	pRS315	This study
pPC1195	3HA-GFP-PGC1	pRS314	This study
pPC1196	ADH1pr-DGA1-GFP	pRS415	This study
pPC1227	ADH1pr-DGA1-GFP	pRS314	This study
pPC1270	GFP- PGC1$_{1-274}$-Scs2TM	pRS315	This study
pPC1271	GFP-PGC1$_{1-274}$-Bos1TM	pRS315	This study
pPC1272	3HA-PGC1$_{1-274}$-GPAT4$_{160-216}$	pRS315	This study
pPC1273	GFP-PGC1$_{1-274}$-GPAT4$_{160-216}$	pRS315	This study
pPC1295	3HA-GFP-PGC1-NKT	pRS315	This study
pPC1296	3HA-PGC1-NKT	pRS315	This study
pPC1297	3HA-PGC1$_{1-274}$-Bos1TM-NKT	pRS315	This study
pPC1298	3HA-PGC1-2Flag	pRS315	This study
pPC1299	Yeh1-3HA	pRS316	This study

Table 3

Number	Name	Sequence
185	Yos9R5	CTATTGTACTCGAGCGAGGCAAGCTAAACAGATC
1515	Pgc1-F2	ccagtgtccaGAGCTCCTAAGTACCCAACAGAGGTT
1516	Pgc1pr-3HA FusionRv	GAACATCGTATGGGTAACCCATCCTCGTGTCCTTGTTGTTATC
1517	Pgc1pr-3HA FusionFw	GATAACAACAAGGACACGAGGATGGGTTACCCATACGATGTTC
1518	Pgc1-R1	gcagttcagtcCTGCAGGGAGAATGGCATACACATATC
1779	ADH1 F2	CTCAGAGGACAACACCTGTTG
2091	Pgc1pr-GFP FusionRv	ATAATTCTTCACCTTTAGACATgcggccgcCCTCGTGTCCTTGTTGTTATC
2092	Pgc1pr-GFP FusionFw	GATAACAACAAGGACACGAGGgcggccgcATGTCTAAAGGTGAAGAATTAT
2148	Yeh1-F3	CAATGTCGAAAGCGGCCGCCCAATATACATTCTCAAGTGTGC

A


wt
doa10Δ
ubc6Δ
ubc7Δ
hrd1Δ
chx(min)
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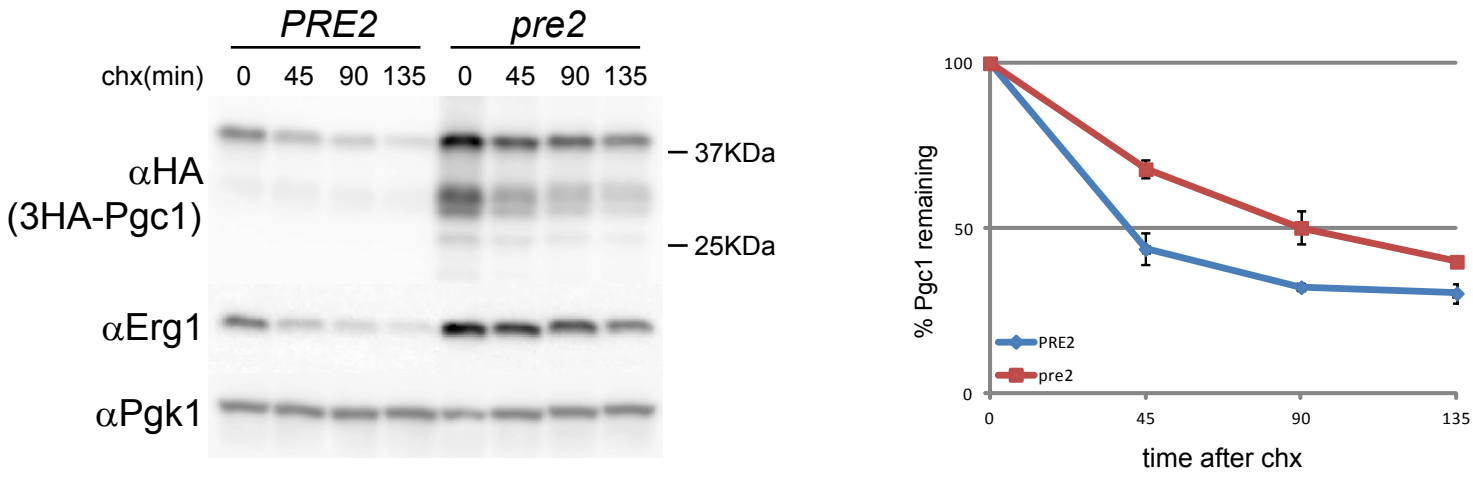
αHA
(3HA-Pgc1)
αKar2
37KDa
75KDa
wt
doa10D
ubc6D
ubc7D
hrd1D
% Pgc1 remaining
100
50
0
0
45
90
135
time after chx

B


PRE2
pre2
chx(min)
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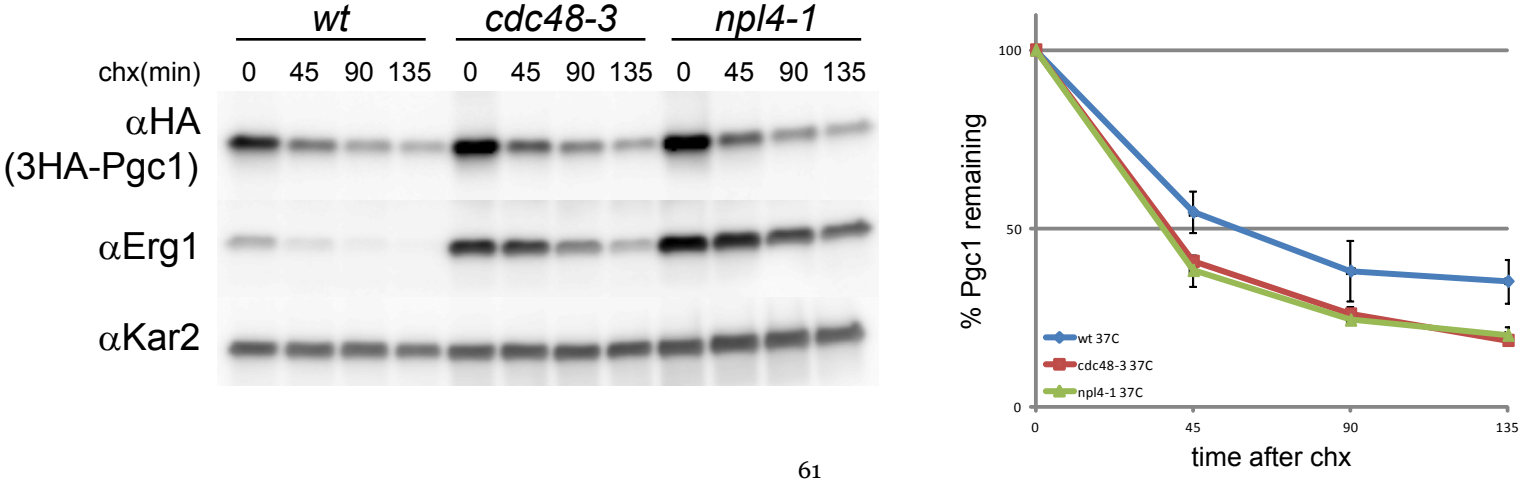
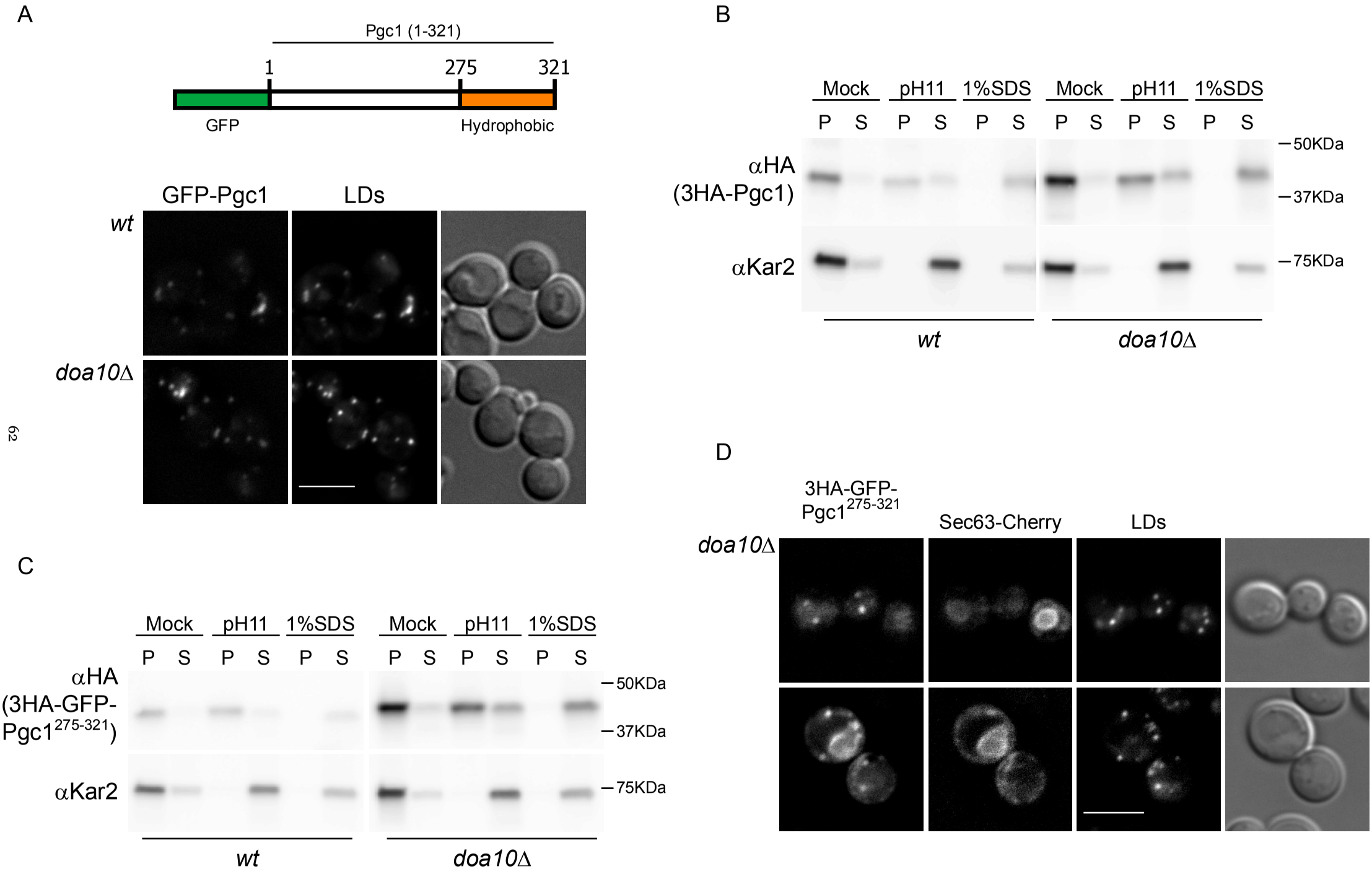
αHA
(3HA-Pgc1)
αErg1
αPgk1
37KDa
25KDa
% Pgc1 remaining
100
50
0
PRE2
pre2
0
45
90
135
time after chx

C


wt
cdc48-3
npl4-1
chx(min)
0 45 90 135 0 45 90 135 0 45 90 135
αHA
(3HA-Pgc1)
αErg1
αKar2
% Pgc1 remaining
100
50
0
wt 37C
cdc48-3 37C
npl4-1 37C
0
45
90
135
time after chx


A
Pgc1 (1-321)
1
275
321
GFP
Hydrophobic
GFP-Pgc1
LDs
wt
doa10Δ
B
Mock
pH11
1%SDS
Mock
pH11
1%SDS
P S P S P S P S P S P S
αHA
(3HA-Pgc1)
αKar2
50KDa
37KDa
75KDa
wt
doa10Δ
C
Mock
pH11
1%SDS
Mock
pH11
1%SDS
P S P S P S P S P S P S
αHA
(3HA-GFP-
Pgc1275-321)
αKar2
50KDa
37KDa
75KDa
wt
doa10Δ
D
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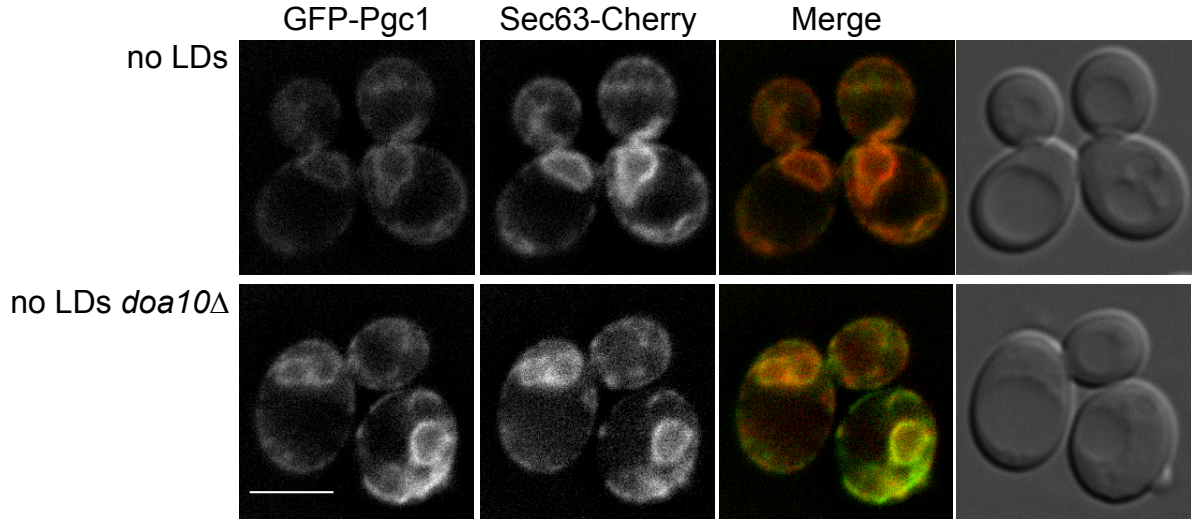
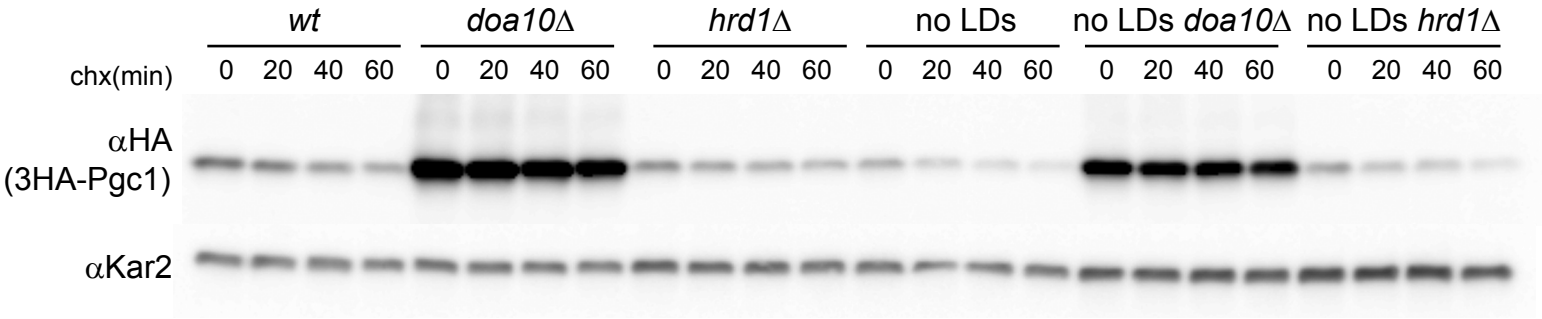
Pgc1275-321
Sec63-Cherry
LDs
doa10Δ

A


GFP-Pgc1
Sec63-Cherry
Merge
no LDs
no LDs doa10Δ

B


wt
doa10Δ
hrd1Δ
no LDs
no LDs doa10Δ
no LDs hrd1Δ
chx(min)
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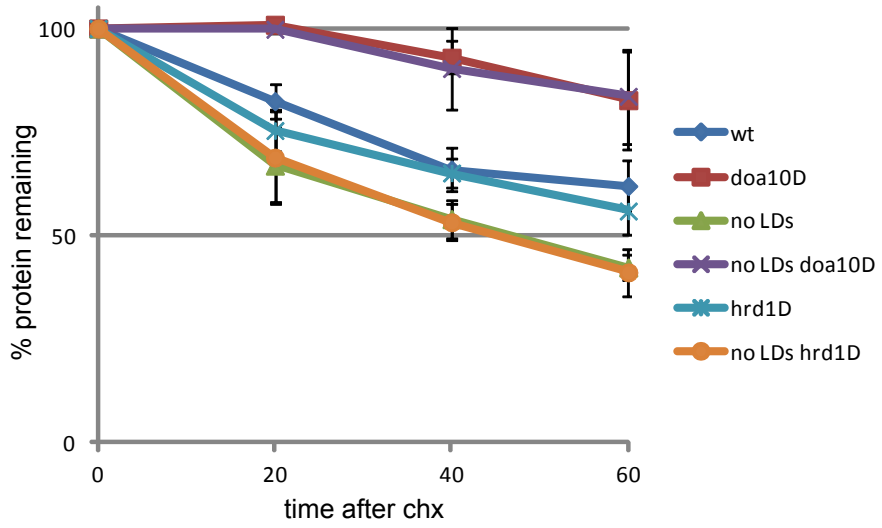
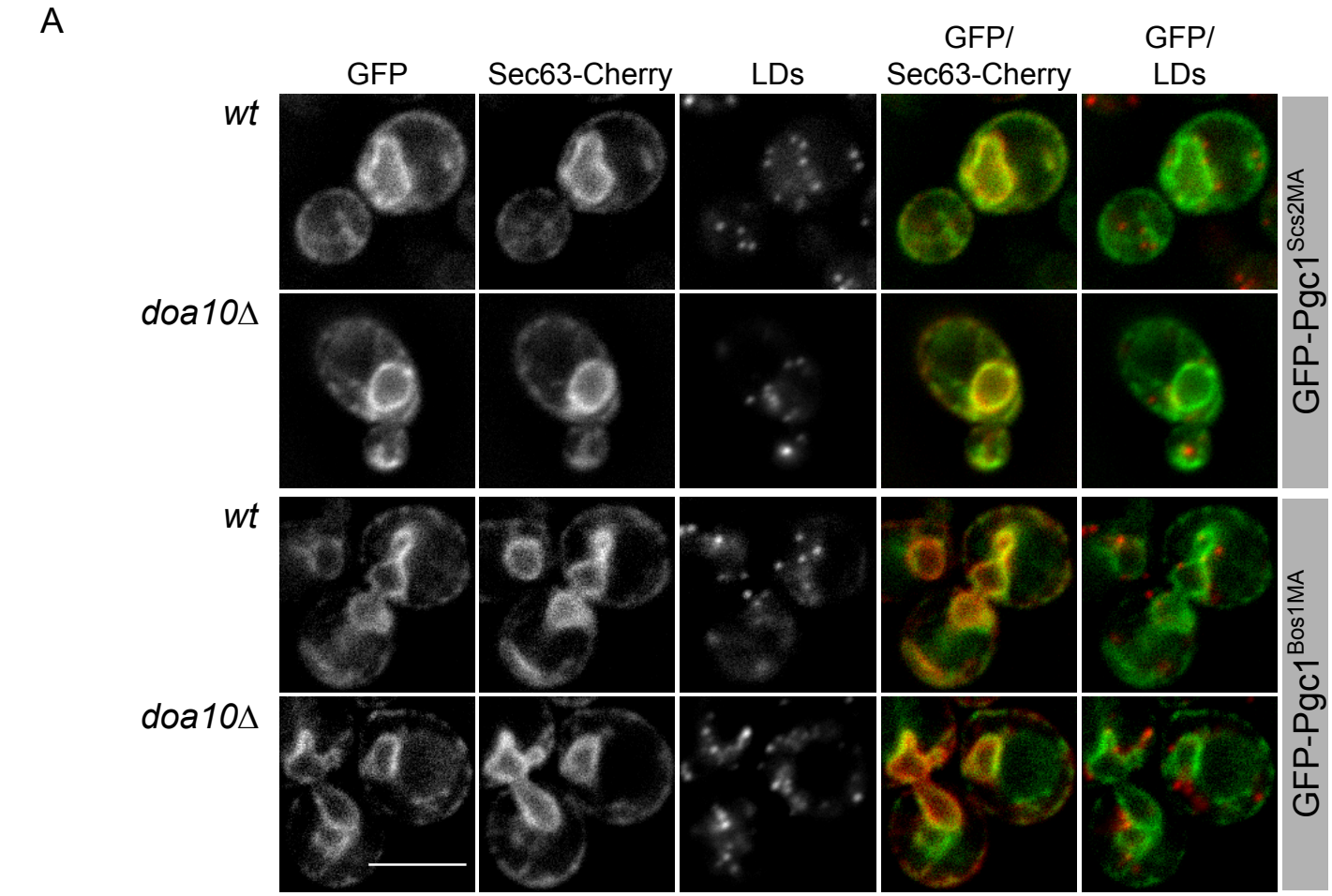
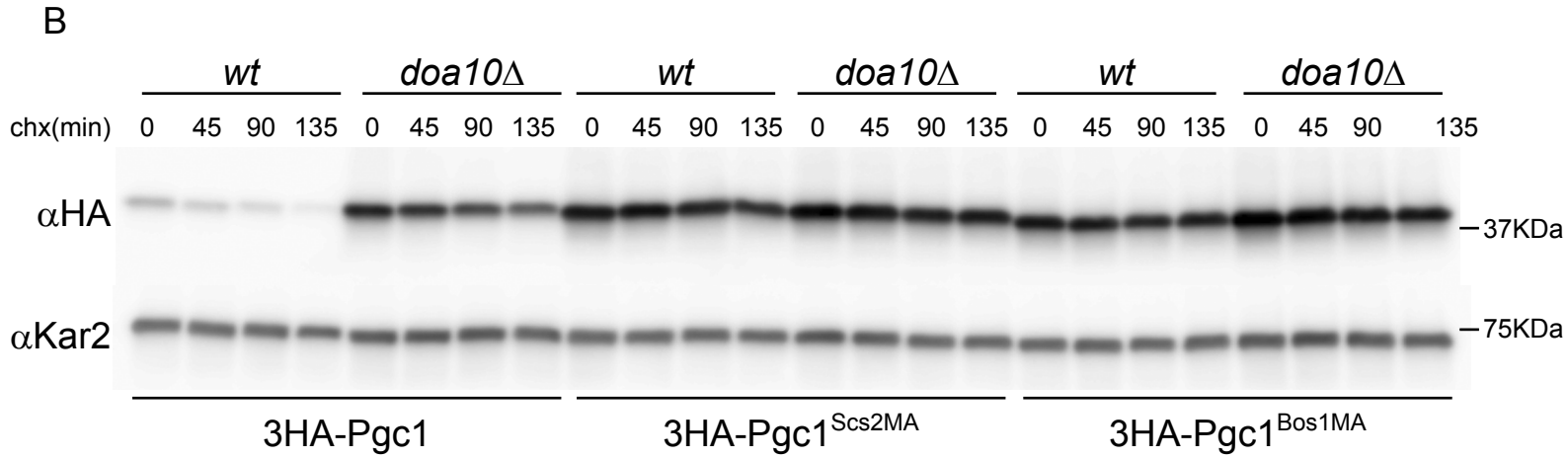
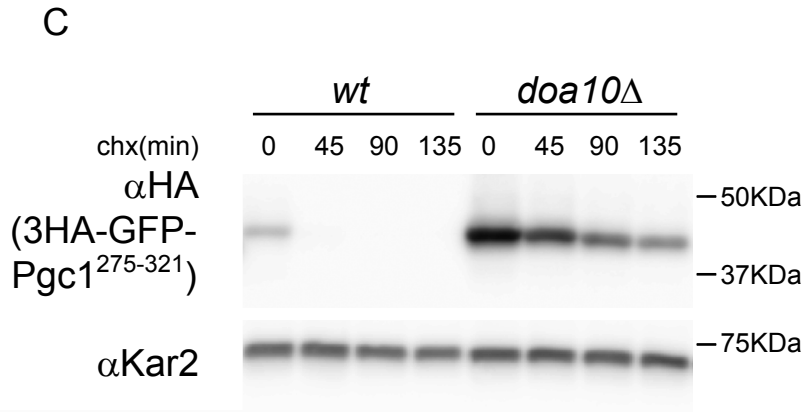
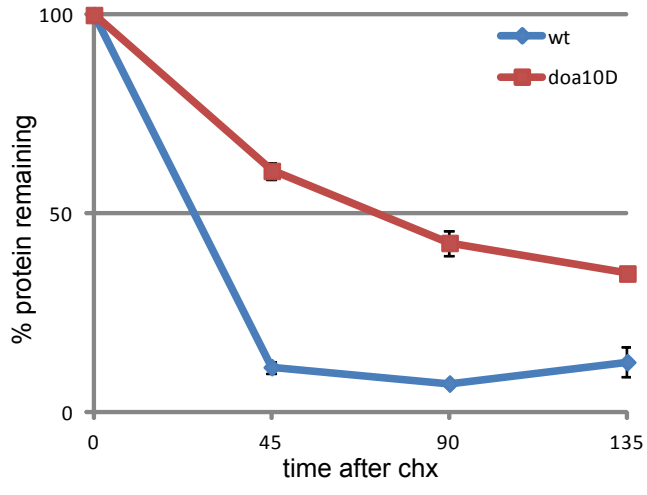
αHA
(3HA-Pgc1)
αKar2

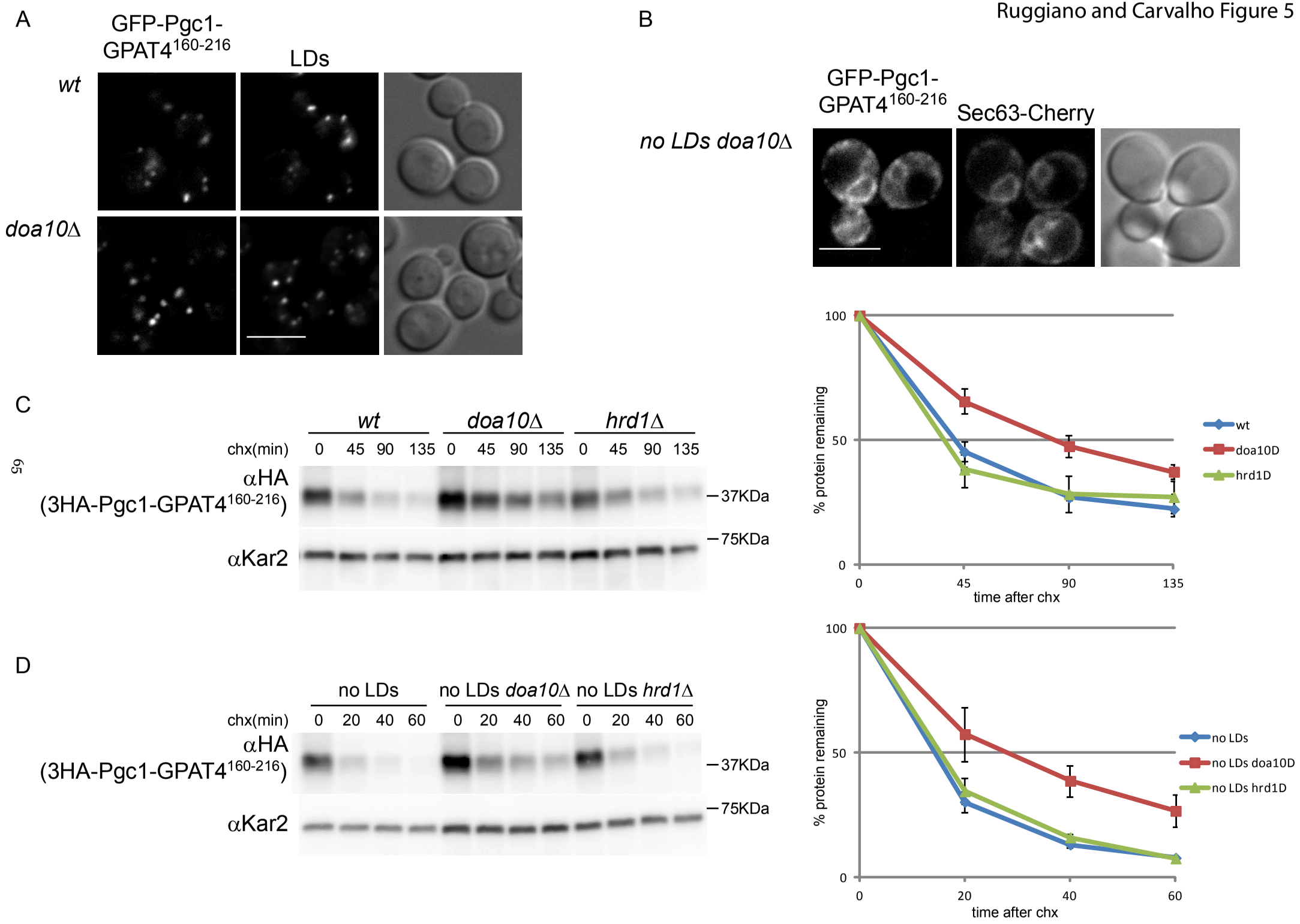

100
50
0
% protein remaining
0
20
40
60
time after chx
wt
doa10D
no LDs
no LDs doa10D
hrd1D
no LDs hrd1D


A
GFP
Sec63-Cherry
LDs
GFP/
Sec63-Cherry
GFP/
LDs
wt
doa10Δ
wt
doa10Δ
GFP-Pgc1Scs2MA
GFP-Pgc1Bos1MA

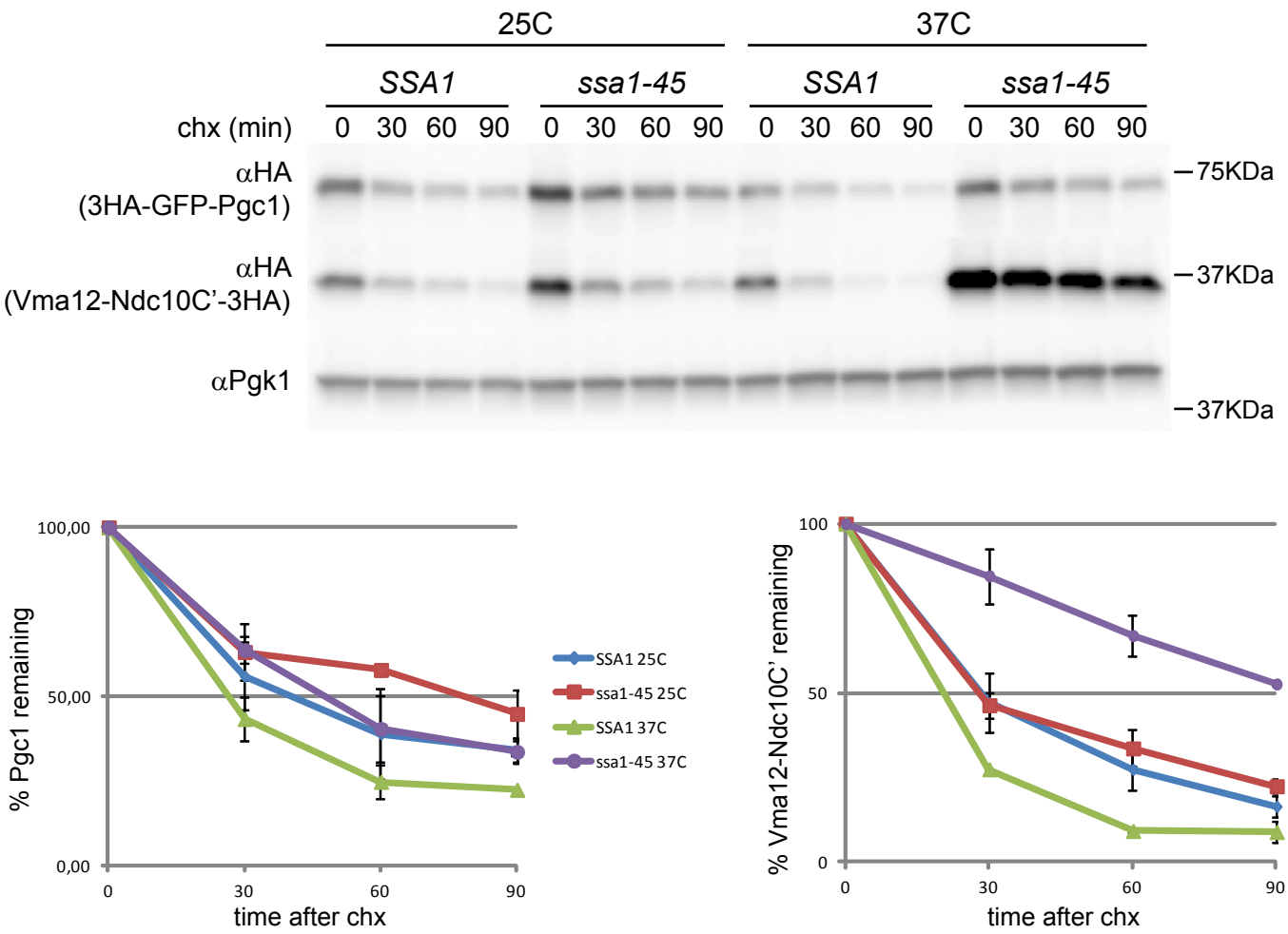

B
wt
doa10Δ
wt
doa10Δ
wt
doa10Δ
chx(min) 0 45 90 135 0 45 90 135 0 45 90 135 0 45 90 135 0 45 90 135 0 45 90 135
αHA
37KDa
αKar2
75KDa
3HA-Pgc1
3HA-Pgc1Scs2MA
3HA-Pgc1Bos1MA


C
wt
doa10Δ
chx(min) 0 45 90 135 0 45 90 135
αHA
(3HA-GFP-
Pgc1275-321)
50KDa
37KDa
αKar2
75KDa

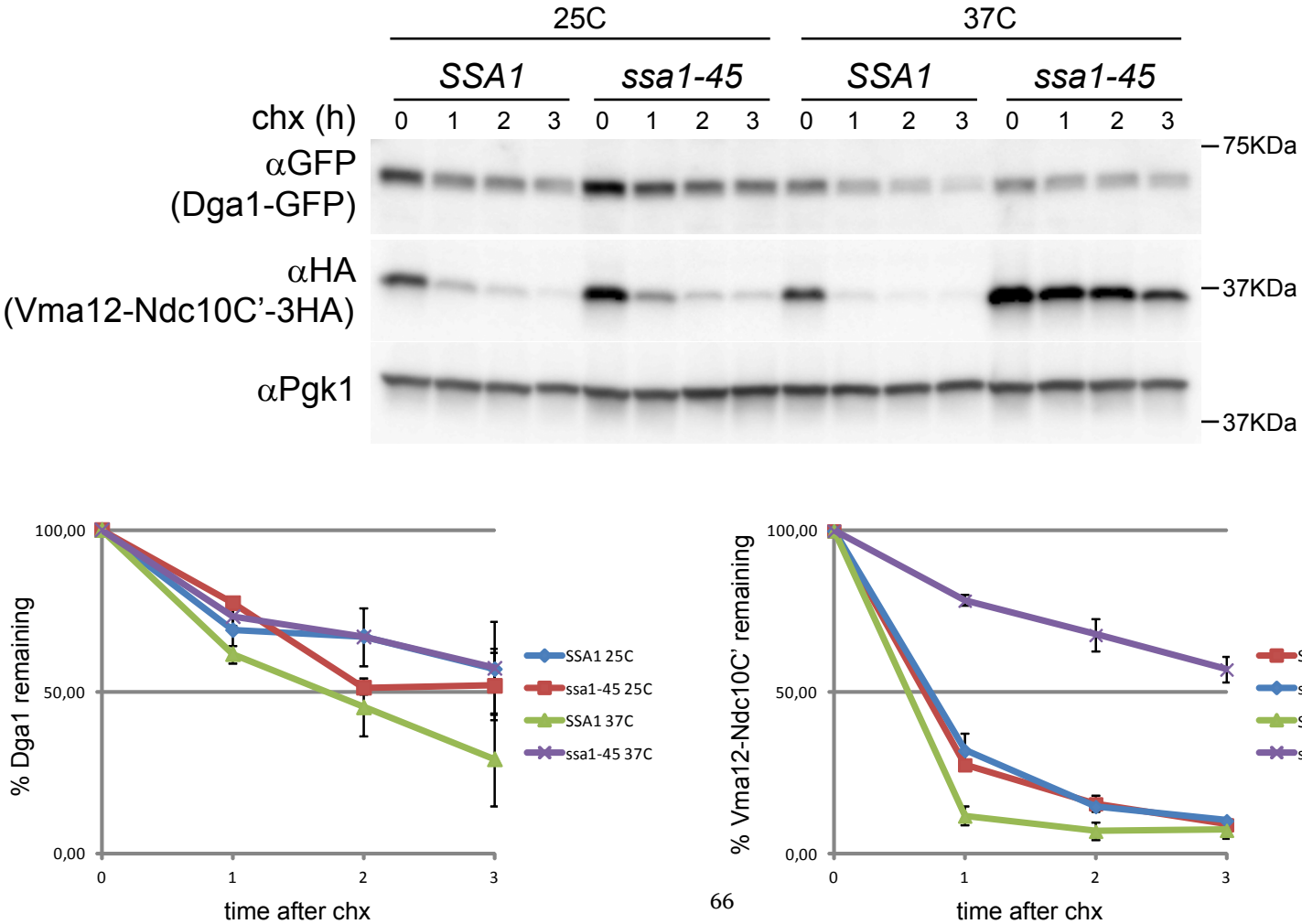

100
50
0
% protein remaining
0
45
90
135
time after chx
wt
doa10D


A
GFP-Pgc1-GPAT4160-216
LDs
wt
doa10Δ
B
GFP-Pgc1-GPAT4160-216
Sec63-Cherry
no LDs doa10Δ
C
wt
doa10Δ
hrd1Δ
chx(min)
0 45 90 135 0 45 90 135 0 45 90 135
αHA
(3HA-Pgc1-GPAT4160-216)
αKar2
37KDa
75KDa
% protein remaining
time after chx
wt
doa10D
hrd1D
D
no LDs
no LDs doa10Δ
no LDs hrd1Δ
chx(min)
0 20 40 60 0 20 40 60 0 20 40 60
αHA
(3HA-Pgc1-GPAT4160-216)
αKar2
37KDa
75KDa
% protein remaining
time after chx
no LDs
no LDs doa10D
no LDs hrd1D

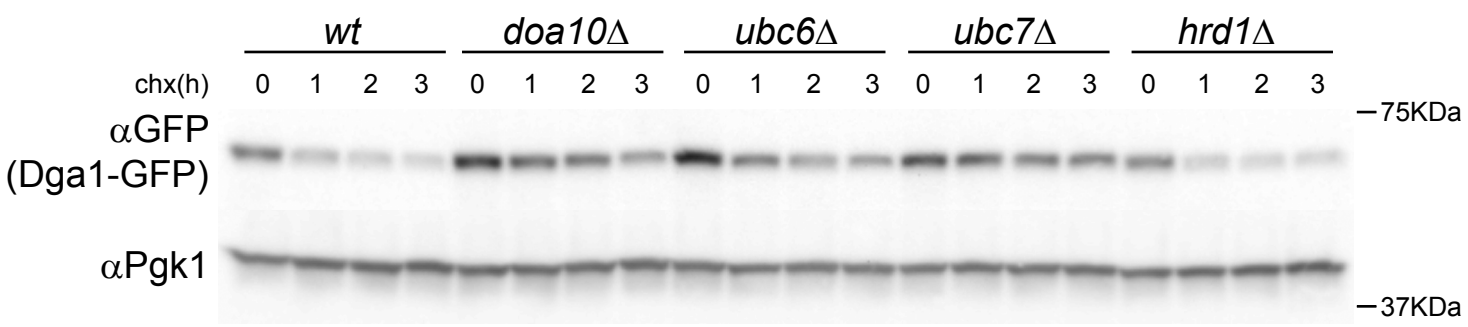
A


25C
37C
SSA1
ssa1-45
SSA1
ssa1-45
chx (min)
0 30 60 90 0 30 60 90 0 30 60 90 0 30 60 90
αHA
(3HA-GFP-Pgc1)
αHA
(Vma12-Ndc10C'-3HA)
αPgk1
75KDa
37KDa
37KDa
% Pgc1 remaining
100,00
50,00
0,00
0
30
60
90
time after chx
SSA1 25C
ssa1-45 25C
SSA1 37C
ssa1-45 37C
% Vma12-Ndc10C' remaining
100
50
0
0
30
60
90
time after chx
SSA1 25C
ssa1-45 25C
SSA1 37C
ssa1-45 37C

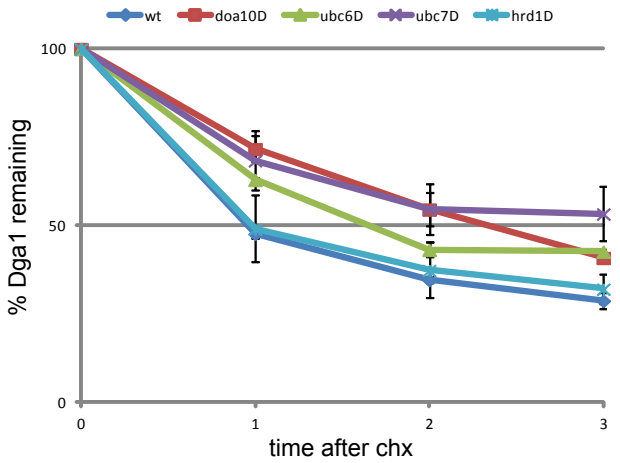
B


25C
37C
SSA1
ssa1-45
SSA1
ssa1-45
chx (h)
0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3
αGFP
(Dga1-GFP)
αHA
(Vma12-Ndc10C'-3HA)
αPgk1
75KDa
37KDa
37KDa
% Dga1 remaining
100,00
50,00
0,00
0
1
2
3
time after chx
SSA1 25C
ssa1-45 25C
SSA1 37C
ssa1-45 37C
% Vma12-Ndc10C' remaining
100,00
50,00
0,00
0
1
2
3
time after chx
SSA1 25C
ssa1-45 25C
SSA1 37C
ssa1-45 37C

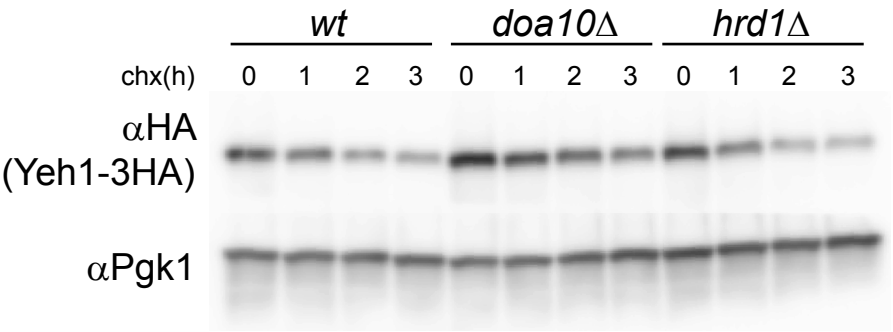
A


wt
doa10Δ
ubc6Δ
ubc7Δ
hrd1Δ
chx(h) 0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3
αGFP (Dga1-GFP)
αPgk1
75KDa
37KDa

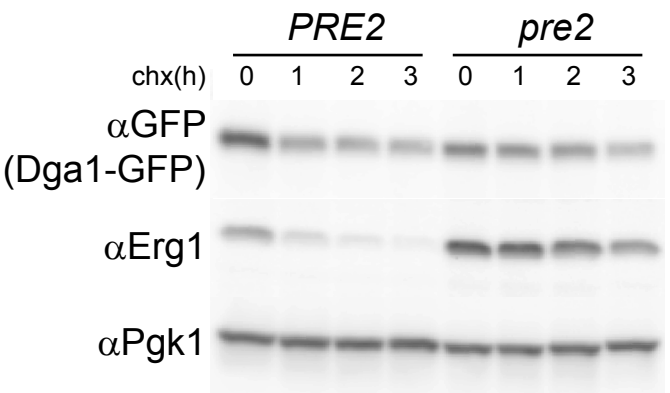
B

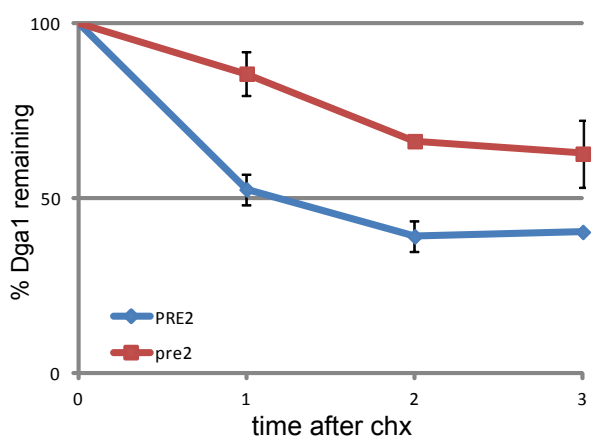

wt
doa10D
ubc6D
ubc7D
hrd1D
% Dga1 remaining
time after chx

C

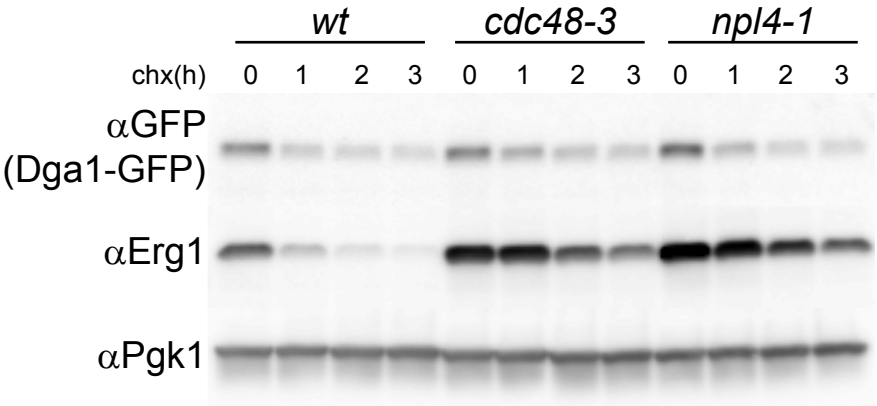

wt
doa10Δ
hrd1Δ
chx(h) 0 1 2 3 0 1 2 3 0 1 2 3
αHA (Yeh1-3HA)
αPgk1

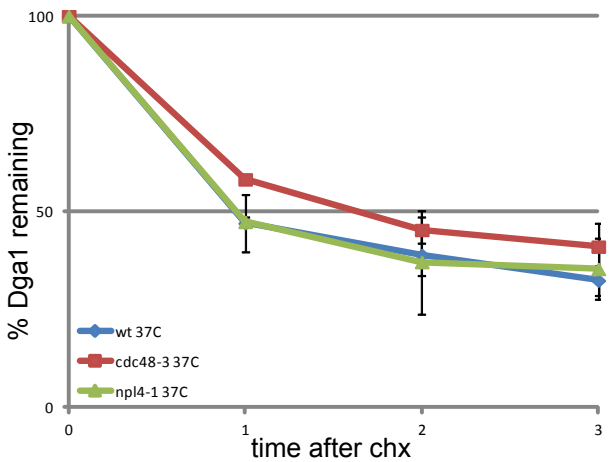
D


PRE2
pre2
chx(h) 0 1 2 3 0 1 2 3
αGFP (Dga1-GFP)
αErg1
αPgk1

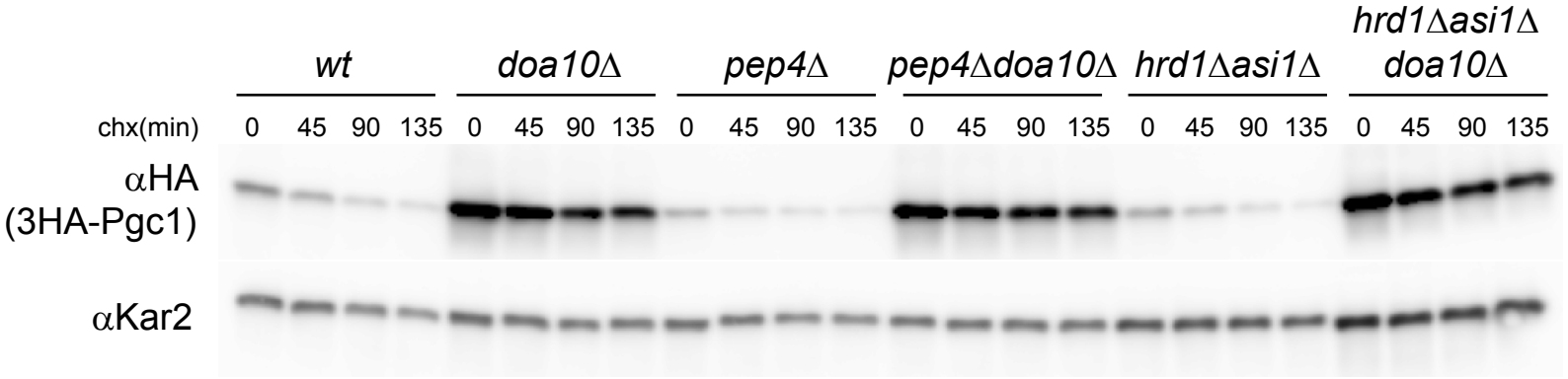

% Dga1 remaining
PRE2
pre2
time after chx

E

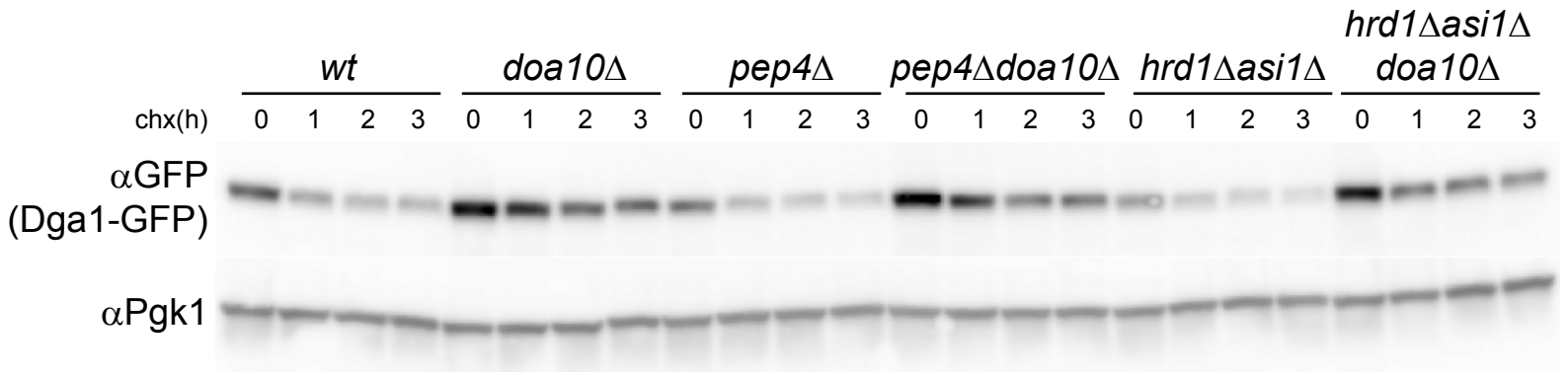

wt
cdc48-3
npl4-1
chx(h) 0 1 2 3 0 1 2 3 0 1 2 3
αGFP (Dga1-GFP)
αErg1
αPgk1


% Dga1 remaining
wt 37C
cdc48-3 37C
npl4-1 37C
time after chx

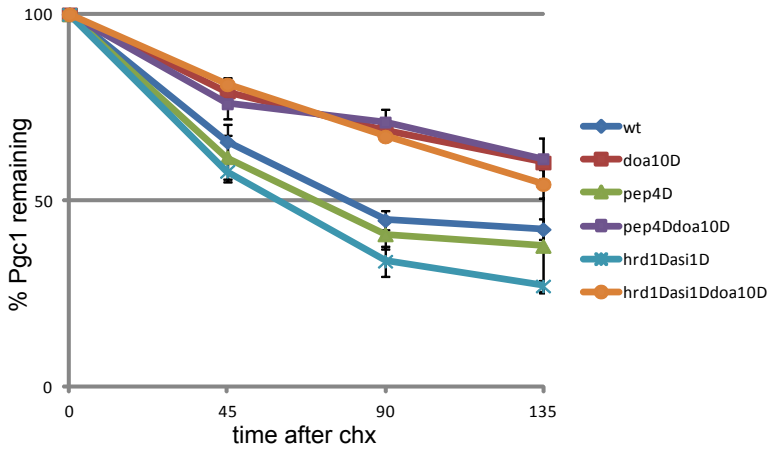
A

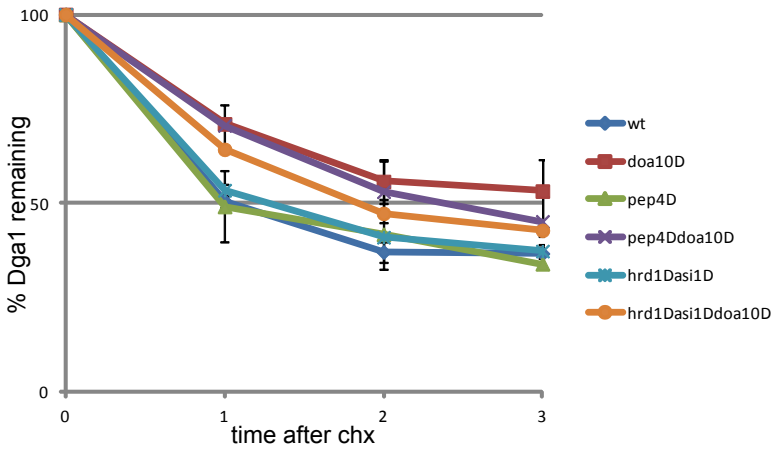

wt
doa10Δ
pep4Δ
pep4Δdoa10Δ
hrd1Δasi1Δ
hrd1Δasi1Δ doa10Δ
chx(min)
0 45 90 135 0 45 90 135 0 45 90 135 0 45 90 135 0 45 90 135 0 45 90 135
αHA (3HA-Pgc1)
αKar2

B


wt
doa10Δ
pep4Δ
pep4Δdoa10Δ
hrd1Δasi1Δ
hrd1Δasi1Δ doa10Δ
chx(h)
0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3
αGFP (Dga1-GFP)
αPgk1

C


100
50
0
% Pgc1 remaining
0
45
90
135
time after chx
wt
doa10D
pep4D
pep4Ddoa10D
hrd1Dasi1D
hrd1Dasi1Ddoa10D


100
50
0
% Dga1 remaining
0
1
2
3
time after chx
wt
doa10D
pep4D
pep4Ddoa10D
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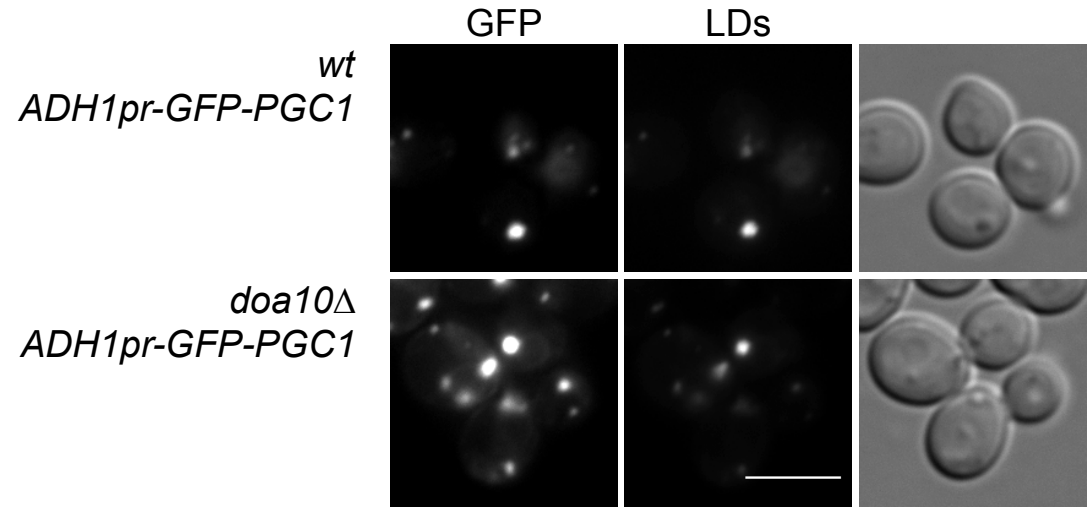
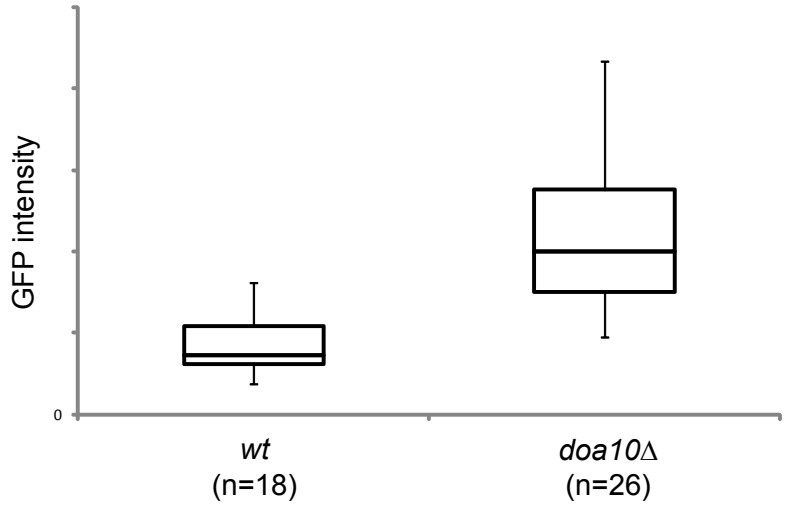
hrd1Dasi1Ddoa10D

A


GFP
LDs
wt
ADH1pr-GFP-PGC1
doa10Δ
ADH1pr-GFP-PGC1

B


GFP intensity
0
wt
(n=18)
doa10Δ
(n=26)

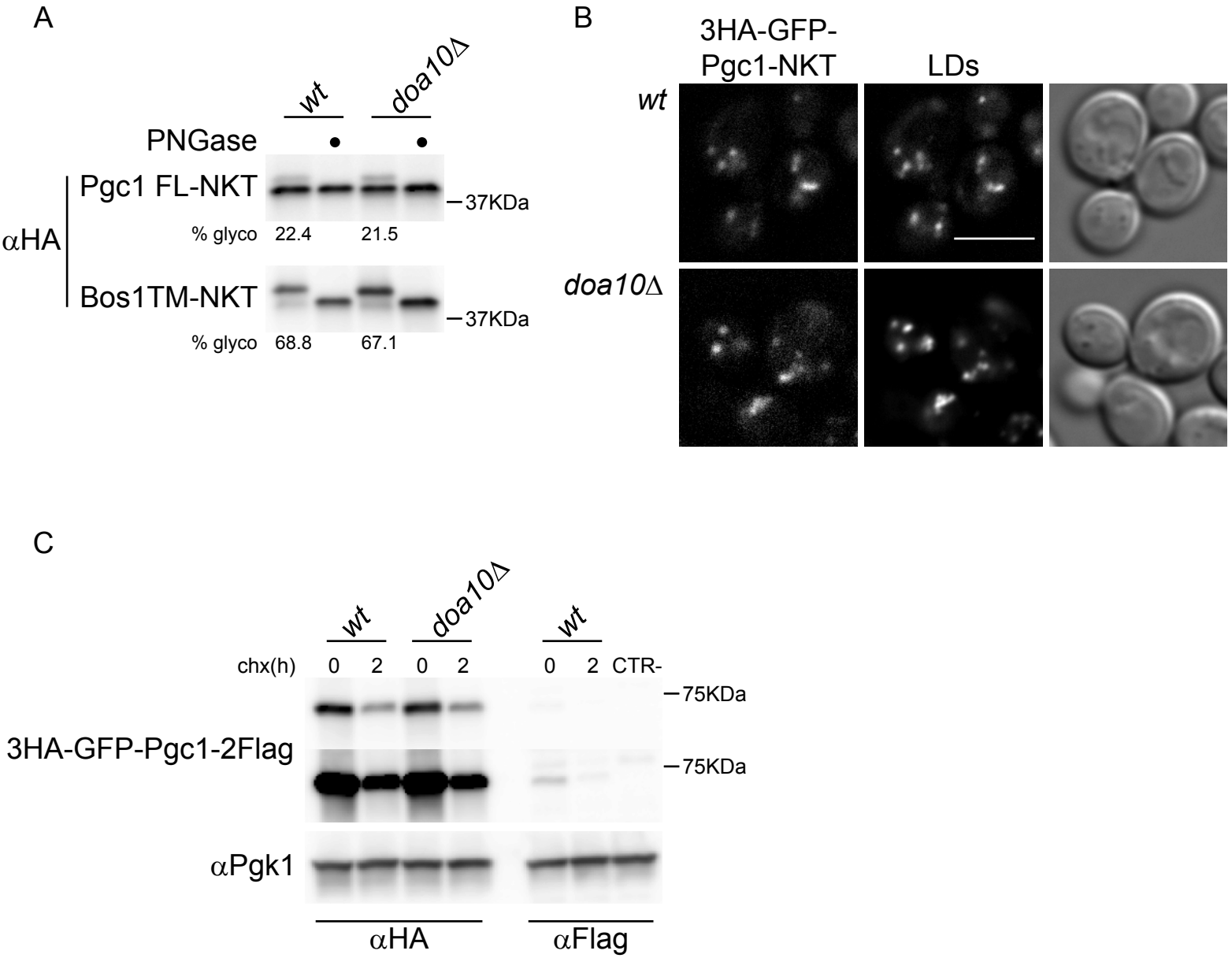

A
wt
doa10Δ
PNGase
αHA
Pgc1 FL-NKT
37KDa
% glyco 22.4 21.5
Bos1TM-NKT
37KDa
% glyco 68.8 67.1
B
3HA-GFP-Pgc1-NKT
LDs
wt
doa10Δ
C
wt
doa10Δ
wt
chx(h) 0 2 0 2 0 2 CTR-
75KDa
3HA-GFP-Pgc1-2Flag
75KDa
αPgk1
αHA
αFlag

Figure legends

Figure 1: Pgc1 is an ERAD substrate. A, degradation of plasmid-borne 3HA-Pgc1 expressed under the endogenous promoter in the indicated mutant backgrounds was followed after inhibition of protein synthesis by cycloheximide; quantification of two independent experiments is plotted. B, degradation of 3HA-Pgc1 in the proteasome mutant *pre2* after addition of cycloheximide; quantification of two independent experiments is plotted. C, degradation of 3HA-Pgc1 in mutants of the Cdc48 complex at 37C was followed after addition of cycloheximide (two hours after temperature shift from 25 to 37 degrees); quantification of two independent experiments is plotted. Kar2 and Pgk1 have been used as loading controls; Erg1 has been used as a control ERAD substrate.

Figure 2: Pgc1 localizes to LDs and behaves as an integral membrane protein. A, top, schematic of Pgc1 showing the location of the predicted hydrophobic domain; bottom: localization of GFP-Pgc1 overlaps with LDs stained with the neutral lipid dye MDH. Scale bar: 5 μm. Images were acquired using the same settings and brightness and contrast were processed comparatively. B, membranes from *wild-type* and *doa10*Δ mutant cells expressing 3HA-Pgc1 were subjected to mock, 0.2M Na_2CO_3 pH11 or 1% SDS treatment and subsequently fractionated into membrane pellet (P) and supernatant (S) fractions; membrane pellets and TCA precipitates from the supernatant were resuspended in equal volumes and loaded in equal volumes. C, Na_2CO_3 membrane extraction of 3HA-GFP-Pgc1$^{275\text{-}321}$ was performed as in (B). D, localization of 3HA-GFP-Pgc1$^{275\text{-}321}$ in *doa10*Δ mutant cells; Sec63-Cherry has been used as an ER marker protein. Scale bar: 5 μm.

Figure 3: Pgc1 is degraded by Doa10 at the ER. A, ER localization of GFP-Pgc1 in "no LDs" mutant and this strain with the additional *DOA10* deletion (no LDs *doa10*Δ). Scale bar: 5 μm. Images were acquired using the same settings and brightness and contrast were processed comparatively. B, degradation of

3HA-Pgc1 in the indicated mutant backgrounds was followed after inhibition of protein synthesis by cycloheximide; quantification of three independent experiments is plotted.

Figure 4: Membrane anchor is necessary and sufficient for Doa10-dependent degradation. A, ER localization of GFP-tagged chimeric proteins where Pgc1 membrane anchor (aa275-321) has been replaced by Scs2 or Bos1 transmembrane helices. Scale bar: 5 μm. B, degradation of 3HA-tagged full-length Pgc1 or chimeras upon replacement of Pgc1 membrane anchor by Scs2 or Bos1 transmembrane helices. C, degradation of 3HA-GFP-Pgc1$^{275-321}$ in wild-type and *doa10*Δ mutant cells; quantification of two independent experiments is plotted.

Figure 5: The hairpin is the degradation signal for Doa10. A, LD localization of a GFP-tagged chimeric protein where Pgc1 membrane anchor (aa275-321) has been replaced by *Dm*GPAT4 hairpin (aa160-216). Scale bar: 5 μm. B, ER localization of GFP-Pgc1-GPAT$^{160-216}$ in the "no LDs" *doa10*Δ mutant. Degradation of 3HA-Pgc1-GPAT$^{160-216}$ in the indicated mutant backgrounds in presence (C) or absence (D) of LDs was followed after inhibition of protein synthesis by cycloheximide; quantifications of three independent experiments are plotted.

Figure 6: Recognition of LD proteins does not require cytosolic chaperones. Degradation of 3HA-GFP-Pgc1 (A) and Dga1-GFP (B) in *ssa1-45* mutant at 25C or 37C was followed after addition of cycloheximide (45 minutes after temperature shift at 37C); quantification of three (A) and two (B) independent experiments is plotted.

Figure S1: The LD proteins Dga1 and Yeh1 are ERAD substrates. A, degradation of constitutively expressed (*ADH1* promoter) Dga1-GFP in the indicated backgrounds was followed after addition of cycloheximide. B, quantification of two independent experiments as in (A). C, degradation of plasmid-borne Yeh1-3HA expressed under the endogenous promoter in the

indicated mutant backgrounds was followed after inhibition of protein synthesis by cycloheximide. D, degradation of Dga1-GFP in the proteasome mutant *pre2* after addition of cycloheximide; quantification of two independent experiments has been plotted. E, degradation of Dga1-GFP in mutants of the Cdc48 complex at 37C was followed after addition of cycloheximide (two hours after temperature shift from 25 to 37 degrees); quantification of two independent experiments is plotted. Pgk1 has been used as loading control; Erg1 has been used as a control ERAD substrate.

Figure S2: Pgc1 and Dga1 degradation is independent from the vacuolar protease Pep4 and ubiquitin ligases other than Doa10. Degradation of 3HA-Pgc1 (A) and Dga1-GFP (B) in the indicated backgrounds was followed after addition of cycloheximide. C, quantifications of two independent experiments as in (A) and (B) have been plotted.

Figure S3: GFP-Pgc1 localization upon constitutive expression. A, localization of GFP-Pgc1 expressed under the *ADH1* promoter. B, distribution of the GFP intensity values measured at the nuclear envelope in *wild-type* and *doa10*Δ cells.

Figure S4: Pgc1 topology through C-terminal tagging. A, full-length 3HA-Pgc1 or a chimera where Pgc1 membrane anchor (aa275-321) has been replaced by Bos1 transmembrane with a glycosylation consensus sequence (NKT) at their C-terminus were subjected to PNGase treatment. B, LD localization of 3HA-GFP-Pgc1 carrying a glycosylation consensus sequence at its C-terminus. Scale bar: 5 μm. C, degradation of a C-terminally flag-tagged version of 3HA-GFP-Pgc1 was followed after addition of cycloheximide.

3. DISCUSSION

The structure and the function of an organelle are greatly dependent on its proteome and lipidome. A large variety of lipids (phospholipids, sphingolipids and sterols) defines the biophysical properties of the organelle membrane, which in turn also influences protein targeting. This is well illustrated by membrane protein sorting among the organelles along the secretory pathway (Holthuis and Menon, 2014; van Meer et al., 2008). Another example is insertion of tail-anchored proteins in mitochondria and ER: decrease in ER membrane sterol content diverts mitochondria specific tail-anchored proteins to ER (Krumpe et al., 2012). On lipid droplets, proteins (enzymes or structural proteins) greatly influence lipid composition (phospholipid in the monolayer and/or neutral lipids in the core), which is turn also affects LD size and morphology (*e.g.* through coalescence) (Brasaemle et al., 2000; Guo et al., 2008).

Proteins alone can dictate membrane thickness (Mitra et al., 2004) or force membrane remodeling (Antonny, 2006). Remarkable examples of the latter case are reticulon proteins, which shape ER tubules by imposing a high membrane curvature (Hu et al., 2008; Voeltz et al., 2006).

Environmental changes can perturb organelle homeostasis. However, cells react in order to preserve organelle compositional identity, for example through transcriptional reprogramming and changes in protein stability. In the ER, protein quality control mechanisms contribute to maintenance of both protein and lipid homeostasis. The most relevant examples will be discussed below.

3.1 Connections between protein quality control and lipid homeostasis

ERAD is a major contributor to protein homeostasis. It eliminates defective ER proteins, which are aggregation-prone and therefore potentially toxic; not surprisingly, perturbations of the ERAD system cause accumulation of misfolded species and activate transcriptional responses collectively termed

unfolded protein response, or UPR (Gardner et al., 2013). In order to manage protein folding stress, UPR reinforces the pool of ER chaperones; in addition, it also leads to new lipid synthesis and ER expansion, as a measure to relief stress in the lumen (Schuck et al., 2009; Travers et al., 2000). Changes in ER membrane biogenesis also impact the amount of lipids stored in lipid droplets, closely related to the ER. From yeast to mammals, ER stress (*e.g*. caused by mutations in ERAD genes or treatment with ER stressors) increases TAG levels and LD number and size (Fei et al., 2009; Yamamoto et al., 2010); however, the underlying mechanisms for these observations remain poorly understood.
Interestingly, perturbations in lipid homeostasis also induce UPR, leading to proteome remodeling in order to restore protein homeostasis and ER functions (Han et al., 2010; Thibault et al., 2012).
Altogether, these evidences suggest a strong connection between protein quality control and lipid homeostasis.

a) Impact of ERAD on lipid homeostasis

Lipid metabolism is regulated through gene expression patterns and modulation of enzymatic activity, *e.g*. by phosphorylation (Carman and Han, 2011; Raychaudhuri et al., 2012; Sharpe and Brown, 2013). Nevertheless, control of protein stability is an effective and irreversible way to shape the proteome in response to physiological or environmental changes. Evidences for a direct role of ERAD in lipid homeostasis have been previously reported (DeBose-Boyd, 2008).
Membrane fluidity is greatly determined by saturated fatty acid pool and sterol amount (Holthuis and Menon, 2014). In yeast, fatty acid desaturation is catalyzed by the ER-bound enzyme Ole1. Membrane fluidity must readily adapt to growth conditions; thus, Ole1 levels are stringently controlled through transcriptional and post-transcriptional regulation. *OLE1* expression is induced by the transcription factors Spt23 and Mga2, which are processed from ER-bound inactive precursors to enter the nucleus when desaturated fatty acid pool is limited (Hoppe et al., 2000; Rape et al., 2001). In addition, Ole1 abundance is

controlled at the level of mRNA and protein stability. Indeed, Ole1 disposal depends on the ERAD system (Braun et al., 2002).

Degradation of HMGR/Hmg2, a rate-limiting enzyme in the mevalonate pathway, represents another exquisite example of how ERAD-mediated proteolysis fine-tunes lipid abundance. The mevalonate pathway has two major branches for the production of isoprenoids and sterols, the final product being cholesterol in mammals and ergosterol in yeast. Given their impact on membrane properties, sterol levels must be strictly monitored. Therefore, ERAD adjusts the flux through the mevalonate pathway according to intermediate availability.

Notably, the metabolite signal for HMGR and Hmg2 degradations are different. In mammals, it is primarily 24, 25-dihydrolanosterol (Song et al., 2005a). In yeast, Hmg2 destabilization is primarily triggered by GGPP, an early isoprenoid (Garza et al., 2009). However, also in yeast Hmg2 ERAD is coordinated with sterol production. Because sterol synthesis is oxygen-consuming, GGPP levels are typically high in anaerobiosis, as a consequence of a scarce flux in the sterol branch (Figure 5 in the "Introduction"). In this condition, isoprenoid production must slow down because the sterol branch is blocked. Conversely, during aerobiotic growth, lanosterol, the first sterol intermediate, is present and Hmg2 is protected from degradation by virtue of its interaction with Insig; therefore, flux through the pathway and sterol synthesis can continue (Theesfeld and Hampton, 2013).

When Hmg2 is stable and sterols accumulate, a second regulatory step is required to control their production. We discovered that Erg1 (squalene monooxygenase), catalyzing the first committed step in the sterol-specific branch of the mevalonate pathway, is subjected to regulated degradation by the ERAD ubiquitin ligase Doa10 when lanosterol accumulates (Foresti et al., 2013). Thus, signal-mediated Erg1 degradation responds to a feedback mechanism to prevent further sterol build-up. It represents the second example of ERAD contribution to sterol synthesis regulation.

Sterols are converted to inert sterol esters (SE) and stored in LDs. We and others showed that *DOA10* deletion results in a significant increase in sterol esters (Fei et al., 2009; Foresti et al., 2013). It was reported that *doa10*Δ cells,

among other ERAD mutants, display an increase in LD number, possibly a consequence of a raise in SE. However, while the cause for such increase remains to be clarified, these evidences create a strong connection between DOA10-mediated protein degradation and LD regulation.

Further strengthening this relationship, we found that some lipid droplet proteins are Doa10 clients. LD protein localization competes with degradation by Doa10 at the ER, thus facilitating net LD accumulation of such proteins. Our data suggest that quality control contributes to ER compartmentalization and maintenance of membrane identity by eliminating mistargeted LD proteins.

LD metabolism greatly depends on the proteins at their surface; importantly, this pool is susceptible to the metabolic state of the cell. For instance, metabolic state can change ER/LD relative accumulation of proteins that exhibit dual localization (Wang and Lee, 2012; Wilfling et al., 2013). Protein abundance at the LDs can be modulated by regulating their association (*e.g.* from the cytosol) or their stability (Hooper et al., 2010). Intriguingly, several factors involved in ERAD have been previously assigned to LDs, including the p97/Cdc48-recruiting factor UBXD8/Ubx2 (Olzmann et al., 2013; Wang and Lee, 2012). However, its role is independent from proteolysis: UBXD8 recruits the ATPase p97, which negatively regulates the lipase ATGL at the LDs by uncoupling it from its activator (Olzmann et al., 2013). Here we provide the evidence for a direct role of ERAD in LD protein degradation.

We showed that a heterologous LD targeting signal from *Drosophila melanogaster* GPAT4 replaces Pgc1 membrane anchor with respect to Doa10-mediated degradation. Interestingly, one of the Dga1 homologues in mammals, DGAT2, has been shown to be a short-lived protein disposed by the ERAD E3 gp78; however, the physiological significance of this is not clear (Choi et al., 2014). These observations suggest that spatial control of LD proteins might not be exclusive for the yeast system. Future studies should address the evolutionary conservation of LD protein disposal by ERAD.

A recent finding from our laboratory established a role for ERAD in maintaining the identity of the INM, an ER subdomain, by clearing it from mistargeted proteins (Foresti et al., 2014). This evidence, together with data presented here,

supports the notion that protein quality control integrates spatial cues for preserving ER homeostasis.

3.2 Recognition of Doa10 physiological substrates

An unsolved question is how physiological Doa10 clients are recognized and engaged in the ERAD pathway.

Previous unpublished work from our laboratory and data presented in this thesis indicate that a common element in the degradation of Erg1 and LD proteins is the requirement of an intact membrane anchor. Similarly, HMGR possesses a sterol sensing domain (SSD) in its membrane region which is necessary for degradation by Hrd1 and which is thought to display signal-dependent structural instability (Shearer and Hampton, 2005; Theesfeld et al., 2011). A similar mechanism could account for Erg1 regulated degradation.

In the case of LD proteins, we speculate that the hairpin might be unstably inserted in the bilayer; thereby, ER localization might be sufficient to trigger ERAD. Therefore, in all these cases, substrate engagement could depend on a conditionally misfolded region.

Previous unpublished work from our laboratory and data presented in this thesis show that the Ssa1 family of Hsp70 chaperones does not play a role in recognition of Erg1 and LD proteins. It is possible that Hsp70 chaperones only contribute to recognition of canonical ERAD-C substrates; other adaptor proteins might mediate delivery to the Doa10 complex in the case of physiological ERAD substrates.

Doa10 is a multispanning membrane protein with a cytosolic catalytic RING domain. One of the membrane helices is required for interaction with the ubiquitin-conjugating enzyme Ubc6 (Kreft and Hochstrasser, 2011); however, the function of its large transmembrane domain has remained elusive. The long-standing assumption for a role in direct binding/recognition of membrane substrates is intriguing but totally speculative at present. It remains formally possible that recognition of these clients occurs in the membrane bilayer by the

ubiquitin ligase itself. This is an attractive hypothesis to explore in future studies.

Conclusion

Proteolysis through the ubiquitin-proteasome system has an enormous regulatory potential. For example, timed degradation of cyclins is crucial for cell cycle progression. The ER, a central organelle with many diverse functions, possesses its own machineries for ubiquitination; these are conveniently exploited not only for protein quality control but also to regulate other processes. It is not clear whether ERAD originally evolved to degrade defective proteins or whether it originally developed for regulatory purposes, but there is now complete awareness that misfolded proteins are not the sole substrates.

We used yeast as a model system to further investigate the impact of ERAD on general ER homeostasis. Given the well-studied function of this pathway in degradation of misfolded proteins, we explored its role in regulated degradation. In this work, we focused on the ubiquitin ligase Doa10. Our results highlighted a previously unappreciated function in the degradation of physiological substrates and a tight connection with lipid homeostasis.

We are far from having a complete spectrum of ERAD regulated substrates, but the available tools in yeast and higher eukaryotes will certainly lead to the identification of more of them.

Bibliography

Agrawal, G., Joshi, S., and Subramani, S. (2011). Cell-free sorting of peroxisomal membrane proteins from the endoplasmic reticulum. Proceedings of the National Academy of Sciences of the United States of America *108*, 9113-9118.

Antonny, B. (2006). Membrane deformation by protein coats. Current opinion in cell biology *18*, 386-394.

Aviram, N., and Schuldiner, M. (2014). Embracing the void--how much do we really know about targeting and translocation to the endoplasmic reticulum? Current opinion in cell biology *29*, 8-17.

Bartz, R., Zehmer, J.K., Zhu, M., Chen, Y., Serrero, G., Zhao, Y., and Liu, P. (2007). Dynamic activity of lipid droplets: protein phosphorylation and GTP-mediated protein translocation. Journal of proteome research *6*, 3256-3265.

Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A., and Hampton, R.Y. (2001a). Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nature cell biology *3*, 24-29.

Bays, N.W., Wilhovsky, S.K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R.Y. (2001b). HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Molecular biology of the cell *12*, 4114-4128.

Benyair, R., Ogen-Shtern, N., and Lederkremer, G.Z. (2014). Glycan regulation of ER-associated degradation through compartmentalization. Seminars in cell & developmental biology.

Bernardi, K.M., Williams, J.M., Inoue, T., Schultz, A., and Tsai, B. (2013). A deubiquitinase negatively regulates retro-translocation of nonubiquitinated substrates. Molecular biology of the cell *24*, 3545-3556.

Bhamidipati, A., Denic, V., Quan, E.M., and Weissman, J.S. (2005). Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen. Molecular cell *19*, 741-751.

Blanchette-Mackie, E.J., Dwyer, N.K., Barber, T., Coxey, R.A., Takeda, T., Rondinone, C.M., Theodorakis, J.L., Greenberg, A.S., and Londos, C. (1995). Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. Journal of lipid research *36*, 1211-1226.

Bordallo, J., Plemper, R.K., Finger, A., and Wolf, D.H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Molecular biology of the cell *9*, 209-222.

Borgese, N., Colombo, S., and Pedrazzini, E. (2003). The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. The Journal of cell biology *161*, 1013-1019.

Borgese, N., and Fasana, E. (2011). Targeting pathways of C-tail-anchored proteins. Biochimica et biophysica acta *1808*, 937-946.

Braakman, I., and Hebert, D.N. (2013). Protein folding in the endoplasmic reticulum. Cold Spring Harbor perspectives in biology *5*, a013201.

Brasaemle, D.L., Dolios, G., Shapiro, L., and Wang, R. (2004). Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. The Journal of biological chemistry *279*, 46835-46842.

Brasaemle, D.L., Rubin, B., Harten, I.A., Gruia-Gray, J., Kimmel, A.R., and Londos, C. (2000). Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. The Journal of biological chemistry *275*, 38486-38493.

Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002). Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. The EMBO journal *21*, 615-621.

Brodsky, J.L. (2007). The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic-reticulum-associated degradation). The Biochemical journal *404*, 353-363.

Brown, M.S., and Goldstein, J.L. (2009). Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. Journal of lipid research *50 Suppl*, S15-27.

Buhman, K.K., Chen, H.C., and Farese, R.V., Jr. (2001). The enzymes of neutral lipid synthesis. The Journal of biological chemistry *276*, 40369-40372.

Carman, G.M., and Han, G.S. (2011). Regulation of phospholipid synthesis in the yeast Saccharomyces cerevisiae. Annual review of biochemistry *80*, 859-883.

Carvalho, P., Goder, V., and Rapoport, T.A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell *126*, 361-373.

Carvalho, P., Stanley, A.M., and Rapoport, T.A. (2010). Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. Cell *143*, 579-591.

Currie, E., Guo, X., Christiano, R., Chitraju, C., Kory, N., Harrison, K., Haas, J., Walther, T.C., and Farese, R.V., Jr. (2014). High confidence proteomic analysis of yeast LDs identifies additional droplet proteins and reveals connections to dolichol synthesis and sterol acetylation. Journal of lipid research *55*, 1465-1477.

Czabany, T., Athenstaedt, K., and Daum, G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. Biochimica et biophysica acta *1771*, 299-309.

Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993). Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. Cell *74*, 357-369.

Choi, K., Kim, H., Kang, H., Lee, S.Y., Lee, S.J., Back, S.H., Lee, S.H., Kim, M.S., Lee, J.E., Park, J.Y., *et al.* (2014). Regulation of diacylglycerol acyltransferase 2 protein stability by gp78-associated endoplasmic-reticulum-associated degradation. The FEBS journal *281*, 3048-3060.

Christianson, J.C., and Green, W.N. (2004). Regulation of nicotinic receptor expression by the ubiquitin-proteasome system. The EMBO journal *23*, 4156-4165.

Christianson, J.C., and Ye, Y. (2014). Cleaning up in the endoplasmic reticulum: ubiquitin in charge. Nature structural & molecular biology *21*, 325-335.

de Godoy, L.M., Olsen, J.V., de Souza, G.A., Li, G., Mortensen, P., and Mann, M. (2006). Status of complete proteome analysis by mass spectrometry: SILAC labeled yeast as a model system. Genome biology *7*, R50.

DeBose-Boyd, R.A. (2008). Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. Cell research *18*, 609-621.

Denic, V., Quan, E.M., and Weissman, J.S. (2006). A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. Cell *126*, 349-359.

Elsabrouty, R., Jo, Y., Dinh, T.T., and DeBose-Boyd, R.A. (2013). Sterol-induced dislocation of 3-hydroxy-3-methylglutaryl coenzyme A reductase from membranes of permeabilized cells. Molecular biology of the cell *24*, 3300-3308.

Ernst, R., Mueller, B., Ploegh, H.L., and Schlieker, C. (2009). The otubain YOD1 is a deubiquitinating enzyme that associates with p97 to facilitate protein dislocation from the ER. Molecular cell *36*, 28-38.

Fei, W., Wang, H., Fu, X., Bielby, C., and Yang, H. (2009). Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in Saccharomyces cerevisiae. The Biochemical journal *424*, 61-67.

Feige, M.J., and Hendershot, L.M. (2013). Quality control of integral membrane proteins by assembly-dependent membrane integration. Molecular cell *51*, 297-309.

Fink, A.L. (1998). Protein aggregation: folding aggregates, inclusion bodies and amyloid. Folding & design *3*, R9-23.

Flury, I., Garza, R., Shearer, A., Rosen, J., Cronin, S., and Hampton, R.Y. (2005). INSIG: a broadly conserved transmembrane chaperone for sterol-sensing domain proteins. The EMBO journal *24*, 3917-3926.

Fonseca, S.G., Gromada, J., and Urano, F. (2011). Endoplasmic reticulum stress and pancreatic beta-cell death. Trends in endocrinology and metabolism: TEM *22*, 266-274.

Foresti, O., Rodriguez-Vaello, V., Funaya, C., and Carvalho, P. (2014). Quality control of inner nuclear membrane proteins by the Asi complex. Science *346*, 751-755.

Foresti, O., Ruggiano, A., Hannibal-Bach, H.K., Ejsing, C.S., and Carvalho, P. (2013). Sterol homeostasis requires regulated degradation of squalene monooxygenase by the ubiquitin ligase Doa10/Teb4. eLife *2*, e00953.

Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J., and Voeltz, G.K. (2011). ER tubules mark sites of mitochondrial division. Science *334*, 358-362.

Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Mori, M., Higashi, Y., Kojima, S., and Takano, T. (2004). Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7. Biochimica et biophysica acta *1644*, 47-59.

Fujita, K., Omura, S., and Silver, J. (1997). Rapid degradation of CD4 in cells expressing human immunodeficiency virus type 1 Env and Vpu is blocked by proteasome inhibitors. The Journal of general virology *78 (Pt 3)*, 619-625.

Furth, N., Gertman, O., Shiber, A., Alfassy, O.S., Cohen, I., Rosenberg, M.M., Doron, N.K., Friedler, A., and Ravid, T. (2011). Exposure of bipartite hydrophobic signal triggers nuclear quality control of Ndc10 at the endoplasmic reticulum/nuclear envelope. Molecular biology of the cell *22*, 4726-4739.

Gardner, B.M., Pincus, D., Gotthardt, K., Gallagher, C.M., and Walter, P. (2013). Endoplasmic reticulum stress sensing in the unfolded protein response. Cold Spring Harbor perspectives in biology *5*, a013169.

Gardner, R.G., Swarbrick, G.M., Bays, N.W., Cronin, S.R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R.Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. The Journal of cell biology *151*, 69-82.

Garza, R.M., Tran, P.N., and Hampton, R.Y. (2009). Geranylgeranyl pyrophosphate is a potent regulator of HRD-dependent 3-Hydroxy-3-methylglutaryl-CoA reductase degradation in yeast. The Journal of biological chemistry *284*, 35368-35380.

Greenberg, A.S., Egan, J.J., Wek, S.A., Garty, N.B., Blanchette-Mackie, E.J., and Londos, C. (1991). Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. The Journal of biological chemistry *266*, 11341-11346.

Grillitsch, K., Connerth, M., Kofeler, H., Arrey, T.N., Rietschel, B., Wagner, B., Karas, M., and Daum, G. (2011). Lipid particles/droplets of the yeast Saccharomyces cerevisiae revisited: lipidome meets proteome. Biochimica et biophysica acta *1811*, 1165-1176.

Guo, Y., Walther, T.C., Rao, M., Stuurman, N., Goshima, G., Terayama, K., Wong, J.S., Vale, R.D., Walter, P., and Farese, R.V. (2008). Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. Nature *453*, 657-661.

Hampton, R.Y., Gardner, R.G., and Rine, J. (1996). Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Molecular biology of the cell *7*, 2029-2044.

Hampton, R.Y., and Rine, J. (1994). Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. The Journal of cell biology *125*, 299-312.

Han, S., Lone, M.A., Schneiter, R., and Chang, A. (2010). Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. Proceedings of the National Academy of Sciences of the United States of America *107*, 5851-5856.

Herscovics, A. (1999). Processing glycosidases of Saccharomyces cerevisiae. Biochimica et biophysica acta *1426*, 275-285.

Hettema, E.H., Erdmann, R., van der Klei, I., and Veenhuis, M. (2014). Evolving models for peroxisome biogenesis. Current opinion in cell biology *29*, 25-30.

Hetz, C., and Mollereau, B. (2014). Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nature reviews. Neuroscience *15*, 233-249.

Hiller, M.M., Finger, A., Schweiger, M., and Wolf, D.H. (1996). ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science *273*, 1725-1728.

Holthuis, J.C., and Menon, A.K. (2014). Lipid landscapes and pipelines in membrane homeostasis. Nature *510*, 48-57.

Hooper, C., Puttamadappa, S.S., Loring, Z., Shekhtman, A., and Bakowska, J.C. (2010). Spartin activates atrophin-1-interacting protein 4 (AIP4) E3 ubiquitin ligase and promotes ubiquitination of adipophilin on lipid droplets. BMC biology *8*, 72.

Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D., and Jentsch, S. (2000). Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell *102*, 577-586.

Hu, J., Shibata, Y., Voss, C., Shemesh, T., Li, Z., Coughlin, M., Kozlov, M.M., Rapoport, T.A., and Prinz, W.A. (2008). Membrane proteins of the endoplasmic reticulum induce high-curvature tubules. Science *319*, 1247-1250.

Hughes, E.A., Hammond, C., and Cresswell, P. (1997). Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. Proceedings of the National Academy of Sciences of the United States of America *94*, 1896-1901.

Huyer, G., Piluek, W.F., Fansler, Z., Kreft, S.G., Hochstrasser, M., Brodsky, J.L., and Michaelis, S. (2004). Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. The Journal of biological chemistry *279*, 38369-38378.

Ingelmo-Torres, M., Gonzalez-Moreno, E., Kassan, A., Hanzal-Bayer, M., Tebar, F., Herms, A., Grewal, T., Hancock, J.F., Enrich, C., Bosch, M.*, et al.* (2009). Hydrophobic and basic domains target proteins to lipid droplets. Traffic *10*, 1785-1801.

Jacquier, N., Choudhary, V., Mari, M., Toulmay, A., Reggiori, F., and Schneiter, R. (2011). Lipid droplets are functionally connected to the endoplasmic reticulum in Saccharomyces cerevisiae. Journal of cell science *124*, 2424-2437.

Jakob, C.A., Bodmer, D., Spirig, U., Battig, P., Marcil, A., Dignard, D., Bergeron, J.J., Thomas, D.Y., and Aebi, M. (2001). Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. EMBO reports *2*, 423-430.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D.H., and Sommer, T. (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nature cell biology *4*, 134-139.

Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., and Riordan, J.R. (1995). Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. Cell *83*, 129-135.

Jo, Y., Lee, P.C., Sguigna, P.V., and DeBose-Boyd, R.A. (2011). Sterol-induced degradation of HMG CoA reductase depends on interplay of two Insigs and two ubiquitin ligases, gp78 and Trc8. Proceedings of the National Academy of Sciences of the United States of America *108*, 20503-20508.

Jungmann, J., Reins, H.A., Schobert, C., and Jentsch, S. (1993). Resistance to cadmium mediated by ubiquitin-dependent proteolysis. Nature *361*, 369-371.

Kalies, K.U., Allan, S., Sergeyenko, T., Kroger, H., and Romisch, K. (2005). The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. The EMBO journal *24*, 2284-2293.

Kearse, K.P., Roberts, J.L., and Singer, A. (1995). TCR alpha-CD3 delta epsilon association is the initial step in alpha beta dimer formation in murine T cells and is limiting in immature CD4+ CD8+ thymocytes. Immunity *2*, 391-399.

Kim, P.K., Mullen, R.T., Schumann, U., and Lippincott-Schwartz, J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. The Journal of cell biology *173*, 521-532.

Kim, W., Spear, E.D., and Ng, D.T. (2005). Yos9p detects and targets misfolded glycoproteins for ER-associated degradation. Molecular cell *19*, 753-764.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635-644.

Krahmer, N., Guo, Y., Wilfling, F., Hilger, M., Lingrell, S., Heger, K., Newman, H.W., Schmidt-Supprian, M., Vance, D.E., Mann, M.*, et al.* (2011). Phosphatidylcholine synthesis for lipid droplet expansion is mediated by localized activation of CTP:phosphocholine cytidylyltransferase. Cell metabolism *14*, 504-515.

Krahmer, N., Hilger, M., Kory, N., Wilfling, F., Stoehr, G., Mann, M., Farese, R.V., Jr., and Walther, T.C. (2013). Protein correlation profiles identify lipid droplet proteins with high confidence. Molecular & cellular proteomics : MCP *12*, 1115-1126.

Kreft, S.G., and Hochstrasser, M. (2011). An unusual transmembrane helix in the endoplasmic reticulum ubiquitin ligase Doa10 modulates degradation of its cognate E2 enzyme. The Journal of biological chemistry *286*, 20163-20174.

Kreft, S.G., Wang, L., and Hochstrasser, M. (2006). Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). The Journal of biological chemistry *281*, 4646-4653.

Krumpe, K., Frumkin, I., Herzig, Y., Rimon, N., Ozbalci, C., Brugger, B., Rapaport, D., and Schuldiner, M. (2012). Ergosterol content specifies targeting of tail-anchored proteins to mitochondrial outer membranes. Molecular biology of the cell *23*, 3927-3935.

Kuerschner, L., Moessinger, C., and Thiele, C. (2008). Imaging of lipid biosynthesis: how a neutral lipid enters lipid droplets. Traffic *9*, 338-352.

Lam, S.K., Yoda, N., and Schekman, R. (2010). A vesicle carrier that mediates peroxisome protein traffic from the endoplasmic reticulum. Proceedings of the National Academy of Sciences of the United States of America *107*, 21523-21528.

Lilley, B.N., Gilbert, J.M., Ploegh, H.L., and Benjamin, T.L. (2006). Murine polyomavirus requires the endoplasmic reticulum protein Derlin-2 to initiate infection. Journal of virology *80*, 8739-8744.

Lilley, B.N., and Ploegh, H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. Nature *429*, 834-840.

Lin, J.H., and Lavail, M.M. (2010). Misfolded proteins and retinal dystrophies. Advances in experimental medicine and biology *664*, 115-121.

Lippincott-Schwartz, J., Bonifacino, J.S., Yuan, L.C., and Klausner, R.D. (1988). Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. Cell *54*, 209-220.

Lipson, C., Alalouf, G., Bajorek, M., Rabinovich, E., Atir-Lande, A., Glickman, M., and Bar-Nun, S. (2008). A proteasomal ATPase contributes to dislocation of endoplasmic reticulum-associated degradation (ERAD) substrates. The Journal of biological chemistry *283*, 7166-7175.

Liu, Q., Siloto, R.M., Snyder, C.L., and Weselake, R.J. (2011). Functional and topological analysis of yeast acyl-CoA:diacylglycerol acyltransferase 2, an endoplasmic reticulum enzyme essential for triacylglycerol biosynthesis. The Journal of biological chemistry *286*, 13115-13126.

Loureiro, J., Lilley, B.N., Spooner, E., Noriega, V., Tortorella, D., and Ploegh, H.L. (2006). Signal peptide peptidase is required for dislocation from the endoplasmic reticulum. Nature *441*, 894-897.

Magadan, J.G., Perez-Victoria, F.J., Sougrat, R., Ye, Y., Strebel, K., and Bonifacino, J.S. (2010). Multilayered mechanism of CD4 downregulation by HIV-1 Vpu involving distinct ER retention and ERAD targeting steps. PLoS pathogens *6*, e1000869.

Mehnert, M., Sommer, T., and Jarosch, E. (2014). Der1 promotes movement of misfolded proteins through the endoplasmic reticulum membrane. Nature cell biology *16*, 77-86.

Menendez-Benito, V., van Deventer, S.J., Jimenez-Garcia, V., Roy-Luzarraga, M., van Leeuwen, F., and Neefjes, J. (2013). Spatiotemporal analysis of organelle and macromolecular complex inheritance. Proceedings of the National Academy of Sciences of the United States of America *110*, 175-180.

Metzger, M.B., Maurer, M.J., Dancy, B.M., and Michaelis, S. (2008). Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. The Journal of biological chemistry *283*, 32302-32316.

Mitra, K., Ubarretxena-Belandia, I., Taguchi, T., Warren, G., and Engelman, D.M. (2004). Modulation of the bilayer thickness of exocytic pathway membranes by membrane proteins rather than cholesterol. Proceedings of the National Academy of Sciences of the United States of America *101*, 4083-4088.

Morris, L.L., Hartman, I.Z., Jun, D.J., Seemann, J., and DeBose-Boyd, R.A. (2014). Sequential actions of the AAA-ATPase valosin-containing protein (VCP)/p97 and the proteasome 19 S regulatory particle in sterol-accelerated, endoplasmic reticulum (ER)-associated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. The Journal of biological chemistry *289*, 19053-19066.

Motley, A.M., and Hettema, E.H. (2007). Yeast peroxisomes multiply by growth and division. The Journal of cell biology *178*, 399-410.

Mueller, B., Lilley, B.N., and Ploegh, H.L. (2006). SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER. The Journal of cell biology *175*, 261-270.

Nakatsukasa, K., Huyer, G., Michaelis, S., and Brodsky, J.L. (2008). Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. Cell *132*, 101-112.

Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (2005). Ubx2 links the Cdc48 complex to ER-associated protein degradation. Nature cell biology *7*, 993-998.

Nian, Z., Sun, Z., Yu, L., Toh, S.Y., Sang, J., and Li, P. (2010). Fat-specific protein 27 undergoes ubiquitin-dependent degradation regulated by triacylglycerol synthesis and lipid droplet formation. The Journal of biological chemistry *285*, 9604-9615.

Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J.T., and Sturley, S.L. (2002). The DGA1 gene determines a second triglyceride synthetic pathway in yeast. The Journal of biological chemistry *277*, 8877-8881.

Okuda-Shimizu, Y., and Hendershot, L.M. (2007). Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. Molecular cell *28*, 544-554.

Olzmann, J.A., Richter, C.M., and Kopito, R.R. (2013). Spatial regulation of UBXD8 and p97/VCP controls ATGL-mediated lipid droplet turnover. Proceedings of the National Academy of Sciences of the United States of America *110*, 1345-1350.

Pilon, M., Schekman, R., and Romisch, K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. The EMBO journal *16*, 4540-4548.

Plemper, R.K., Bohmler, S., Bordallo, J., Sommer, T., and Wolf, D.H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature *388*, 891-895.

Pol, A., Gross, S.P., and Parton, R.G. (2014). Review: biogenesis of the multifunctional lipid droplet: lipids, proteins, and sites. The Journal of cell biology *204*, 635-646.

Quan, E.M., Kamiya, Y., Kamiya, D., Denic, V., Weibezahn, J., Kato, K., and Weissman, J.S. (2008). Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. Molecular cell *32*, 870-877.

Rabinovich, E., Kerem, A., Frohlich, K.U., Diamant, N., and Bar-Nun, S. (2002). AAA-ATPase p97/Cdc48p, a Cytosolic Chaperone Required for Endoplasmic Reticulum-Associated Protein Degradation. Molecular and cellular biology *22*, 626-634.

Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (2001). Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. Cell *107*, 667-677.

Ravid, T., Doolman, R., Avner, R., Harats, D., and Roitelman, J. (2000). The ubiquitin-proteasome pathway mediates the regulated degradation of mammalian 3-hydroxy-3-methylglutaryl-coenzyme A reductase. The Journal of biological chemistry *275*, 35840-35847.

Ravid, T., Kreft, S.G., and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. The EMBO journal *25*, 533-543.

Raychaudhuri, S., and Prinz, W.A. (2008). Nonvesicular phospholipid transfer between peroxisomes and the endoplasmic reticulum. Proceedings of the National Academy of Sciences of the United States of America *105*, 15785-15790.

Raychaudhuri, S., Young, B.P., Espenshade, P.J., and Loewen, C., Jr. (2012). Regulation of lipid metabolism: a tale of two yeasts. Current opinion in cell biology *24*, 502-508.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell *120*, 73-84.

Robenek, H., Hofnagel, O., Buers, I., Robenek, M.J., Troyer, D., and Severs, N.J. (2006). Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. Journal of cell science *119*, 4215-4224.

Rowland, A.A., Chitwood, P.J., Phillips, M.J., and Voeltz, G.K. (2014). ER contact sites define the position and timing of endosome fission. Cell *159*, 1027-1041.

Rowland, A.A., and Voeltz, G.K. (2012). Endoplasmic reticulum-mitochondria contacts: function of the junction. Nature reviews. Molecular cell biology *13*, 607-625.

Ruggiano, A., Foresti, O., and Carvalho, P. (2014). Quality control: ER-associated degradation: protein quality control and beyond. The Journal of cell biology *204*, 869-879.

Rumpf, S., and Jentsch, S. (2006). Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. Molecular cell *21*, 261-269.

Sandager, L., Gustavsson, M.H., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., and Stymne, S. (2002). Storage lipid synthesis is non-essential in yeast. The Journal of biological chemistry *277*, 6478-6482.

Sato, B.K., Schulz, D., Do, P.H., and Hampton, R.Y. (2009). Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. Molecular cell *34*, 212-222.

Schelhaas, M., Malmstrom, J., Pelkmans, L., Haugstetter, J., Ellgaard, L., Grunewald, K., and Helenius, A. (2007). Simian Virus 40 depends on ER protein folding and quality control factors for entry into host cells. Cell *131*, 516-529.

Schubert, U., Anton, L.C., Bacik, I., Cox, J.H., Bour, S., Bennink, J.R., Orlowski, M., Strebel, K., and Yewdell, J.W. (1998). CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. Journal of virology *72*, 2280-2288.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., and Bennink, J.R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature *404*, 770-774.

Schuberth, C., and Buchberger, A. (2005). Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. Nature cell biology *7*, 999-1006.

Schuck, S., Prinz, W.A., Thorn, K.S., Voss, C., and Walter, P. (2009). Membrane expansion alleviates endoplasmic reticulum stress independently of the unfolded protein response. The Journal of cell biology *187*, 525-536.

Schumacher, M.M., Elsabrouty, R., Seemann, J., Jo, Y., and DeBose-Boyd, R.A. (2015). The prenyltransferase UBIAD1 is the target of geranylgeraniol in degradation of HMG CoA reductase. eLife *4*.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. The EMBO journal *9*, 543-550.

Seufert, W., McGrath, J.P., and Jentsch, S. (1990). UBC1 encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation. The EMBO journal *9*, 4535-4541.

Sever, N., Song, B.L., Yabe, D., Goldstein, J.L., Brown, M.S., and DeBose-Boyd, R.A. (2003a). Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. The Journal of biological chemistry *278*, 52479-52490.

Sever, N., Yang, T., Brown, M.S., Goldstein, J.L., and DeBose-Boyd, R.A. (2003b). Accelerated degradation of HMG CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. Molecular cell *11*, 25-33.

Shao, S., and Hegde, R.S. (2011). Membrane protein insertion at the endoplasmic reticulum. Annual review of cell and developmental biology *27*, 25-56.

Sharpe, L.J., and Brown, A.J. (2013). Controlling cholesterol synthesis beyond 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). The Journal of biological chemistry *288*, 18707-18715.

Shearer, A.G., and Hampton, R.Y. (2005). Lipid-mediated, reversible misfolding of a sterol-sensing domain protein. The EMBO journal *24*, 149-159.

Shibata, Y., Voeltz, G.K., and Rapoport, T.A. (2006). Rough sheets and smooth tubules. Cell *126*, 435-439.

Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., Tanaka, K., Cuervo, A.M., and Czaja, M.J. (2009). Autophagy regulates lipid metabolism. Nature *458*, 1131-1135.

Sommer, T., and Jentsch, S. (1993). A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature *365*, 176-179.

Song, B.L., Javitt, N.B., and DeBose-Boyd, R.A. (2005a). Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. Cell metabolism *1*, 179-189.

Song, B.L., Sever, N., and DeBose-Boyd, R.A. (2005b). Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. Molecular cell *19*, 829-840.

Sorger, D., Athenstaedt, K., Hrastnik, C., and Daum, G. (2004). A yeast strain lacking lipid particles bears a defect in ergosterol formation. The Journal of biological chemistry *279*, 31190-31196.

Sorger, D., and Daum, G. (2002). Synthesis of Triacylglycerols by the Acyl-Coenzyme A:Diacyl-Glycerol Acyltransferase Dga1p in Lipid Particles of the Yeast Saccharomyces cerevisiae. Journal of Bacteriology *184*, 519-524.

Sorger, D., and Daum, G. (2003). Triacylglycerol biosynthesis in yeast. Applied microbiology and biotechnology *61*, 289-299.

Stagg, H.R., Thomas, M., van den Boomen, D., Wiertz, E.J., Drabkin, H.A., Gemmill, R.M., and Lehner, P.J. (2009). The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. The Journal of cell biology *186*, 685-692.

Stein, A., Ruggiano, A., Carvalho, P., and Rapoport, T.A. (2014). Key steps in ERAD of luminal ER proteins reconstituted with purified components. Cell *158*, 1375-1388.

Stolz, A., Besser, S., Hottmann, H., and Wolf, D.H. (2013). Previously unknown role for the ubiquitin ligase Ubr1 in endoplasmic reticulum-associated protein degradation. Proceedings of the National Academy of Sciences of the United States of America *110*, 15271-15276.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes & development *15*, 2660-2674.

Szathmary, R., Bielmann, R., Nita-Lazar, M., Burda, P., and Jakob, C.A. (2005). Yos9 protein is essential for degradation of misfolded glycoproteins and may function as lectin in ERAD. Molecular cell *19*, 765-775.

Tabas, I., and Ron, D. (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nature cell biology *13*, 184-190.

Tam, Y.Y., Fagarasanu, A., Fagarasanu, M., and Rachubinski, R.A. (2005). Pex3p initiates the formation of a preperoxisomal compartment from a subdomain of the endoplasmic reticulum in Saccharomyces cerevisiae. The Journal of biological chemistry *280*, 34933-34939.

Tanaka, T., Kuroda, K., Ikeda, M., Wakita, T., Kato, N., and Makishima, M. (2013). Hepatitis C virus NS4B targets lipid droplets through hydrophobic residues in the amphipathic helices. Journal of lipid research *54*, 881-892.

Tannous, A., Pisoni, G.B., Hebert, D.N., and Molinari, M. (2014). N-linked sugar-regulated protein folding and quality control in the ER. Seminars in cell & developmental biology.

Theesfeld, C.L., and Hampton, R.Y. (2013). Insulin-induced gene protein (INSIG)-dependent sterol regulation of Hmg2 endoplasmic reticulum-associated degradation (ERAD) in yeast. The Journal of biological chemistry *288*, 8519-8530.

Theesfeld, C.L., Pourmand, D., Davis, T., Garza, R.M., and Hampton, R.Y. (2011). The sterol-sensing domain (SSD) directly mediates signal-regulated endoplasmic reticulum-associated degradation (ERAD) of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase isozyme Hmg2. The Journal of biological chemistry *286*, 26298-26307.

Thibault, G., Shui, G., Kim, W., McAlister, G.C., Ismail, N., Gygi, S.P., Wenk, M.R., and Ng, D.T. (2012). The membrane stress response buffers lethal effects of lipid disequilibrium by reprogramming the protein homeostasis network. Molecular cell *48*, 16-27.

Ting, J.T., Balsamo, R.A., Ratnayake, C., and Huang, A.H. (1997). Oleosin of plant seed oil bodies is correctly targeted to the lipid bodies in transformed yeast. The Journal of biological chemistry *272*, 3699-3706.

Travers, K.J., Patil, C.K., Wodicka, L., Lockhart, D.J., Weissman, J.S., and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell *101*, 249-258.

Tsai, Y.C., Leichner, G.S., Pearce, M.M., Wilson, G.L., Wojcikiewicz, R.J., Roitelman, J., and Weissman, A.M. (2012). Differential regulation of HMG-CoA reductase and Insig-1 by enzymes of the ubiquitin-proteasome system. Molecular biology of the cell *23*, 4484-4494.

Tzen, J.T., and Huang, A.H. (1992). Surface structure and properties of plant seed oil bodies. The Journal of cell biology *117*, 327-335.

van de Weijer, M.L., Bassik, M.C., Luteijn, R.D., Voorburg, C.M., Lohuis, M.A., Kremmer, E., Hoeben, R.C., LeProust, E.M., Chen, S., Hoelen, H., *et al.* (2014). A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation. Nature communications *5*, 3832.

van den Boomen, D.J., Timms, R.T., Grice, G.L., Stagg, H.R., Skodt, K., Dougan, G., Nathan, J.A., and Lehner, P.J. (2014). TMEM129 is a Derlin-1 associated ERAD E3 ligase essential for virus-induced degradation of MHC-I. Proceedings of the National Academy of Sciences of the United States of America *111*, 11425-11430.

van der Zand, A., Braakman, I., and Tabak, H.F. (2010). Peroxisomal membrane proteins insert into the endoplasmic reticulum. Molecular biology of the cell *21*, 2057-2065.

van der Zand, A., Gent, J., Braakman, I., and Tabak, H.F. (2012). Biochemically distinct vesicles from the endoplasmic reticulum fuse to form peroxisomes. Cell *149*, 397-409.

van Meer, G., Voelker, D.R., and Feigenson, G.W. (2008). Membrane lipids: where they are and how they behave. Nature reviews. Molecular cell biology *9*, 112-124.

van Zutphen, T., Todde, V., de Boer, R., Kreim, M., Hofbauer, H.F., Wolinski, H., Veenhuis, M., van der Klei, I.J., and Kohlwein, S.D. (2014). Lipid droplet autophagy in the yeast Saccharomyces cerevisiae. Molecular biology of the cell *25*, 290-301.

Vashist, S., and Ng, D.T. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. The Journal of cell biology *165*, 41-52.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., 3rd, Koonin, E.V., and Deshaies, R.J. (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science *298*, 611-615.

Voeltz, G.K., Prinz, W.A., Shibata, Y., Rist, J.M., and Rapoport, T.A. (2006). A class of membrane proteins shaping the tubular endoplasmic reticulum. Cell *124*, 573-586.

Walther, T.C., and Farese, R.V., Jr. (2009). The life of lipid droplets. Biochimica et biophysica acta *1791*, 459-466.

Walther, T.C., and Farese, R.V., Jr. (2012). Lipid droplets and cellular lipid metabolism. Annual review of biochemistry *81*, 687-714.

Wang, C.W., and Lee, S.C. (2012). The ubiquitin-like (UBX)-domain-containing protein Ubx2/Ubxd8 regulates lipid droplet homeostasis. Journal of cell science *125*, 2930-2939.

Wang, Q., Li, L., and Ye, Y. (2006). Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3. The Journal of cell biology *174*, 963-971.

Ward, C.L., and Kopito, R.R. (1994). Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. The Journal of biological chemistry *269*, 25710-25718.

Ward, C.L., Omura, S., and Kopito, R.R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. Cell *83*, 121-127.

Werner, E.D., Brodsky, J.L., and McCracken, A.A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proceedings of the National Academy of Sciences of the United States of America *93*, 13797-13801.

Wiertz, E.J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A., and Ploegh, H.L. (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature *384*, 432-438.

Wilfling, F., Thiam, A.R., Olarte, M.J., Wang, J., Beck, R., Gould, T.J., Allgeyer, E.S., Pincet, F., Bewersdorf, J., Farese, R.V., Jr.*, et al.* (2014). Arf1/COPI machinery acts directly on lipid droplets and enables their connection to the ER for protein targeting. eLife *3*, e01607.

Wilfling, F., Wang, H., Haas, J.T., Krahmer, N., Gould, T.J., Uchida, A., Cheng, J.X., Graham, M., Christiano, R., Frohlich, F.*, et al.* (2013). Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. Developmental cell *24*, 384-399.

Wolins, N.E., Brasaemle, D.L., and Bickel, P.E. (2006). A proposed model of fat packaging by exchangeable lipid droplet proteins. FEBS letters *580*, 5484-5491.

Xu, G., Sztalryd, C., and Londos, C. (2006). Degradation of perilipin is mediated through ubiquitination-proteasome pathway. Biochimica et biophysica acta *1761*, 83-90.

Xu, G., Sztalryd, C., Lu, X., Tansey, J.T., Gan, J., Dorward, H., Kimmel, A.R., and Londos, C. (2005). Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. The Journal of biological chemistry *280*, 42841-42847.

Yamamoto, K., Takahara, K., Oyadomari, S., Okada, T., Sato, T., Harada, A., and Mori, K. (2010). Induction of liver steatosis and lipid droplet formation in ATF6alpha-knockout mice burdened with pharmacological endoplasmic reticulum stress. Molecular biology of the cell *21*, 2975-2986.

Yang, M., Omura, S., Bonifacino, J.S., and Weissman, A.M. (1998). Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. The Journal of experimental medicine *187*, 835-846.

Yao, T., and Cohen, R.E. (2002). A cryptic protease couples deubiquitination and degradation by the proteasome. Nature *419*, 403-407.

Ye, Y., Meyer, H.H., and Rapoport, T.A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature *414*, 652-656.

Ye, Y., Meyer, H.H., and Rapoport, T.A. (2003). Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. The Journal of cell biology *162*, 71-84.

Ye, Y., Shibata, Y., Kikkert, M., van Voorden, S., Wiertz, E., and Rapoport, T.A. (2005). Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. Proceedings of the National Academy of Sciences of the United States of America *102*, 14132-14138.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T.A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature *429*, 841-847.

Zehmer, J.K., Bartz, R., Bisel, B., Liu, P., Seemann, J., and Anderson, R.G. (2009). Targeting sequences of UBXD8 and AAM-B reveal that the ER has a direct role in the emergence and regression of lipid droplets. Journal of cell science *122*, 3694-3702.

Zehmer, J.K., Bartz, R., Liu, P., and Anderson, R.G. (2008). Identification of a novel N-terminal hydrophobic sequence that targets proteins to lipid droplets. Journal of cell science *121*, 1852-1860.

Zhang, Z.R., Bonifacino, J.S., and Hegde, R.S. (2013). Deubiquitinases sharpen substrate discrimination during membrane protein degradation from the ER. Cell *154*, 609-622.