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RESEARCH ARTICLE

SPECIAL ISSUE: CELL BIOLOGY OF LIPIDS

Lipid droplets form a network interconnected by the endoplasmic reticulum through which their proteins equilibrate

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ABSTRACT

Lipid droplets (LDs) are globular intracellular structures dedicated to the storage of neutral lipids. They are closely associated with the endoplasmic reticulum (ER) and are delineated by a monolayer of phospholipids that is continuous with the cytoplasmic leaflet of the ER membrane. LDs contain a specific set of proteins, but how these proteins are targeted to the LD surface is not fully understood. Here, we devised a yeast mating-based microscopic readout to monitor the transfer of LD proteins upon zygote formation. The results of this analysis indicate that ER fusion between mating partners is required for transfer of LD proteins and that this transfer is continuous, bidirectional and affects most LDs simultaneously. These observations suggest that LDs do not fuse upon mating of yeast cells, but that they form a network that is interconnected through the ER membrane. Consistent with this, ER-localized LD proteins rapidly move onto LDs of a mating partner and this protein transfer is affected by seipin, a protein important for proper LD biogenesis and the functional connection of LDs with the ER membrane.

KEY WORDS: Lipid droplets, Endoplasmic reticulum, *Saccharomyces cerevisiae*, Protein targeting, Mating, Membrane fusion, Seipin, Triacylglycerol, Steryl esters

INTRODUCTION

Lipid droplets (LDs) are intracellular compartments dedicated to the storage of neutral lipids, particularly triacylglycerol (TAG) and steryl esters. They form globular structures composed of a hydrophobic core of neutral lipids that is shielded from the aqueous cytoplasm by a monolayer of phospholipids onto which a specific set of proteins associate. The structure of LDs is thus reminiscent of that of lipoproteins; however, unlike lipoproteins, LDs are not secreted. LD biogenesis is driven by the synthesis of neutral lipids by enzymes that are localized in the membrane of the endoplasmic reticulum (ER) (Olzmann and Carvalho, 2018; Thiam et al., 2013). LDs thus form from the ER membrane and they remain closely associated with ER throughout their life cycle, both in yeast and mammalian cells (Choudhary et al., 2015, 2020; Choudhary and Schneiter, 2021; Jacquier et al., 2011; Wilfling et al., 2013).

LD growth and expansion can occur through different pathways (Barneda and Christian, 2017). LDs expand through the localized synthesis of neutral lipids by enzymes such as Dga1 (yeast) or DGAT2 (mammals) on the surface of or in the immediate vicinity to LDs (Jacquier et al., 2011; Kassan et al., 2013; Kuerschner et al., 2008; Wilfling et al., 2013; Xu et al., 2012). In addition, small LDs can fuse with each other to yield larger LDs (Bostrom et al., 2007; Gao et al., 2017a). Alternatively, LDs can grow through a ripening process, that is, the incorporation of neutral lipids from either the ER or from adjacent LDs into pre-existing LDs (Jüngst et al., 2013; Salo et al., 2019; Thiam and Forêt, 2016).

Proteins localize to the surface of LDs through two distinct targeting determinants, amphipathic helices or hairpin-type of membrane domains (Dhiman et al., 2020; Kory et al., 2016). Hairpin-anchored proteins, such as the diacylglycerol acyltransferase Dga1 in yeast, DGAT2 in mammalian cells or the glycerol-3-phosphate acyltransferase 4 (GPAT4), are first targeted to the ER membrane from where they then localize to the surface of LDs (Jacquier et al., 2011; Olarte et al., 2020; Wilfling et al., 2013; Xu et al., 2012). Therefore, in the absence of LDs, these proteins are localized to the ER bilayer membrane but they prefer a TAG-covering monolayer membrane over a pure phospholipid bilayer membrane *in vitro* (Caillon et al., 2020; Jacquier et al., 2011). The second class of LD-localized proteins, on the other hand, are more soluble, and their amphipathic helices target the surface of LDs from the cytoplasm, possibly by recognizing lipid packing defects on the limiting phospholipid monolayer of LDs. They include the abundant LD scaffolding perilipin family of proteins (PLINs), their yeast ortholog Pet10 and also the Parkinson's disease-associated protein α-synuclein (Bulankina et al., 2009; Chorlay and Thiam, 2020; Čopič et al., 2018; Gao et al., 2017b; Jacquier et al., 2013; Prévost et al., 2018; Rowe et al., 2016; Sztalryd and Brasaemle, 2017).

Here, we analyze the *in vivo* dynamics of proteins targeting to LDs using a yeast mating-based microscopic assay in which one of the mating partners expresses an mCitrine-tagged LD protein and the other cell expresses an mCherry-tagged LD protein together with a cytoplasmic cyan fluorescent protein (CFP). Three color time-lapse imaging allows us to analyze the redistribution of these marker proteins upon zygote formation. The results of these analyses indicate that LD proteins redistribute between LDs of mating cells in a continuous and bidirectional process that involves the majority of LDs in the mating pair. In cells where otherwise LD-localized proteins are mis-localized to the ER, we observe that these proteins reach the LDs of the mating partner through the interconnecting ER. LD-targeting of such proteins is delayed in mutants affecting ER fusion. Efficient ER to LD targeting requires seipin, an ER protein that is important for proper LD biogenesis and localizes to ER–LD contact sites. These results support a model in which LDs form a network of discrete compartments that are interconnected through the ER membrane through which they exchange their proteins with each other.

RESULTS

Experimental design to monitor the transfer of proteins between LDs *in vivo*

To monitor the targeting of proteins to LDs, we adapted a yeast mating-based experimental setup (Anwar et al., 2012). In this setup,

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LD marker proteins, such as Erg6, an enzyme of the late part of the ergosterol biosynthetic pathway, and Dga1, a diacylglycerol acyltransferase, which catalyzes the formation of TAG, were fused to fluorescent proteins and their expression was controlled by a galactose inducible promoter (*GAL1*^{prom}) (Gaber et al., 1989; Oelkers et al., 2002; Sorger and Daum, 2002). Plasmids encoding *GAL1*^{prom}*-ERG6-mCITRINE* or *GAL1*^{prom}*-DGA1-3mCHERRY* were then transformed into wild-type cells of different mating-type. *MAT*a cells expressing *DGA1-3mCHERRY* also co-expressed a soluble cytoplasmic CFP, which served to monitor mixing of the cytoplasmic content between the two mating partners upon cell fusion, thereby defining the zero timepoint (t=0 min) of the mating reaction. Cells of both mating types were cultivated overnight in galactose-containing medium to induce expression of the fluorescently tagged LD marker proteins. They were then resuspended in glucose-containing medium to repress expression of the marker proteins, and mixed in a 1:1 ratio to initiate the mating reaction. After 2 h of co-cultivation, cells were mounted under an agarose patch on a glass coverslip and progression of mating reactions was monitored by time-lapse fluorescence microscopy (Fig. 1A). Before cell fusion occurred, Dga1–3mCherry was localized to punctuate LDs in cells expressing cytosolic CFP whereas Erg6–mCitrine marked punctuate LDs in cells that were negative for CFP fluorescence (Fig. 1A, t=−1 min). A line scan through a mating pair confirmed that Erg6–mCitrine fluorescence was confined to one half of the pre-zygote and Dga1–3mCherry and CFP fluorescence were both restricted to the other half. Upon cytoplasmic mixing, however, CFP fluorescence re-distributed throughout the zygote, while the two LD markers, Dga1–3mCherry and Erg6–mCitrine remained spatially separated (Fig. 1A, t=0 min). At 40 min after cell fusion had occurred, the two LD markers colocalized on punctuate intracellular structures representing LDs as indicated by the overlap between red and green signals in the line scan and the appearance of a yellow signal in the merge image (Fig. 1A, t=40 min). Thus, LD-localized marker proteins started to colocalize on LDs of mating partners in a process that is much slower than cytoplasmic mixing.

To examine whether LD localization of the fluorescent LD marker proteins was possibly due to their transfer through the aqueous space from a mating donor to an acceptor LD or due to their synthesis within the resulting zygote, we determined the half-life of these marker proteins upon glucose-dependent repression of the *GAL1*^{prom} and examined the solubility of these proteins by differential centrifugation. While both Dga1–3mCherry and Erg6–mCitrine were stably expressed in cells grown in the presence of galactose, glucose repression of their transcription resulted in a rapid decline of steady-state levels of Dga1–3mCherry, with a half-life of 31 min. Erg6–mCitrine was more stable even in glucose-repressed cells, with half-life of 270 min (Fig. 1B). Both proteins co-fractionated with Sec63, a component of the ER translocon, indicating that they are membrane-associated and thus are not likely to transfer through the shared cytoplasm of mating partners (Fig. 1C). Taken together these data indicate that pre-existing LD-localized proteins redistribute between donor and acceptor LDs upon cell fusion, possibly through membrane-mediated protein transfer or through direct fusion of LDs between the mating partners.

Exchange of LD proteins between mating partners is continuous and reciprocal

Next, we analyzed the time dependence of transfer of LD proteins between donor and acceptor. To do this, time-lapse images of mating cells were recorded at 1 min intervals. The changes in fluorescence intensities of the fluorophores was measured in both mating partners and expressed as a percentage of that in the whole cell averaged over five time points prior to cytoplasmic mixing (t=−5 min to t=−1 min). This analysis revealed that the fluorescence of both Dga1–3mCherry and Erg6–mCitrine continuously increased over a period of ∼20 min in the respective acceptor mating partner and then began to level off. This indicates that these LD marker proteins redistribute between LDs of the two mating partners until they reach a new steady-state distribution in which all of the LDs in the resulting zygote were covered by both marker proteins (Fig. 2). Both marker proteins displayed similar behavior in this assay, suggesting that this transfer kinetics is not protein specific but possibly a more general property of membrane-associated LD proteins. However, their relative distribution between the ER and the LD might vary between different proteins. Redistribution of these LD markers was reciprocal between the two mating partners as the increase in one of the marker proteins in the acceptor cell was paralleled by a concurrent decrease in fluorescence in the donor cell (Fig. 2C). At the qualitative level, this reciprocal exchange of LD proteins between LDs of the two mating partners was also observed in time-lapse images, which revealed that individual LDs acquired the respective LD marker from the mating partner with similar kinetics (Fig. 2B, time points 10 and 14 min; Movie 1). Analysis of the transfer of a genomically tagged version of Erg6 during mating revealed a dynamic similar to that of the galactose promoter-controlled Erg6–mCitrine. In this case, however, a continuous increase of the fluorescence on the acceptor LDs was observed at later time points, because the synthesis of the protein was not repressed (Fig. S1A,B). The continuous and reciprocal exchange of LD proteins between the two partners suggests that transfer occurs through membrane bridges connecting the LDs within the zygote rather than by LD–LD fusion. Fusion between LDs would likely result in a non-homogenous, mosaic-like, distribution of the marker proteins over different LDs because not all the LDs would be expected to fuse simultaneously, resulting in LDs decorated with both fluorophores next to LDs that would show only red or only green fluorescence. This was not observed, supporting the notion that LDs did not fuse with each other upon mating of cells, but that they exchanged and re-equilibrate their protein content. Although the time resolution of imaging does not allow for unambiguously tracking the fate of a single LD, their dynamics and presence at the fusion neck suggest that LDs can also move from one half of the zygote to the other (Movie 1). To monitor this possible redistribution of LDs, rather than the transfer of LD markers, upon zygote formation, we labeled LDs with Pet10–mScarlet in one of the mating partners. When compared to the membrane-anchored LD proteins Erg6 or Dga1, the soluble Pet10–mScarlet was less efficiently targeted to the LD surface of the mating partner. As a consequence, two separate populations of LDs were discernible even 40 min after cytoplasmic mixing had occurred (Fig. S1C,D). These observations indicate that at early time points of zygote formation, LDs appear to largely remain in the part of the zygote where they originate from.

Transfer of ER proteins precedes the exchange of LD proteins upon zygote formation, while the exchange of mitochondrial markers is comparatively slow

To examine whether the exchange of proteins between LDs of the newly formed zygote could be mediated by formation of a common ER membrane, we first analyzed the redistribution of an ER membrane protein, Sec63–mCherry, upon zygote formation.

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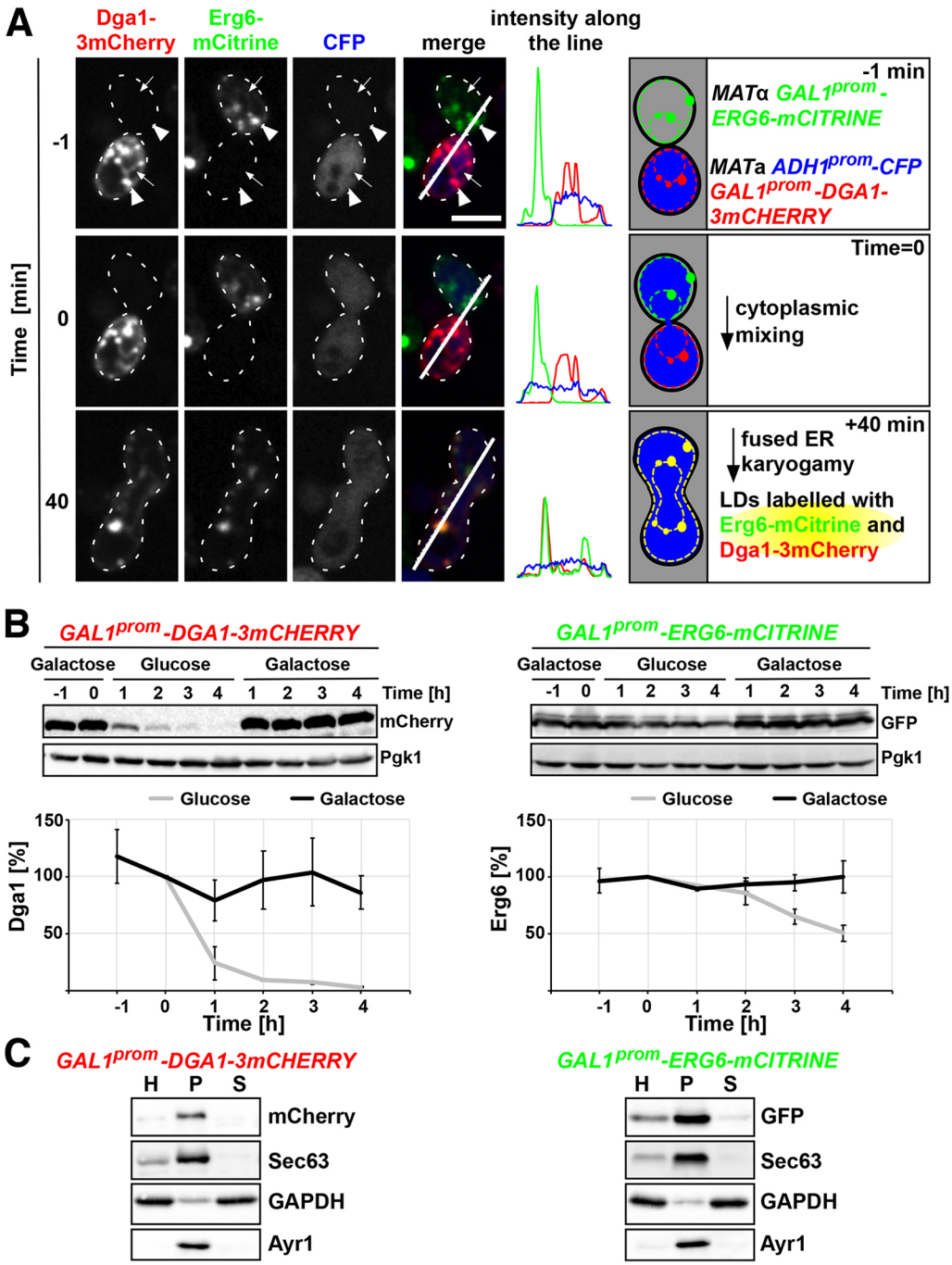

Fig. 1. Experimental design to monitor the exchange of LD proteins upon cell fusion. (A) A yeast mating-based assay to follow the exchange of LD-localized proteins. *MAT*a cells co-expressing Dga1–3mCherry (*GAL1*^{prom}*-DGA1-3mCHERRY*) with a soluble cytosolic CFP (*ADH1*^{prom}*-CFP*) and *MAT*α cells expressing Erg6–mCitrine (*GAL1*^{prom}*-ERG6-mCITRINE*) were grown in galactose-supplemented medium to induce expression of the markers. Cells were then mixed in a 1:1 ratio and shifted to glucose medium for 2 h before imaging. Cells were collected, mounted on a glass slide and covered with an agarose patch to allow live-cell imaging over an extended period of time. Time 0 of the mating event was defined as the time point of cytoplasmic mixing, monitored by the dispersion of CFP into the newly formed zygote. Shown are single confocal sections for each of the three fluorophores 1 min before (t=−1), at the time of cytoplasmic mixing (t=0), and 40 min after cell fusion had occurred. The fluorescence intensity profile along the white line shown in the merge is plotted. Colocalization of the two LD markers, Dga1–3mCherry and Erg6–mCitrine is indicated by yellow in the merge. A schematic illustration of the mating event and transfer of marker proteins is shown to the right. Arrows point to the ER membrane, arrowheads to LDs. Dashed white lines indicate cell outlines. Scale bar: 5 µm. (B) Turnover of Dga1–3mCherry and Erg6–mCitrine upon glucose repression. Cells expressing the LD marker proteins Dga1–3mCherry or Erg6–mCitrine under control of a galactose inducible promoter (*GAL1*^{prom}) were cultivated in galactose-containing medium and then shifted into fresh medium containing either glucose or galactose, samples were taken every hour and protein levels were analyzed by western blotting using the constitutively expressed 3-phosphoglycerate kinase (Pgk1) as loading standard. Protein levels, normalized to t=0, are plotted in the graphs. Values represent mean±s.d. of three independent experiments. (C) The LD marker proteins Dga1–3mCherry and Erg6–mCitrine are membrane associated. The fractionation properties of Dga1–3mCherry and Erg6–mCitrine was assessed by differential centrifugation. Equal amounts of proteins from the cell homogenate (H), the membrane pellet fraction (P, 30,000 ***g***), and the soluble supernatant (S) were separated by SDS-PAGE and probed with antibodies against mCherry, GFP, the soluble glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the LD-resident protein Ayr1, or Sec63, a component of the ER translocon.

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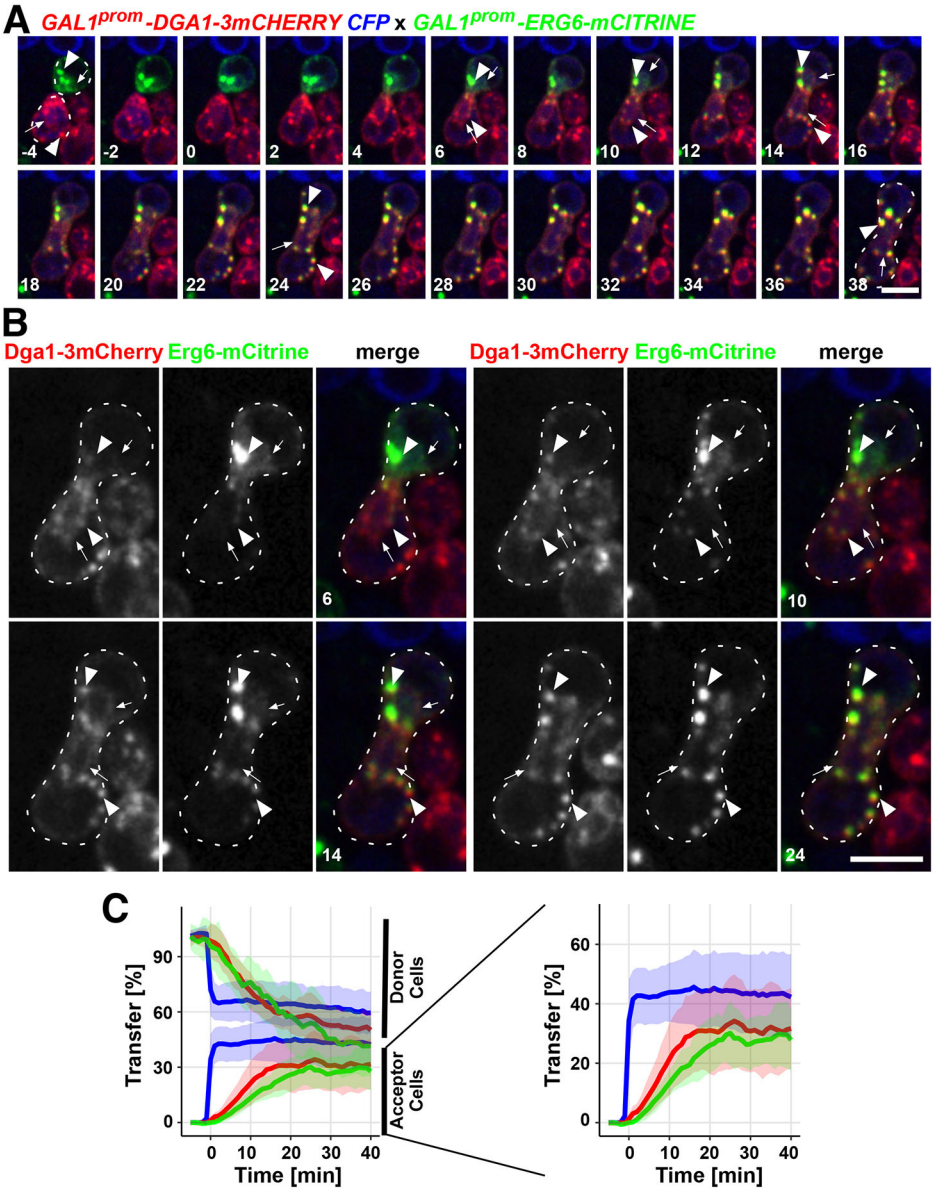

Fig. 2. Exchange of proteins between LDs of mating partners is continuous, reciprocal, and occurs on many LDs concurrently. (A) Exchange of LD proteins between mating partners. Representative time-lapse images showing the transfer of Dga1–3mCherry and Erg6–mCitrine into the mating partner. Images were acquired every 1 min, but only even time points are depicted starting 4 min before cytoplasmic mixing as monitored by spreading of the soluble CFP into the fusion partner (*t*=0). Arrows point to the ER membrane, arrowheads to LDs. Scale bar: 5 µm. (B) Enlarged views of the 6, 10, 14 and 24 min time points shown in A. Note the simultaneous and uniform acquisition of Erg6–mCitrine on multiple LDs containing Dga1–3mCherry, and vice versa. Arrows point to the ER membrane, arrowheads to LDs. Dashed white lines indicate cell outlines. Scale bar: 5 µm. (C) The exchange of LD proteins into the mating partner occurs through a continuous and reciprocal process. The graph shows the time-dependent decrease (upper part) and increase (lower part) in fluorescence in the mating partners. Transfer of Dga1–3mCherry is indicated by the red lines, that of Erg6–mCitrine by the green lines. The fast re-distribution of CFP fluorescence upon cytoplasmic mixing at *t*=0 is shown by the blue lines. The increase in fluorescence in the acceptor cells is enlarged in the graph to the right. Values represent mean±s.d. (shaded area) of ten individual mating events.

Sec63–mCherry fluorescence continuously increased in the acceptor cell and reached a plateau after ∼20 min of cytoplasmic mixing (Fig. 3A–C; Movie 2). This time-dependence and the redistribution of Sec63–mCherry between donor and acceptor cells was slightly faster compared to the transfer of the LD marker Erg6–mCitrine. The soluble cytoplasmic marker CFP, on the other hand, very rapidly mixed with the cytoplasm of the mating partner to reach a new steady-state distribution in the zygote within ∼1 min of cell fusion (Fig. 3C). Mitochondria, as monitored with a soluble MITO–3mCherry matrix protein, on the other hand, did not fuse to a significant degree with the organelle of the mating partner within this 20 min timeframe. However, at later time points, mitochondria started to appear in some of the acceptor cells, as indicated by a sudden increase of the standard deviation derived from monitoring 10 individual mating reactions (Fig. 3D–F; Movie 3) (Nunnari et al., 1997). Taken together, these observations suggest that LD proteins continuously and reciprocally exchange between the LDs of a newly formed zygote with a time dependence that is more similar to the exchange of ER proteins than that of mitochondrial fusion and/or transfer.

The exchange of LD proteins between mating partners is strongly reduced in mutants that affect ER fusion

Next, we tested whether ER fusion between the mating partners is required for the exchange of LD proteins. Therefore, we analyzed the transfer of LD marker proteins in mutant cells known to exhibit a delay in homotypic ER fusion. These mutants bear defects in the yeast ortholog of atlastin, Sey1, a dynamin-like GTPase required for ER fusion, and in the Dsl1 tethering complex. These *sey1*Δ *dsl1*Δ*E* double mutants have previously been shown to reduce ER fusion in bi-parental matings, in which both mating partners are deficient for Sey1 and Dsl1 (Rogers et al., 2014). Mating of *sey1*Δ *dsl1*Δ*E* mutant cells expressing Dga1–3mCherry with *sey1*Δ *dsl1*Δ*E* mutant cells expressing Erg6–mCitrine resulted in a significantly slower initial rate of exchange of the two LD proteins between the mating partners than with wild-type cells. Moreover, time-lapse images indicate that the marker proteins did not significantly colocalize even after 20 min of cytoplasmic mixing (Fig. 4A,C; Movie 4). Colocalization of the two LD proteins was stalled at the mating neck, where ER membranes appeared to accumulate. This is likely due to the severe structural defects of the ER in *sey1*Δ *dsl1*Δ*E* mutant cells, which are

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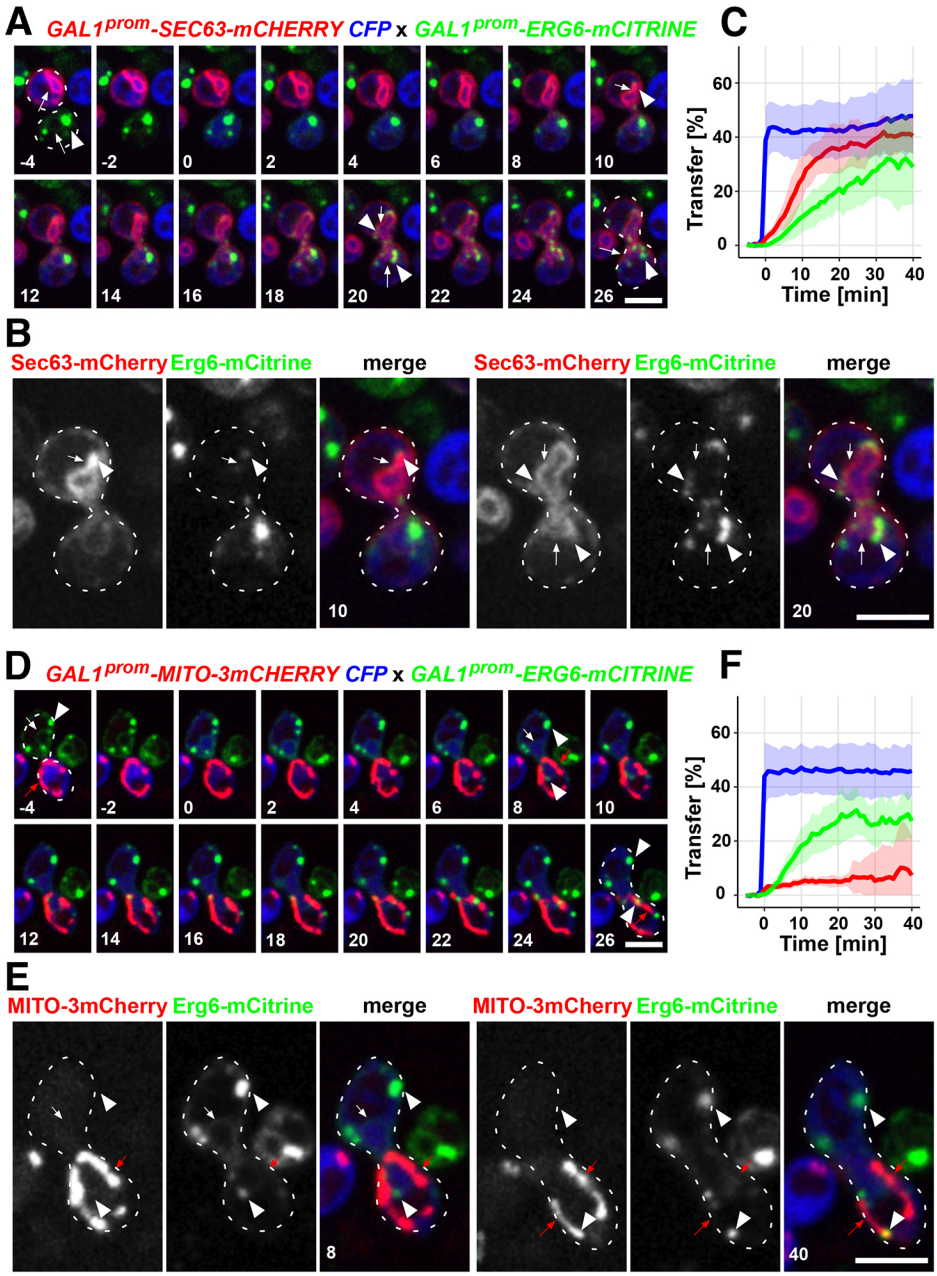

Fig. 3. Comparison of the transfer of LD proteins to that of the ER protein Sec63 and to a mitochondrial marker. (A,D) Comparison of transfer of proteins localized to LDs, the ER membrane or mitochondria into the mating partner. Representative time-lapse images showing the transfer of the ER translocon component Sec63–mCherry (A), or the mitochondrial marker MITO–3mCherry (D) into the mating partner expressing the LD marker Erg6–mCitrine. Images were acquired every 1 min, but only even time points are depicted starting 4 min before cytoplasmic mixing, as monitored by spreading of the soluble CFP into the fusion partner (*t*=0). White arrows point to the ER membrane, red arrows to mitochondria, and arrowheads to LDs. Scale bars: 5 µm. (B,E) Enlarged views of the 10 and 20 min time points shown in A (B) and of the 8 and 40 min time points after cytoplasmic mixing represented in D (E). Note the close apposition of Erg6–mCitrine to the ER (B). White arrows point to the ER membrane, red arrows to mitochondria and arrowheads to LDs. Scale bars: 5 µm. (C,F) The graphs show the time-dependent increase in fluorescence in the mating partners. Transfer of Sec63–mCherry (C) and MITO–3mCherry (F), respectively, is indicated by the red lines. Transfer of Erg6-mCitrine onto acceptor LDs is indicated by the green lines. The fast increase in CFP fluorescence upon cytoplasmic mixing at *t*=0 is shown by the blue lines. Note that MITO–3mCherry is not transferred continuously to the mating partner, as is Sec63–mCherry and Erg6–mCitrine. Values represent mean±s.d. (shaded area) of ten mating events. Dashed white lines indicate cell outlines.

known to accumulate ER at the bud neck (Fig. 4B) (Rogers et al., 2014). These results indicate that mutations that delay ER fusion also reduce the exchange of LD-localized proteins between mating partners, suggesting that ER fusion is required for efficient exchange of these LD proteins.

The delay in ER fusion in *sey1*Δ *dsl1*Δ*E* mutant cells was confirmed by analyzing the transfer of the ER luminal protein ss-mCherry-HDEL, a signal-sequence (ss)-containing mCherry bearing a C-terminal ER retention signal (HDEL) (Fig. 4D) (Pelham et al., 1988). The averaged transfer curve of ss-mCherry-HDEL showed only a moderate delay in these mutant cells, probably due to the heterogeneity in the initiation of ER fusion during mating (Rogers et al., 2014). However, in recordings of single mating events the delay in transfer of the ER marker is more clearly visible, confirming the ER fusion phenotype (Fig. 4E). Taken together, these results indicate that the efficient transfer of proteins between LDs of mating partners is delayed in mutants that affect ER fusion and thus likely depends of the formation of a common and continuous ER membrane between the mating partners upon zygote formation.

LD proteins are transferred from the ER membrane of one mating partner to LDs of a recipient

To examine whether the exchange of proteins between LDs of mating cells occurs via the ER membrane, we first tested whether LD proteins could re-localize from the ER membrane of a donor cell onto LDs of a mating partner. Cells lacking the four enzymes for neutral lipid biosynthesis, Are1 and Are2 for the synthesis of steryl esters, and Dga1 and Lro1 for the synthesis of TAG, have no detectable LDs (Sandager et al., 2002). In these quadruple mutant cells (*are1*Δ *are2*Δ *dga1*Δ *lro1*Δ), membrane-associated proteins that are normally present on LDs are localized to the ER membrane (Jacquier et al., 2011). To test whether these ER-localized LD residents would re-localize from the ER of a donor onto LDs of a

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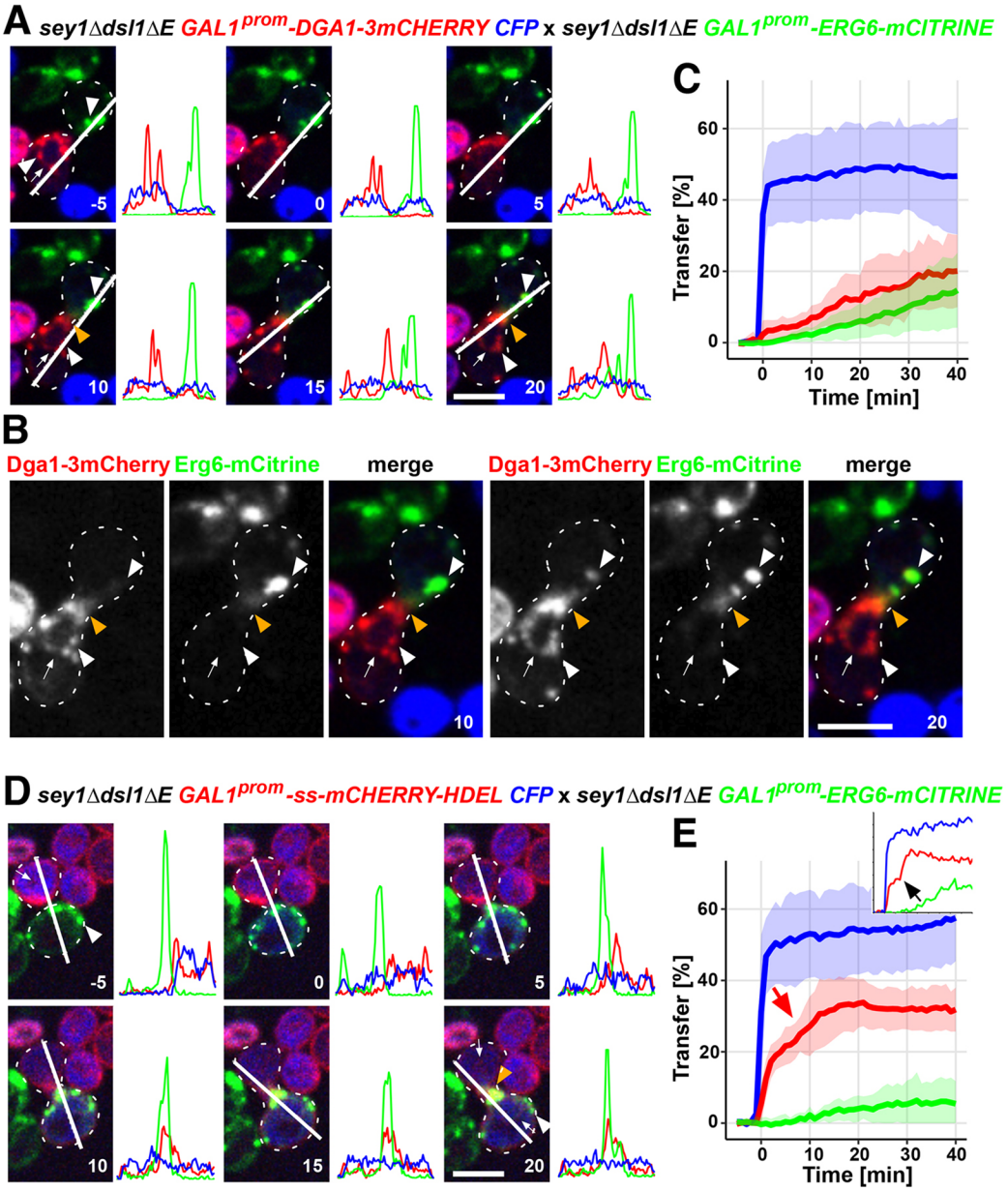

Fig. 4. Transfer of LD proteins between mating partners in mutants with defects in ER fusion. (A) Time-lapse images of mating reactions to monitor the exchange of Dga1–mCherry and Erg6–mCitrine between mutant cells having defects in ER fusion (*sey1*Δ *dsl1*Δ*E*). Images shown are separated by 5 min intervals over a period of 25 min, starting 5 min (*t*=−5) before cytoplasmic mixing (*t*=0), but mating progression was recorded every minute. Fluorescence intensity profiles along the white line crossing through the mating partners are plotted. Arrows point to the ER membrane, arrowheads to LDs. Scale bar: 5 µm. (B) Enlarged views of the mating reaction after 10 min and 20 min of cytoplasmic mixing, respectively. Arrows point to the ER membrane, white arrowheads to LDs, and orange arrowheads to membrane accumulation at the fusion neck. Scale bar: 5 µm. (C) The transfers of the marker proteins between the mating cells are plotted in the graph. The red line represents Dga1–3mCherry, the green line represents Erg6–mCitrine and the blue line represents CFP. Values represent mean±s.d. (shaded area) of ten individual mating events with data acquired at 1 min intervals. (D) Transfer of the ER luminal ss-mCherry-HDEL and Erg6–mCitrine between *sey1*Δ *dsl1*Δ*E* mating partners. Imaging conditions were as described for A. Arrows point to the ER membrane, white arrowheads to LDs, and the orange arrowheads to membrane accumulation at the fusion neck. Scale bar: 5 µm. (E) The rates of transfer of ss-mCherry-HDEL between *sey1*Δ *dsl1*Δ*E* mutant partners. The red line corresponds to ss-mCherry-HDEL, the green line to Erg6–mCitrine and CFP is represented by the blue line. The red arrow points to the flattening of the average transfer curve of ss-mCherry-HDEL, indicating a delay in ER fusion. This delay, followed by a sharp increase, is more apparent in single mating events, as shown in the inset and indicated by a black arrow. Values represent mean ±s.d. (shaded area) of ten individual mating events with data acquired at 1 min intervals. Dashed white lines indicate cell outlines.

recipient, we analyzed mating between quadruple mutant cells expressing ER-localized Erg6–mCitrine and wild-type cells expressing Dga1–3mCherry. In the resulting zygotes, Erg6–mCitrine was transferred onto acceptor LDs in a very similar manner to what we previously observed for the exchange of LD proteins between wild-type cells (Fig. 5A,B; Movie 5). Concurrently, Dga1–3mCherry also redistributed from the donor cell into the ER of the recipient lacking LDs, suggesting that LD proteins continuously equilibrate between their LD and ER localization (Fig. 5; Movie 5). Under these conditions, transfer of ER-localized Erg6–mCitrine to the mating partner seemed to precede that of the LD-localized Dga1–3mCherry (Fig. 5C).

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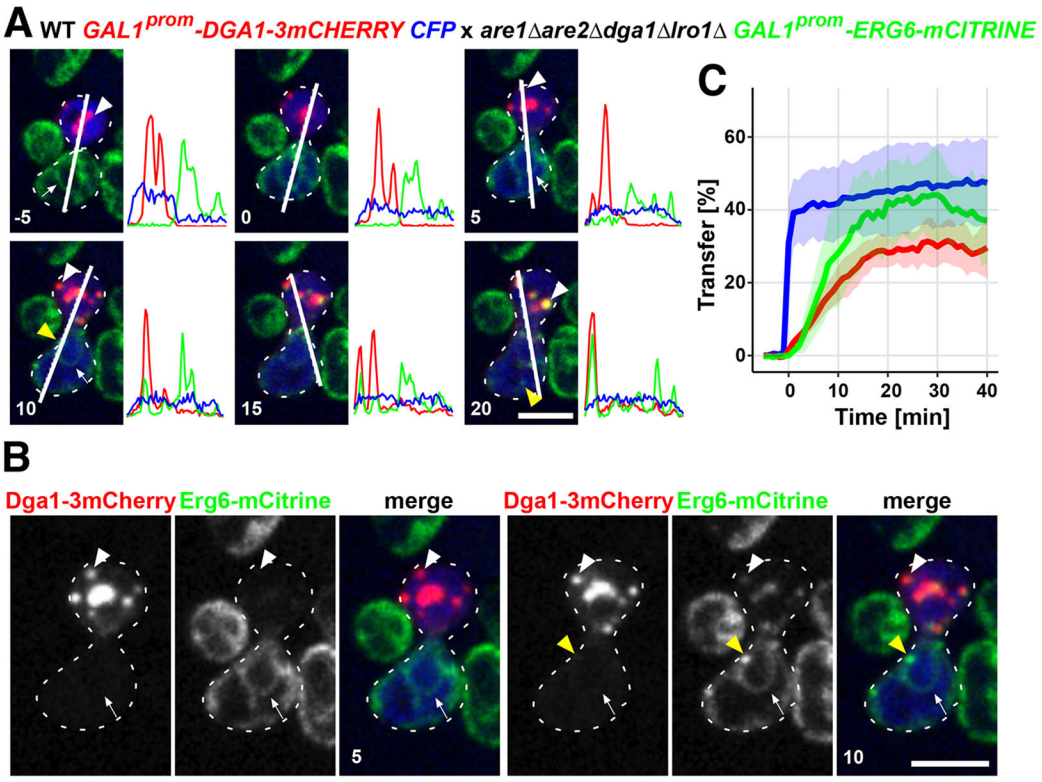

Fig. 5. Transfer of an LD protein from the ER of a donor cell to LDs of an acceptor mating partner. (A) Time-lapse images of mating reactions to monitor the dynamics of Erg6–mCitrine located in the ER of a mutant cell lacking LDs (*4*Δ; *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ) and Dga1–3mCherry labeling of LDs in a wild-type strain (WT). Images shown are separated by 5 min intervals over a period of 25 min, starting 5 min (*t*=−5) before cytoplasmic mixing (*t*=0). Fluorescence profiles along the white line crossing through the mating partners are plotted. The ER membrane is highlighted by arrows, arrowheads point to LDs; white for the LDs in the WT cell and yellow for LDs that appears to form in the *4*Δ mutant. Scale bar: 5 µm. (B) Enlarged views of the mating reaction at 5 min and 10 min after cytoplasmic mixing. Arrows point to the ER membrane, arrowheads to LDs; the yellow arrowheads point to an LD that appears to form in the ER of the *4*Δ mutant mating partner. Scale bar: 5 µm. (C) The rate of transfer of the marker proteins between the mating cells is plotted in the graph. The red line represents Dga1–3mCherry, the green line represents Erg6–mCitrine and the blue line represents CFP. Values represent mean±s.d. (shaded area) of ten individual mating events with data acquired at 1 min intervals. Dashed white lines indicate cell outlines.

Following mating, Erg6–mCitrine-labeled punctate structures quickly appeared in the ER of the recipient quadruple mutant. The rapid appearance of such newly formed LDs is likely due to the redistribution of neutral lipid biosynthetic enzymes from the ER of the wild-type partner into the ER of the quadruple mutant, as is indeed observed with mCherry-labeled Dga1, a TAG biosynthetic enzyme (Fig. 5; Movie 5). In addition, neutral lipids contained within the ER membrane of the wild-type donor could diffuse into the quadruple mutant mating partner upon ER fusion, and thereby contribute to the emergence of LDs. Taken together, these results indicate that LD proteins redistribute between their ER and LD localization upon fusion of the ER membranes of the mating partners.

LD proteins quickly move from the ER to LDs, but only slowly dissociate from LDs

To examine the exchange of LD-localized proteins between their ER localization and their LD association in more detail, we analyzed the exchange of Erg6–mCitrine and that of the ER luminal protein ss-mCherry-HDEL in different mating combinations between wild-type and *4*Δ (*are1*Δ *are2*Δ *dga1*Δ *lro1*Δ) cells (Fig. 6A–D). The resulting transfer curves for individual mating events were plotted and fitted to the Hill equation (Fig. 6E,F). From this mathematical modeling of single transfer curves, we could calculate the half-time of transfer of Erg6–mCitrine and ss-mCherry-HDEL (Fig. 6G). While the median half-times of transfer of ss-mCherry-HDEL were similar for all mating combinations, ranging from 1.5 to 2.4 min, the transfer rates of Erg6-mCitrine fell into two separate groups. The first group contained mating events in which Erg6-mCitrine was transferred from the ER to the ER (ER→ER, 5.2 min; i.e. mating between two *4*Δ mutant cells, *4*Δ×*4*Δ) or from the ER onto LDs (ER→LD, 6.4 min; i.e. mating between *4*Δ mutant cells and wild-type, *4*Δ×WT). The second group was composed of mating events in which Erg6–mCitrine had to dissociate from a donor LD to localize on an acceptor LD (LD→LD, 11.4 min; i.e. mating between two wild-type cells, WT×WT) or in which Erg6–mCitrine moved from LDs into the ER (LD→ER, 12.2 min; i.e. mating between wild-type and *4*Δ mutant, WT×*4*Δ). The differences in the median transfer rates between these two groups were statistically significant, indicating that the association of the protein from the ER onto LDs is about twice as fast as its dissociation from LDs into the ER. These data thus suggest that the relative affinity of Erg6 for LDs is higher than its affinity for the ER membrane.

Seipin affects the exchange of proteins between the ER and LDs

Seipin is an ER membrane protein required for proper formation of LDs. In the absence of seipin, cells either have many small clustered LDs, or they form large supersized LDs. Seipin forms disk-shaped oligomeric structures within the ER membrane at the base of LDs

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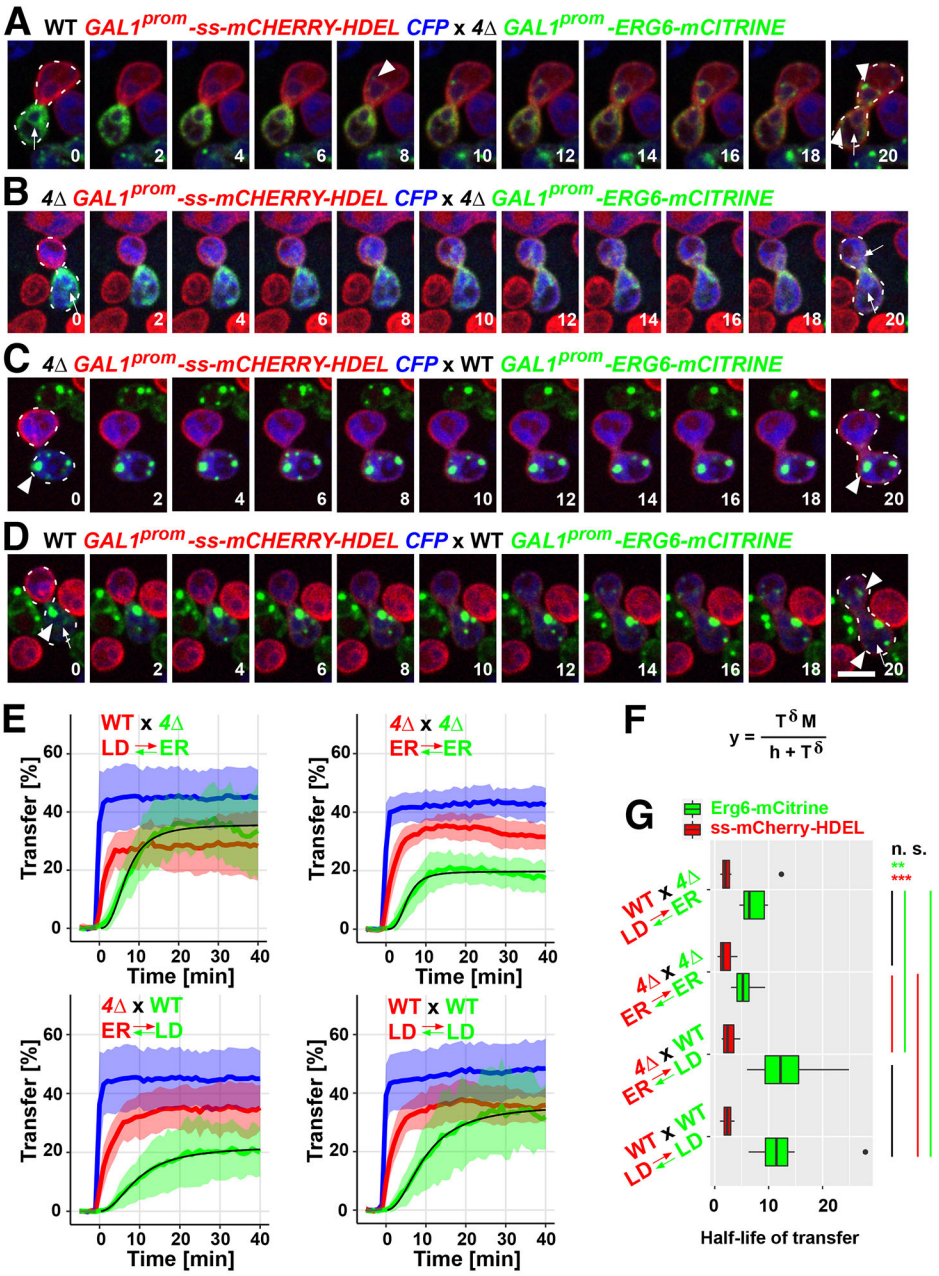

Fig. 6. Comparison between the rates with which proteins associate and dissociate from LDs. (A–D) Time-lapse images of mating reactions to monitor the exchange of the ER luminal marker ss-mCherry-HDEL and Erg6–mCitrine between wild-type (WT) cells containing LDs or mutant cells lacking LDs (4Δ; *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ). Images were recorded at 1 min intervals over a period of 20 min from the time of cytoplasmic mixing (*t*=0). Images shown represent 2 min intervals. Arrows point to the ER membrane, arrowheads to LDs. Dashed white lines indicate cell outlines. Scale bar: 5 µm. (E) The rates of transfer of marker proteins between mating partners of the indicated genotypes are plotted in the graphs. Red lines represent ss-mCherry-HDEL, green lines represent Erg6–mCitrine and blue lines represent CFP. Fitting of the rate of transfer of Erg6–mCitrine to the Hill equation is indicated by the thin black line. Values represent mean±s.d. (shaded area) of nine different mating events with data acquired at 1 min intervals. (F) Hill equation where T=time, M=maximal fluorescence intensity, $h=½T^{\delta}$, δ=slope of the curve. (G) The median half-life of transfer ($h^{1/\delta}$) of Erg6–mCitrine (green) and ss-mCherry-HDEL (red) was calculated by fitting transfer curves to individual mating events and analyzing the data in a box plot. Difference between the median half-life of the transfer of ss-mCherry-HDEL from the donor ER to the recipient ER are statistically non-significant, as indicated by a Kruskal–Wallis *P*-value of 0.52. On the other hand, the *P*-value for the median half-life of transfer of Erg6–mCitrine between the ER and LDs is 0.0001, indicating that these differences are significant. Therefore, for this data set, the statistical significance between the different mating combinations was calculated by a pairwise comparisons using the Wilcoxon rank-sum test. n.s., not significant (black). ***P*<0.01 (green); ****P*<0.001 (red); *n*>9 individual mating events.

and thereby connects LDs to the ER membrane (Salo et al., 2020). To examine whether seipin is also important for the exchange of proteins between the two compartments, we analyzed the exchange of Dga1–3mCherry and Erg6–mCitrine between LDs of mating partners lacking seipin. Both Dga1–3mCherry and Erg6–mCitrine relocated from one seipin mutant (*fld1*Δ) mating partner to the other (Fig. 7A,B), yet they both appeared on LDs of the mating partner with some delay when compared to wild-type cells (Fig. 7A; Movie 6). To quantify this delay, we analyzed the appearance of Dga1–3mCherry on Erg6–mCitrine-containing acceptor LDs by calculating the ratio of Erg6–mCitrine flurorescence to that of Dga1–3mCherry over time (Fig. 7C). Starting from 10 min of cytoplasmic mixing, this ratio was significantly elevated in wild-type compared to seipin mutant cells, indicating that seipin affects transfer of proteins between LDs of the two mating partners. Furthermore, heterogenous labeling of the LD population was observed in the seipin mutant, supporting the hypothesis that cargo delivery was impaired due to aberrant formation of ER–LD contact sites (Grippa et al., 2015; Salo et al., 2016). While exchange of both Dga1–3mCherry and Erg6–mCitrine is reduced in seipin mutant cells, the exchange of the ER marker ss-mCherry-HDEL was not affected during mating of seipin mutant cells (Fig. S2).

Seipin complexes are stable and do not mix upon zygote formation

To monitor the distribution of seipin upon zygote formation, we tagged seipin genomically with two molecules of mCherry (*FLD1-2mCHERRY*) and mated these cells with wild-type cells expressing Erg6-mCitrine. Before zygote formation, seipin was detectable as single puncta. Upon zygote formation, these seipin puncta started to become decorated with Erg6–mCitrine, indicating that Erg6–mCitrine re-equilibrated its distribution to localize to pre-existing LDs from the mating partner (Fig. 8A,B; Movie 7).

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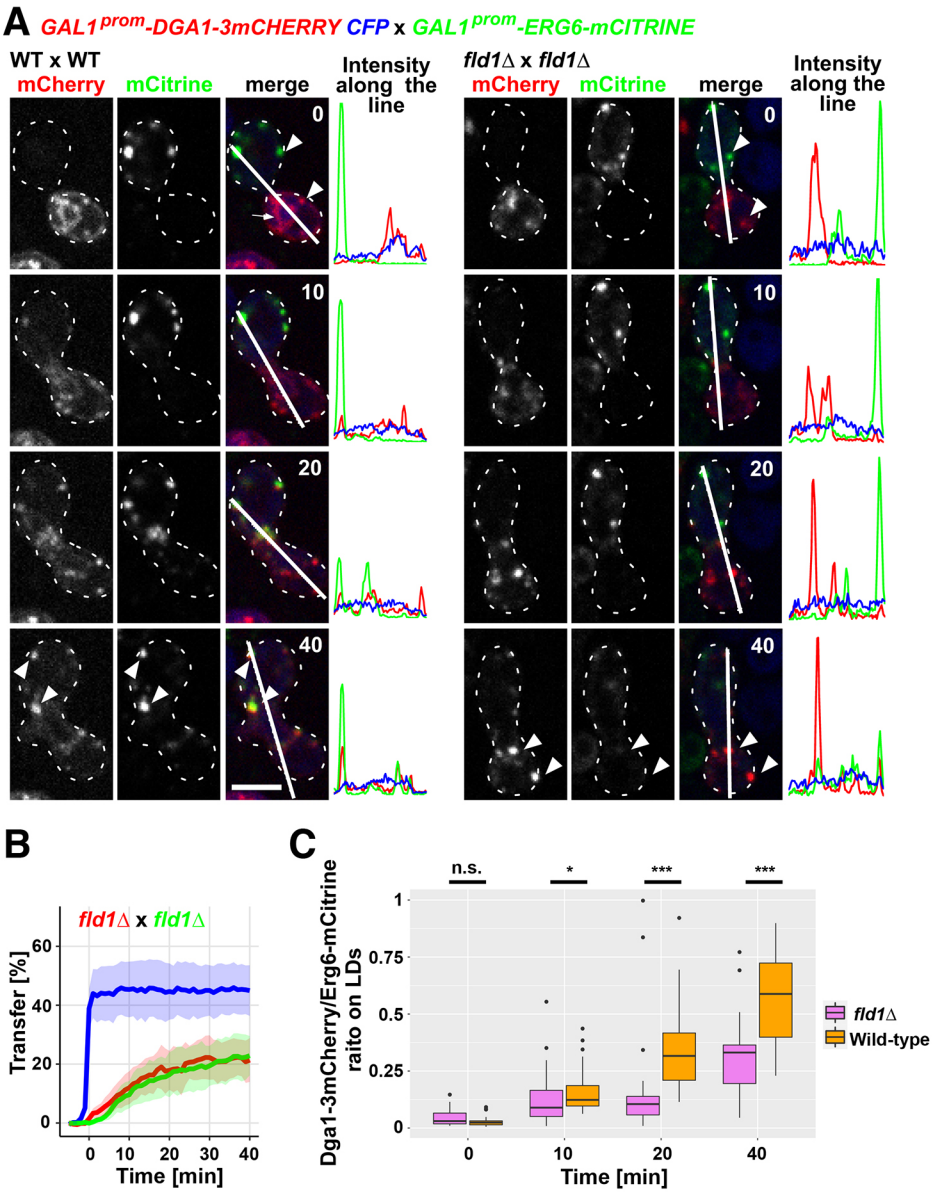

Fig. 7. Seipin is required for efficient targeting of proteins to LDs. (A) Time-lapse images showing the exchange of the LD proteins Dga1–3mCherry and Erg6–mCitrine between wild-type cells (WT, left panels) and seipin mutant cells (*fld1*Δ, right panels). Images shown were acquired at time of cytoplasmic mixing (*t*=0 min), and after 10, 20 and 40 min. Fluorescence intensity profiles along the white line crossing through LDs of both mating partners are plotted to the right of the merge. Red lines represent Dga1–3mCherry, green lines represent Erg6–mCitrine and blue lines show CFP fluorescence. Arrows point to the ER membrane, arrowheads to LDs. Note that the white arrowheads at the 40 min time point indicate LDs with similar ratios of the two marker proteins in WT cells, but differences in marker protein composition in seipin mutant diploids. Dashed white lines indicate cell outlines. Scale bar: 5 µm. (B) Rate of transfer of Dga1–3mCherry and Erg6–mCitrine between seipin mutant (*fld1*Δ) cells. Same color code as in A. Values represent mean ±s.d. (shaded area) of ten individual mating events with data acquired at 1 min intervals. (C) The relative fluorescence of Dga1–3mCherry on Erg6–mCitrine-labeled LDs is shown in the box plot. Wild-type data are shown in orange, *fld1*Δ in pink. Statistical significance was scored with the Wilcoxon rank-sum test (*n*>19 LDs). n.s., not significant; **P*<0.05; ****P*<0.001.

To address whether seipin itself would mix and colocalize with the seipin complex of the mating partner, we tagged seipin genomically with a 7× concatenated split variant of GFP ($GFP11_{x7}$) and expressed the remaining part of GFP (GFP1-10) from a plasmid (Kamiyama et al., 2016). Both color variants of seipin localized to the base of Bodipy-stained LDs and the mCherry-tagged seipin defined the site of LD formation when quadruple mutant cells lacking LDs (4Δ, *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ) were mated with wild-type cells expressing Erg6–mCitrine (Fig. S3). When cells expressing the red fluorescently tagged seipin (Fld1–2mCherry) were mated with cells expressing the split-GFP variant of seipin, the red and green fluorescent puncta appeared to remain stable with hardly any colocalization within a 40 min time-frame after cytoplasmic mixing (Fig. 8C; Movie 8). This degree of non-colocalization in the zygote was similar to that observed when haploid cells of the same mating type, expressing these two color-variants of seipin were mixed (Fig. 8C,D, no mating). On the other hand, when the two color-variants of seipin were simultaneously expressed in a diploid cell, clear colocalization was observed (Fig. 8C,D, diploid). Similarly, zygote formation between cells expressing Dga1–3mCherry and Erg6–mCitrine resulted in rapid colocalization of these two LD markers, but they remained separated when haploid cells of the same mating type were mixed, but colocalized when simultaneously expressed in a diploid (Fig. 8C). Quantification of the colocalization of the two color-variants of seipin in comparison to the two LD markers indicated that seipin spots do not mix between mating partners upon zygote formation (Fig. 8D). Thus, taken together, unlike what is seen for the membrane-anchored LD marker proteins, Dga1 and Erg6, which rapidly re-equilibrate their LD localization upon zygote formation, seipin puncta remain stable and do not exchange monomers upon merging of the ER membrane between the two mating partners.

DISCUSSION

In this study, we analyzed the transfer of LD proteins between mating pairs *in vivo*. This transfer is continuous, and reciprocal and appears to simultaneously occur on the majority of LDs of the newly formed zygote. This indicates that these proteins rapidly re-equilibrate their localization to reach a new steady-state distribution within ~20 min of cytoplasmic mixing (Figs 1 and

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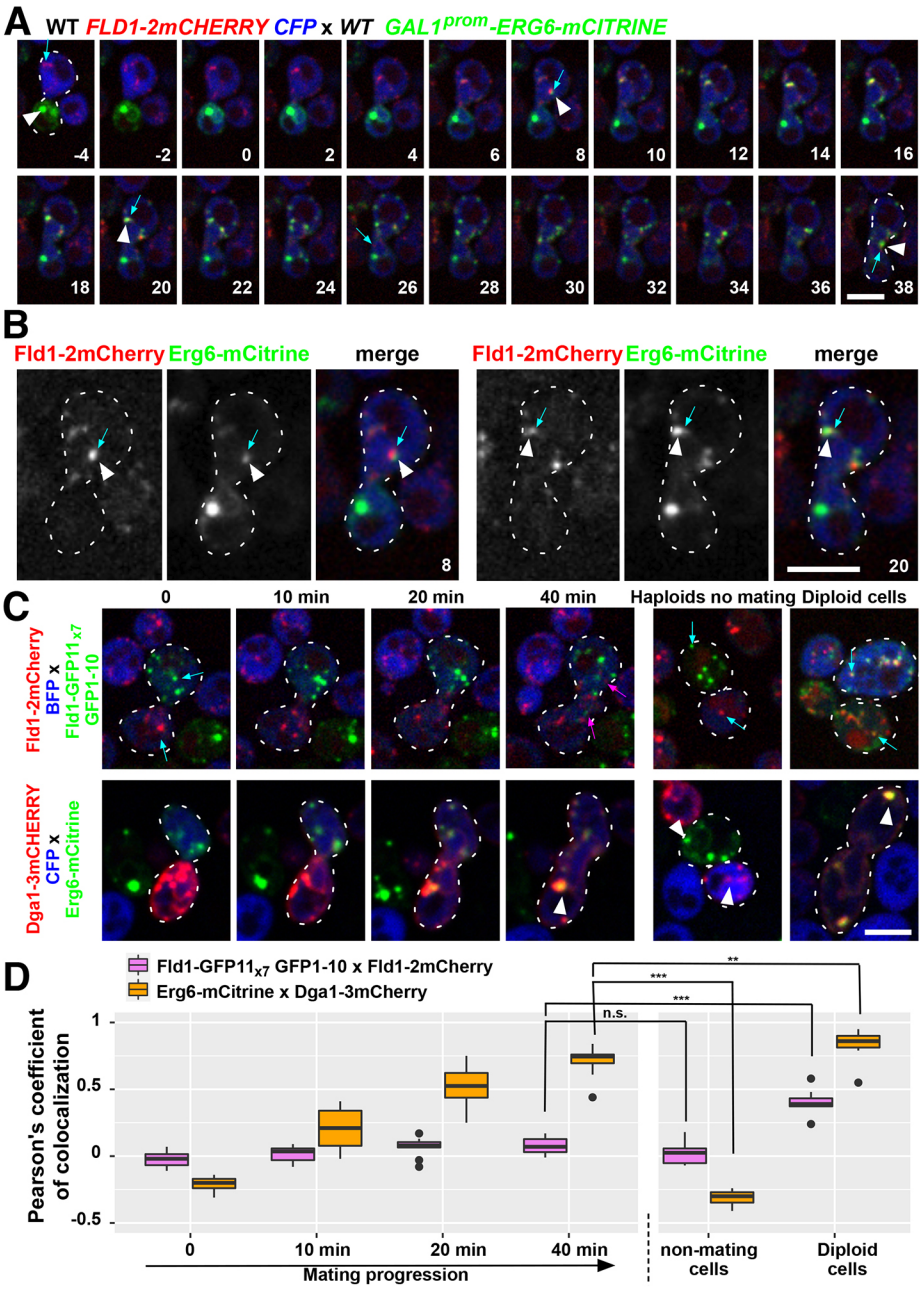

Fig. 8. Seipin is required for efficient targeting of proteins to LDs. (A) Erg6–mCitrine accumulates in the mating partner at ER sites marked by seipin. Representative time-lapse images showing the transfer of Erg6–mCitrine into a mating partner expressing a 2mCherry-tagged seipin, Fld1–2mCherry. Images shown were recorded at 2 min intervals, starting 4 min (*t*=−4) before cytoplasmic mixing (*t*=0), over a period of 42 min. Blue arrows point to the seipin/Fld1 spots, arrowheads to LDs. Scale bar: 5 µm. (B) Enlarged views of the 8 and 20 min time points shown in A. Blue arrows point to the seipin/Fld1 spots, arrowheads to LDs. Scale bar: 5 µm. (C) Seipin domains are stable and do not mix. Representative time-lapse images to monitor the transfer of Fld1–2mCherry and Fld1–GFP11$_{x7}$ GFP1-10 (upper row) or that of Dga1–3mCherry and Erg6–mCitrine (bottom row) upon zygote formation, at 0, 10, 20 and 40 min of cytoplasmic mixing. The same combination of proteins was also imaged in non-mating haploid cells and in diploids. Note the colocalization of mCherry- and GFP-tagged seipin when both variants are simultaneously expressed in diploid cells. Blue arrows point to seipin spots, magenta arrows point to seipin spots that have moved into the mating partner at the 40 min time point and white arrowheads point to LDs. Scale bar: 5 µm. (D) Upon mating, Fld1-tagged proteins do not merge as LD marker proteins do. Boxplot representing Pearson correlation coefficients of colocalization between mCherry- and GFP-tagged seipin variants (Fld1-2mCherry and Fld1-GFP11$_{x7}$ GFP1-10; pink boxes), and between the LD markers Dga1-3mCherry and Erg6-mCitrine (orange boxes), upon progression of zygote formation, i.e., after 0, 10, 20, and 40 min of cytoplasmic mixing. The same analysis is shown for haploid non-mating cells and for diploid cells expressing both fluorophores simultaneously. Statistical significance was scored with the Wilcoxon rank-sum test. n.s., not significant; ***P*<0.01; ****P*<0.001; *n*=10 mating events or cells. Dashed white lines indicate cell outlines.

2). The two membrane-anchored LD proteins Dga1 and Erg6 tested here displayed similar transfer rates, suggesting that the observations made are likely valid for a wider range of proteins containing a hydrophobic hairpin type of LD-targeting determinant (Fig. 2). These LD proteins thus re-equilibrate their distribution at a rate that is slower than the re-equilibration of an ER-resident protein, such as Sec63 (Fig. 3). However, re-equilibration is strongly delayed in mutants with defects in homotypic ER fusion, that is, *sey1*Δ *dsl1*Δ*E*, suggesting that transfer of the LD proteins occurred through an interconnecting ER membrane (Fig. 4). Consistent with this proposition, transfer of ER-localized LD proteins from cells that have no LDs, that is, *4*Δ mutant cells lacking all four enzymes required for neutral lipid synthesis (*are1*Δ *are2*Δ *dga1*Δ *lro1*Δ), to the LDs of a mating partner occurs at a rate that is almost twice as fast as the transfer between LDs (Figs 5 and 6). These data indicate that LD proteins are in a constant equilibrium between their ER and LD localization, and that they associate more readily from the ER membrane with the LD surface than they dissociate back from the LD surface into the ER membrane. Taken together, these observations suggest that LDs form a network of compartments that are interconnected through the ER membrane. The data, on the other hand, do not support a model where LDs stay disconnected from the ER membrane for an extended period of time and in which the transfer of LD proteins would require homotypic LD–LD fusion. The network model is also supported by the observation that seipin is required for efficient transfer of LD proteins, because seipin localizes to and likely even forms the connection between the ER membrane and LDs (Choudhary and Schneiter, 2021; Salo et al., 2020).

A model of LDs forming a network of compartments interconnected by the ER membrane is consistent with previous observations showing uniform incorporation of TAG into existing

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LDs by quantitative electron microscopy (EM) and fluorescent microscopy using fluorescent polyene lipids in living cells (Cheng et al., 2009; Kuerschner et al., 2008). Similarly, polarized flow cytometry indicates that newly synthesized steryl esters are incorporated from the ER into pre-existing LDs rather than forming new droplets (Kellner-Weibel et al., 2001). In addition, label free holo-tomographic microscopy indicates that newly formed LDs are created at the expense of older LDs, indicating that LDs exchange material over relatively short timescales, possibly through an interconnecting ER membrane (Sandoz et al., 2019).

Our data indicate that seipin is required for efficient exchange of LD proteins upon zygote formation (Fig. 7). Seipin has previously been shown to be important to control lipid exchange through a process termed ripening, that is, the transfer of lipids between adjacent LDs through the interconnecting ER membrane. Thereby seipin ensures uniform LD growth and controls the size distribution of the LD population (Salo et al., 2019). In addition, seipin mutant cells have an altered composition of the LD membrane and the LD proteome, and thus fail to properly establish LD identity (Grippa et al., 2015; Salo et al., 2016). Seipin is thus important to control the exchange of both lipids and proteins between the ER membrane and LDs (Salo et al., 2020). In the absence of seipin, LDs are formed at ectopic sites through the ER membrane, indicating that seipin controls both initiation of LD formation and their subsequent growth (Choudhary et al., 2020). The observation that seipin complexes do not mix upon ER fusion and zygote formation indicates that the seipin ring complex is stable and that there is no or little exchange of monomers between the oligomeric ring complexes (Fig. 8). Hairpin-anchored LD proteins, however, do exchange between LDs, suggesting that they can traverse through the stable seipin-mediated connection between the ER membrane and the LD surface. LD-targeted proteins that are anchored to the ER membrane through one or multiple transmembrane domains, however, cannot diffuse onto the LD surface, indicating that the ER–LD interface is 'transparent' for hairpin-anchored proteins only (Khaddaj et al., 2022).

Targeting of protein to the LD surface has been proposed to be limited by protein crowding, that is, the saturation of all available LD surface cues through binding to proteins. Under such crowding conditions, newly made LD proteins could no longer localize to the LD surface and hence would possibly either be degraded or stay in the ER membrane (Ruggiano et al., 2016). We observe a flattening of the transfer curve after ~20 min of zygote formation and believe that this reflects a newly reached steady-state distribution of the LD proteins rather than a saturation of the capacity of the LD surface to acquire more protein. This interpretation is supported by the observation that the acceptor cell continues to acquire more of the LD marker under conditions of continued protein synthesis, that is, when Erg6–mCitrine is controlled by its native promoter (Fig. S1A,B). Both LD marker proteins used in this study are overexpressed from a strong galactose-regulated promoter. The promoter is turned off 2 h before cells are mated, and both markers display a similar fluorescence intensity distribution on LDs at steady state (Figs 1A, 2B) with only a small fraction remaining visible in the ER membrane. Similarly, in mating with 4Δ mutant cells, the LD marker that resides in the ER of the quadruple mutant is rapidly targeted to the LD surface of the recipient mating partner. This suggests that the capacity of the LD surface to host these markers is not limiting. Thus, under these dynamic *in vivo* conditions, molecular crowding on the LD surface does not appear to limit the capacity of the LD to adapt its surface proteome. This situation, however, may change upon induction of lipolysis and shrinkage of the LD surface, resulting in displacement of surface proteins (Kory et al., 2015).

Why and how exactly do these membrane anchored proteins equilibrate their LD localization upon mating and fusion of the ER membranes? While under non-mating conditions, it is likely that the shrinkage of LDs due to lipolysis induces a relocalization of the LD markers back into the ER membrane, the situation under the mating conditions analyzed here, are likely different. The mating reactions are accompanied by homotypic fusion of the ER membranes between the two mating partners. This ER fusion is required for efficient re-equilibration of the LD markers. Could this ER fusion induce conditions that are comparable to those observed under lipolysis? Karyogamy is typically followed by a rapid cell division requiring membrane proliferation. However, even if zygote formation would induce lipolytic conditions, this could only account for the re-localization of the LD marker protein from its LD localization back to an ER localization, but not for the apparent uniform and bidirectional transfer between the LDs of the two mating gametes. It seems possible that not only the membrane anchored LD-localized proteins re-equilibrate upon ER fusion but that this protein exchange is actually accompanied by a similar re-equilibration of the neutral lipid content of these LDs. In that case, both membrane-anchored proteins and neutral lipids could diffuse through the newly formed common ER membrane, which would act as a solvent, to catalyze a new steady-state distribution of the LD content. How this re-equilibration is driven is unclear, but membrane tension and Ostwald ripening might promote such a process (Salo et al., 2019; Thiam and Forêt, 2016).

The mating-based readout established here, will likely be helpful to monitor the time-dependent transfer of lipids and proteins to the ER–LD interface and the LD surface in living cells. Thereby, this *in vivo* assay might help to unravel processes that govern the initiation of LD formation, maintenance of LDs and their turnover, and also the mechanisms that coordinate the expansion and growth of this interconnected network of lipid stores.

MATERIALS AND METHODS

Yeast strains, growth media and plasmid preparation

Yeast strains were cultivated in minimal defined media containing 0.67% yeast nitrogen base without amino acids (US Biological), 0.73 g/l amino acids and 2% carbon source [glucose, raffinose or galactose, depending on the need (US Biological)].

Strains used in this study are listed in Table S1. Starting from the Euroscarf single mutants of the four genes involved in the final step of neutral lipid biosynthesis, *ARE1, ARE2, DGA1, LRO1*, the *MAT*α *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ quadruple mutant strain was obtained after a series of mating, sporulation and assessment of genotypes by PCRs. Mating type was determined by crossing the candidate strains with tester strains *MAT*α *thr* and *MAT*a *thr* and analyzing the auxotrophy of the diploid. *MAT*α cells were then transformed with plasmid pGAL-HO for mating type switching (Herskowitz and Jensen, 1991). Diploid cells were obtained by crossing haploid cells and selected in the appropriate minimal medium. *MAT*a and *MAT*α *sey1*Δ *dsl1*Δ*E* were obtained by sporulation of the strain MY14769 (a kind gift from Mark Rose, Dept. of Molecular Biology, Princeton University, USA) and the genotype of spores was determined by growing cells on selective medium.

ERG6-mCITRINE^{A206K} was obtained by PCR ligation to fuse *ERG6* and *mCITRINE* and introduce the mutation A206K to favor monomeric Citrine (Shaner et al., 2005). The PCR product was recombined into the SalI site of plasmid pGREG506. Then, the whole expression cassette *GAL1*^{prom}*-ERG6-mCITRINE*^{A206K} was amplified by PCR and recombined into plasmid pRS415 to switch the selectable marker from *URA3* to *LEU2*. The endogenously tagged version of *ERG6*-m*CITRINE*^{A206K} was obtained by

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genomic integration of the *mCITRINE^{A206K}-CaURA3* cassette. The *3mCHERRY* cassette was amplified from plasmid p30648 (Dultz and Ellenberg, 2010), and recombined into the XhoI site of pGREG600 to replace GFP and yield *GAL1^{prom}-RecombinationSite-3mCHERRY* (p1079). This plasmid was then used to obtain 3mCherry fusion proteins under the control of *GAL1* promoter for tagging *DGA1* and *SEC63,* yet due to recombination, *SEC63* was only tagged with mCherry. Coding sequences of interest were amplified from yeast DNA. The *Neurospora crassa ATP9* mitochondrial targeting sequence (Westermann and Neupert, 2000) was amplified from the plasmid MITO-RFP, and also recombined into p1079 to label mitochondria. To target mCherry to the ER lumen under the control of a galactose-inducible promoter, *PRC1^{ss}-mCHERRY-HDEL* was PCR-amplified from plasmid MR6474 (Rogers et al., 2014) and recombined into pGREG503 digested with SalI restriction enzymes. Amplification of *sfGFP1-10* from Addgene plasmid #129416 (Salo et al., 2019) and recombination into the XmaI site of plasmid pRS416-ADH1 yielded a plasmid for expression of sfGFP1-10 from the *ADH1* promoter. A similar strategy was used for the expression BFP whose coding sequence was recombined into pRS415-ADH1.

To genomically tag Fld1 with multiple mCherry molecules, a tagging cassette obtained by PCR was recombined into the *FLD1* locus. PCR was used to add recombination arms for insertion into the genome and to PCR-ligate *mCHERRY* sequences amplified from plasmid p1079 with *SpHIS5* selection marker amplified from the pKT vectors (Jansen et al., 2005). With this strategy we obtained strains *MATα FLD1-2mCHERRY*, and *MATα are1Δ are2Δ dga1Δ lro1Δ FLD1-3mCHERRY.* A similar approach was employed to fuse *FLD1* to *sfGFP11$_{x7}$* amplified from Addgene plasmid #70224 (Kamiyama et al., 2016). To visualize Fld1, the *FLD1-sfGFP11$_{x7}$* strain should also expressed sfGFP1-10 (plasmid #p2212) to allow complementation of the two halves of the sfGFP. Plasmids used in this study are listed in Table S2.

Fluorescence microscopy

For microscopic analysis of yeast mating events, cells were first grown in raffinose-supplemented selective medium, and then shifted to galactose medium for overnight cultivation. The next morning, the density of the cells was adjusted to an optical density at 600 nm (OD_{600})=0.7, and cells were further grown in galactose medium (2 h). 1 ml of *MAT*a cells and 1 ml of *MATα* cells were collected together, and washed once with non-selective glucose medium. Cells were then resuspended in 1 ml of the same medium and incubated for 2 h at 30°C without shaking. Finally, cells were collected by centrifugation, resuspended in 50 µl medium, and 2 µl of the cell suspension was placed on a cover slip and overlaid with a SC-glucose-agarose patch to allow live-cell imaging for up to 100 min at 25°C. The same procedure was used for monitoring strains expressing fluorescently tagged Fld1, except that cells were cultured in glucose medium only.

For Bodipy staining, cells were collected at OD_{600}≈2, washed with PBS, and incubated at room temperature, in the dark for 30 min with Bodipy (2 µg/ml). The cells were then washed twice with PBS, and directly imaged when treated with Bodipy 493/503 (Invitrogen), or cultivated for 2 h before imaging for Bodipy-C12 558/568 (Invitrogen).

To image mating events, a Visitron spinning disk CSU-W1 (Visitron Systems, Puchheim, Germany) was employed. This consists of a Nikon Ti-E inverted microscope, supplied with a CSU-W1 spinning disk head with a 50 µm pinhole disk (Yokogawa, Tokyo, Japan), a PLAN APO 10× NA 1.3 oil immersion objective (Nikon), and an Evolve 512 (Photometrics) EM-CCD camera. Single optical sections were acquired every minute, during 101 min, in mCherry, YFP and CFP channels with filter sets for YFP/CFP and mCherry recordings. We account the vibrations induced by the motorized change of filter sets for a slight displacement between color channels that are visible in some highly magnified recordings of live cells labeled with YFP/CFP and mCherry. For imaging strains with fluorescently tagged Fld1, the interval between acquisitions was set to 2 min, and when the 7× split sfGFP was analyzed, the CFP cytosolic marker was replaced by BFP, and the mCherry, GFP and DAPI channels were used for image acquisition. The images were processed and analyzed using FIJI software (Schindelin et al., 2012), and resized in Adobe Photoshop. In line scan analysis, the intensity distribution of each fluorophore along a defined line was quantified, and total intensity was normalized to 1.

To follow the transfer of the different fluorescently tagged proteins, the increase in fluorescence signal in recipient cells was expressed as percentage of total fluorescence in the donor cell before cytoplasmic mixing had occurred. Cytoplasmic mixing was assessed by monitoring the presence of CFP in both mating partners.

To quantify the transfer rate of Erg6–mCitrin, and ss-mCherry-HDEL during mating, the transfer curve (average Fig. 4E, or every mating events Fig. 4F) was fitted to the Hill equation: $y=T^{\delta}\ M/(h+T^{\delta})$, with the help of the Solver add-in in Excel. Half-life of transfer was calculated with the equation: $T½=h^{(1/\delta)}$, as an estimate for the transfer rate of the fluorescent protein. Box plot and statistical analysis were performed with R software (https://www.r-project.org/), and the ggplot2 package (Wickham, 2016). In box plots, the median is indicated in a box that represents the 25–75th percentile range. The whiskers denote the largest and smallest values with 1.5× of the interquartile range from the hinges of the box. Outliers are depicted by black circles. A Wilcoxon rank-sum test was used with Benjamini–Hochberg correction to assess the significance of data.

To estimate the transfer of Erg6–mCitrine to LDs in WT×WT or *fld1Δ×fld1Δ* mating, we plotted the ratio of relative intensity of Dga1-3mCherry on LDs to that of Erg6–mCitrine. The increase in ratio is both due to LDs gaining Dga1–3mCherry and losing Erg6–mCitrine over time.

The Pearson correlation factors were determined with the help of the Coloc2 Fiji plugin (Schindelin et al., 2012). Background was subtracted for each channel, and colocalization was analyzed in a region of interest consisting of a zygote or two adjacent cells. Due to weak signals, we plotted Pearson's *r* value with no threshold, and included positive and negative controls to evaluate the level of colocalization that is detectable in diploid cells expressing both fluorescent proteins (positive control) and in adjacent cells that were not undergoing mating (negative control).

Protein analysis

To analyze protein turnover following promoter shut off, yeast cells were grown essentially as described for microscopic imaging. Namely, preculture in raffinose selective medium, protein induction in galactose selective medium overnight, dilution to OD=0.5 in galactose medium, growth for 2 h before separating the culture into two parts, one held in galactose medium, the other half supplied with glucose to turn expression of *GAL1* promoter driven marker proteins off. Cells were collected every hour, starting 1 h after dilution to OD=0.5 (t=−1). At each time point, 3 OD_{600} units of yeast cells were collected, proteins were extracted, and precipitated with 10% trichloroacetic acid (TCA). For western blot analysis, 0.5 OD_{600} units of cells were loaded, as described previously (Choudhary and Schneiter, 2012). Protein levels were quantified, corrected with respect to the loading control, 3-phosphoglycerate kinase Pgk1, and expressed as a percentage relative to t=0. Protein half-life was calculated by fitting the function $N=N_0e^{-kt}$ to the experimental data with the Solver add-in of Excel (Microsoft Corporation) and then using the equation: $T_{1/2}=\ln(2)/k$ (Belle et al., 2006).

Protein association with membrane was analyzed by differential centrifugation. 100 OD_{600} units of cells were collected after growth in galactose selective medium followed by 2 h in glucose supplemented medium. Cells were lysed in a buffer containing 0.2 M sorbitol, 50 mM potassium acetate, 20 mM HEPES (pH 6.8), 2 mM EDTA (pH 8.0), supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 0.2 mM PMSF. Fractionation was performed as described before, except that the homogenate was only separated into a 30,000 ***g*** pellet (P) and soluble proteins (S) by centrifugation at 30,000 ***g*** for 30 min at 4°C with a Sorvall S55-A rotor (Köffel et al., 2005). Protein concentration in each fraction was determined by a Bradford assay, and 100 µg of proteins were precipitated with TCA and resuspended in loading dye. Subsequently, 10 µg of protein were loaded on SDS-PAGE and analyzed by western blotting.

SDS-PAGE and western blotting were performed according to standard protocols. The primary antibodies employed were: purified anti-mCherry antibody (mouse, 1:1000, BioLegend # 677702), monoclonal anti-GFP antibody (mouse, 1:2000, Roche # 11814460001), which also detected mCitrine-tagged proteins, anti-Pgk1 monoclonal antibody 22C5D8 (mouse, 1:5000, Invitrogen # 459250), anti-Ayr1 (rabbit, 1:5000, Günther Daum, TU-Graz, Austria), anti-GAPDH antibody (rabbit, 1:4000, gift from

Günther Daum), and anti-Sec63 (sheep, 1:10,000, gift from Andreas Conzelmann, University of Fribourg, Switzerland, and Gabrielle Forte, University of Manchester, UK). As secondary antibodies goat anti-mouse IgG (H+L)-HRP conjugate (1:10,000, Bio-Rad # 1706516), goat anti-rabbit IgG (H+L)-HPR conjugate (1:10,000, Bio-Rad # 1706515), and anti-sheep IgG-HRP conjugate (1:10,000, Sigma Aldrich # A3415) were employed.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.C., R.S.; Methodology: S.C., R.S.; Validation: S.C., R.S.; Formal analysis: S.C., R.S.; Investigation: S.C.; Resources: R.S.; Data curation: S.C.; Writing - original draft: S.C., R.S.; Writing - review & editing: S.C., R.S.; Visualization: S.C., R.S.; Supervision: R.S.; Project administration: R.S.; Funding acquisition: R.S.

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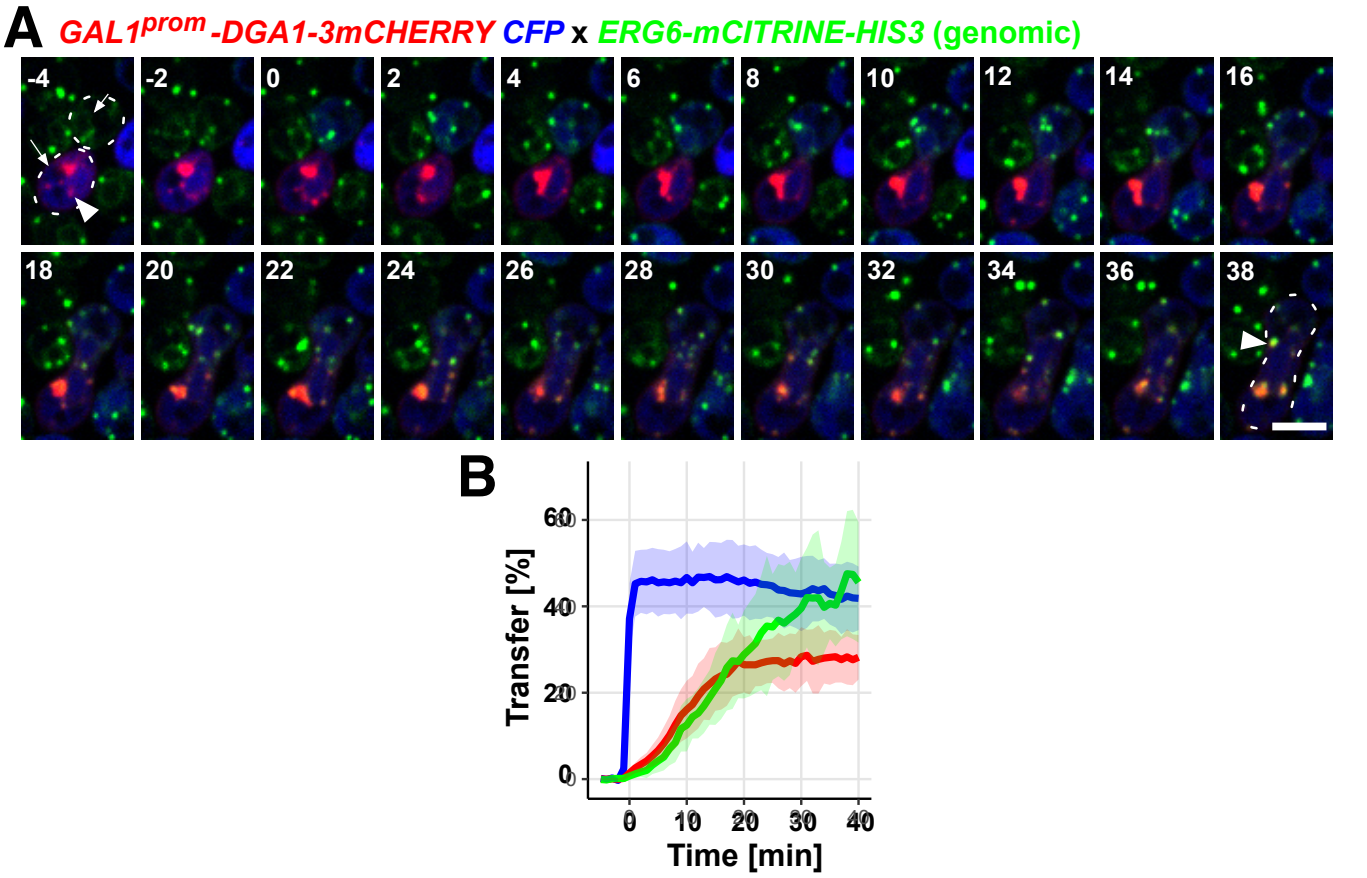
A
GAL1prom-DGA1-3mCHERRY CFP x ERG6-mCITRINE-HIS3 (genomic)
-4
-2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
B
Transfer [%]
60
40
20
0
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20
30
40
Time [min]

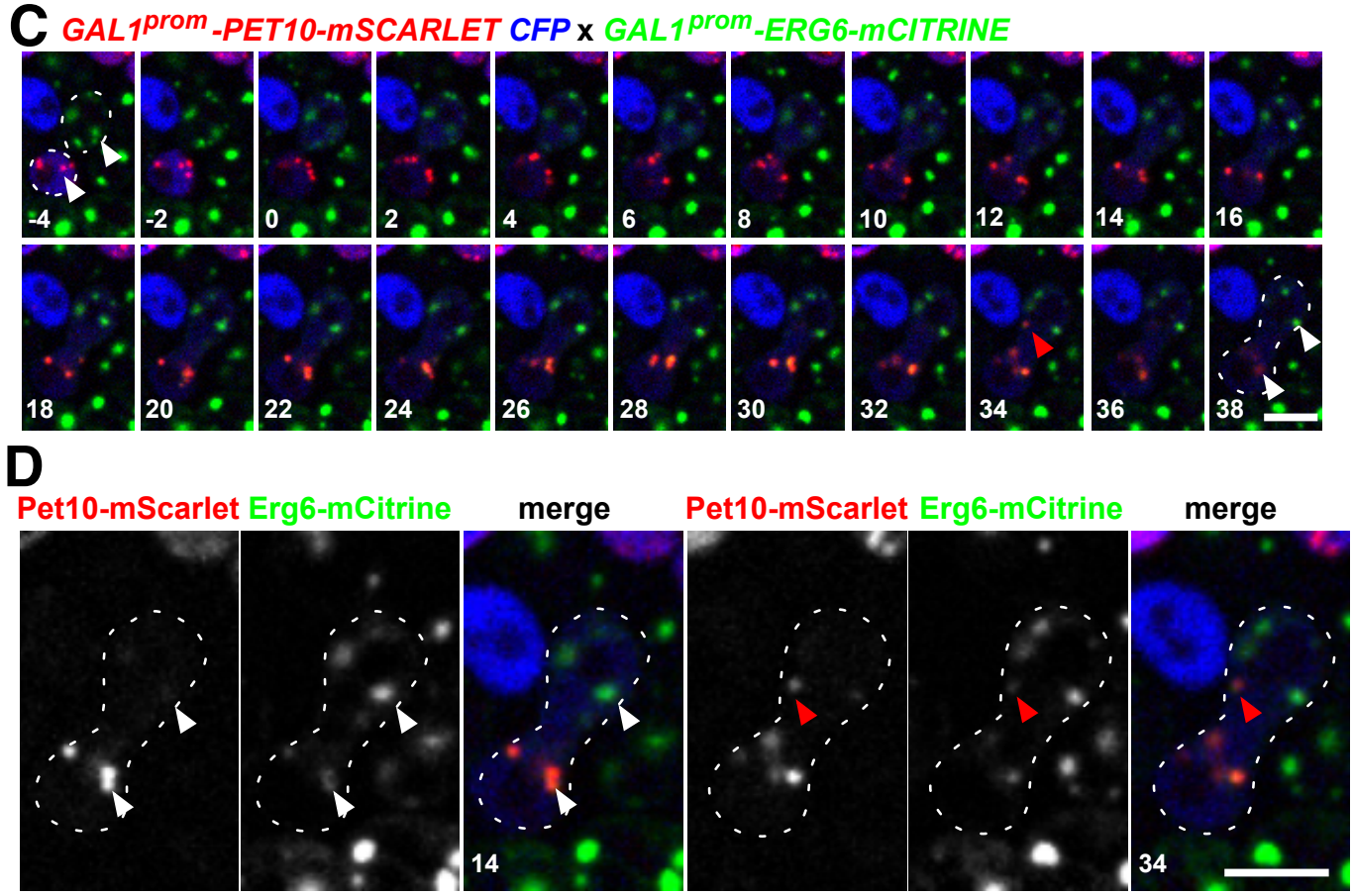

C
GAL1prom-PET10-mSCARLET CFP x GAL1prom-ERG6-mCITRINE
-4
-2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
D
Pet10-mScarlet
Erg6-mCitrine
merge
Pet10-mScarlet
Erg6-mCitrine
merge
14
34

Fig. S1. Transfer of endogenous mCitrine-tagged Erg6 to LDs of the mating partner, and movement of Pet10-mScarlet labelled LDs on zygote formation.

(A) Mating reactions to monitor the exchange of endogenously tagged Erg6-mCitrine and of Dga1-3mCherry, whose expression is control by a galactose inducible promoter, in wild-type cells. Time-lapse images shown are separated by 2 min intervals over a period of 44 min, starting 2 min prior to cytoplasmic mixing (t=0 min). White arrows point to the ER membrane, and arrowheads to LDs. Scale bar, 5 µm.

(B) Rate of transfer of Erg6-mCitrine (green line), Dga1-3mCherry (red line), and the cytosolic marker CFP (blue line) between wild-type mating partners.

(C) Pet10-mScarlet labelled LDs move only slowly into the acceptor half of the zygote upon cytoplasmic mixing. Representative time-lapse images showing the transfer of Pet10-mScarlet and Erg6-mCitrine onto the LDs of mating partners. Images shown are separated by 2 min intervals, starting 4 min (t=-4) before cytoplasmic mixing (t=0), over a period of 42 min. White arrowheads point to LDs, and the red arrowhead marks an LD that has moved into the mating partner. Scale bar, 5 µm.

(D) Enlarged views of the 14 and 34 min time points shown in panel C. White arrowheads point to LDs that are still in the part of the zygote where they originate from, and the red arrowhead points to an LD that is strongly labelled with Pet10-mScarlet and that has moved into the mating partner more than 30 min after fusion had occurred. Scale bar, 5 µm.

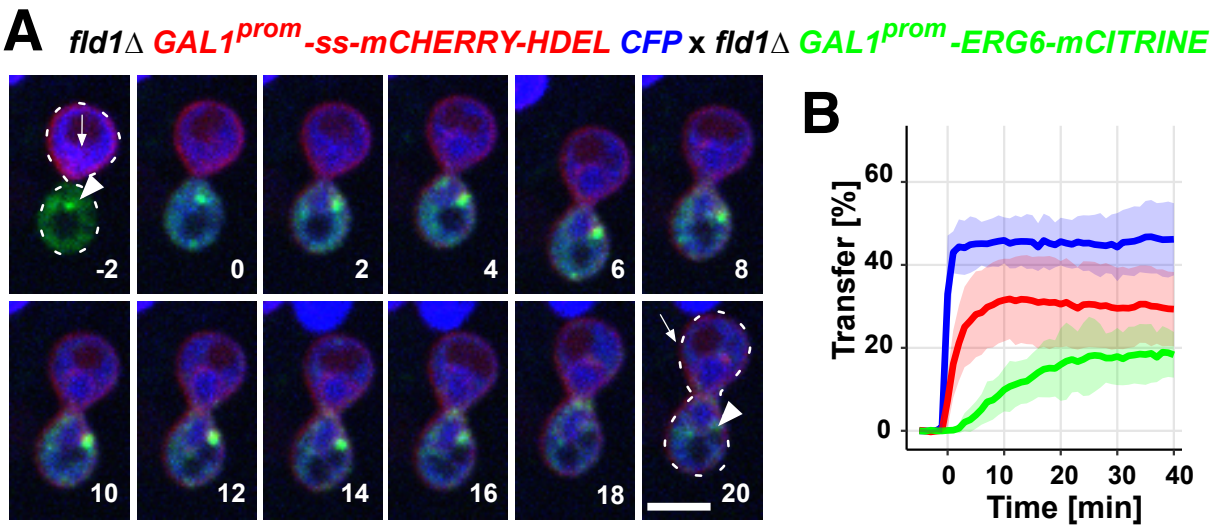
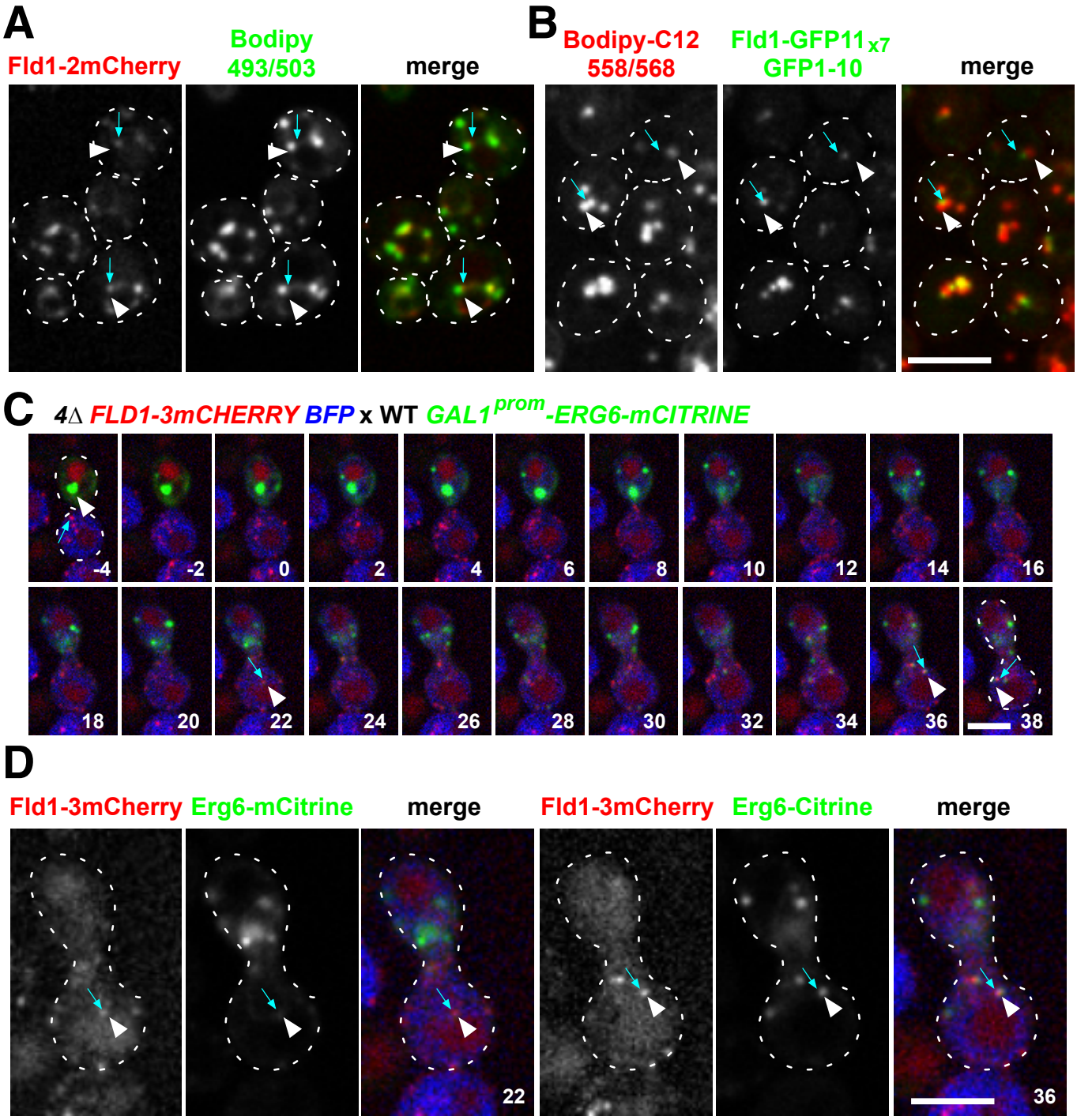


Fig. S2. Seipin affects the exchange of LD proteins but not ER fusion.

(A) Mating reactions to monitor the exchange of ss-mCherry-HDEL and Erg6-mCitrine between seipin (*fld1*Δ) mutant cells. Time-lapse images shown are separated by 2 min intervals over a period of 22 min, starting 2 min prior to cytoplasmic mixing (t=0 min). Arrows point to the ER membrane, arrowheads to LDs. Scale bar, 5 µm.

(B) Rate of transfer of the ER luminal marker ss-mCherry-HDEL and that of the LD marker Erg6-mCitrine between seipin mutant *(fld1*Δ*)* cells. The red line represents ss-mCherry-HDEL, the green line Erg6-mCitrine, and the blue line cytosolic CFP.


A
Fld1-2mCherry
Bodipy 493/503
merge
B
Bodipy-C12 558/568
Fld1-GFP11 x7 GFP1-10
merge
C
4Δ FLD1-3mCHERRY BFP x WT GAL1prom-ERG6-mCITRINE
-4
-2
0
2
4
6
8
10
12
14
16
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20
22
24
26
28
30
32
34
36
38
D
Fld1-3mCherry
Erg6-mCitrine
merge
22
Fld1-3mCherry
Erg6-Citrine
merge
36

Fig. S3. mCherry- and GFP-tagged variants of seipin are functional and mark sites of LD formation.

(A, B) 2mCherry- and the split-GFP- (sfGFP11$_{x7}$+GFP1-10) tagged Fld1 localize at the base of LDs labelled with either BODIPY493/503 (panel A) or BODIPY-C12 (558/568) (panel B), respectively. Blue arrows point to Fld1 spots, arrowheads to LDs. Scale bar, 5 µm.

(C) Fld1-3mCherry marks sites of LD formation in the ER of quadruple mutant cells. Wild-type cells expressing Erg6-mCitrine were mated to quadruple mutant cells, *4*Δ (*are1*Δ *are2*Δ *dga1*Δ *lro1*Δ) expressing Fld1-3mCherry. Images shown were recorded at 2 min intervals, starting 4 min (t=-4) before cytoplasmic mixing (t=0) over a period of 42 min. Blue arrows point to Fld1 spots, arrowheads to LDs. Scale bar, 5 µm.

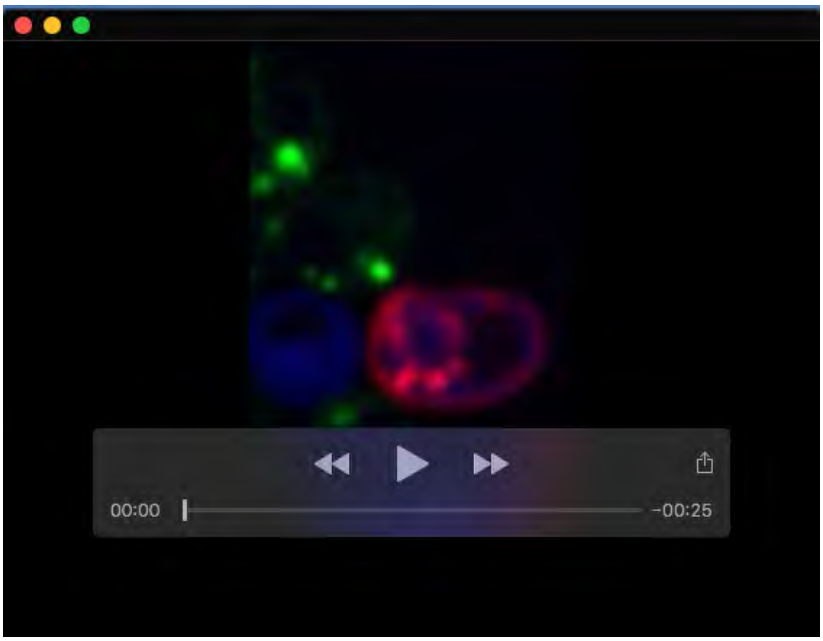
(D) Enlarged views of the 22, and 36 min time points shown in panel A. Note the accumulation of Erg6-mCitrine at ER spots marked by Fld1-3mCherry. Blue arrows point to Fld1 spots, arrowheads to LDs. Scale bar, 5 µm.

Table S1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source	Figure
BY4741	*MAT*a; *his3Δ1 leu2Δ met15Δ0 ura3Δ0*	Euroscarf	1, 2, 3, 5, 6, 7
BY4742	*MAT*α; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf	1, 2, 3, 6, 7, 8
RSY5669	*MAT*α; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1Δ::KanMX4 are2Δ::KanMX4 dga1Δ::KanMX4 lro1Δ::KanMX4*	This study	5, 6
RSY5795	*MAT*a; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1Δ::KanMX4 are2Δ::KanMX4 dga1Δ::KanMX4 lro1Δ::KanMX4*	This study	6
MATa thr⁻	*MAT*a; *thr*	Lab collection	
MATα thr⁻	*MAT*α; *thr*	Lab collection	
MY14769	*MAT*a/α; *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 sey1Δ::Hyg/SEY1 yop1Δ::URA3/YOP1 dsl1ΔE-NatMX/DSL1*	Mark Rose	
RSY5619	*MAT*α; *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sey1Δ::Hyg dsl1ΔE-NatMX*	This study	4
RSY5621	*MAT*a; *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sey1Δ::Hyg dsl1ΔE-NatMX*	This study	4
MATa fld1Δ	*MAT*a; *his3Δ1 leu2Δ met15Δ0 ura3Δ0 fld1Δ::KanMX4*	Euroscarf	7, S2
MATα fld1Δ	*MAT*α; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 fld1Δ::KanMX4*	Euroscarf	7, S2
RSY6911	*MAT*α; *his3Δ1 leu2Δ lys2Δ0 ura3Δ0 FLD1-2mCHERRY::SpHIS5*	This study	8, S2
RSY6970	*MAT*a; *his3Δ1 leu2Δ met15Δ0 ura3Δ FLD1-GFP11x7::SpHIS5*	This study	8, S3
RSY6963	*MAT*α; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1Δ::KanMX4 are2Δ::KanMX4 dga1Δ::KanMX4 lro1Δ::KanMX4 FLD1-3mCHERRY::SpHIS5*	This study	S3
RSY6756	*MAT*α; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ERG6-mCITRINE::SpHIS5*	This study	S1

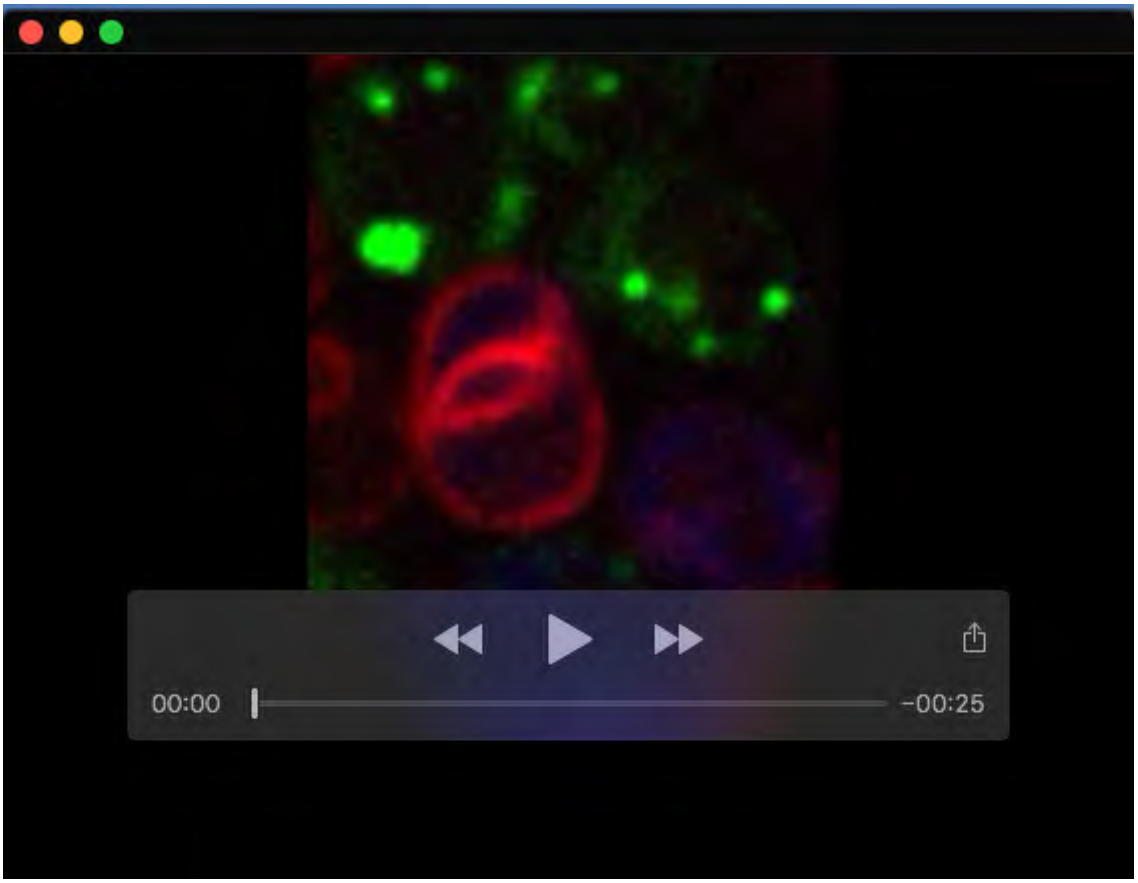
Table S2. Plasmids used in this study

Plasmids	Genotype	Source	Figure
pRS415-ADH1	*CEN/ARS, ADH1^{prom}, LEU2*	Mumberg et al., 1995	
p1174	[pRS415] *ADH1^{prom}-yECFP*	Cottier et al., 2020	1, 2, 3, 4, 5, 6, 7, 8, S1, S2
pGREG506	*CEN/ARS, GAL1^{prom}, URA3*	Jansen et al., 2005	
pKT211	[pFA6a] *link-yEmCITRINE, SpHIS5*	Sheff and Thorn, 2004	
p1307	[pGREG506] *GAL1^{prom}-ERG6-mCITRINE^{A206K}*	This study	1, 2, 3, 4, 5, 6, 7, 8, S2, S3
p30648	*POM121-3mCHERRY*	Dultz and Ellenberg, 2010	
pGREG600	*CEN/ARS, GAL1^{prom}-RecombinationSite-GFP, URA3*	Jansen et al., 2005	
p1079	[pGREG600] *GAL1^{prom}-RecombinationSite-3mCHERRY*	This study	
p1102	[p1079] *GAL1^{prom}-DGA1-3mCHERRY*	This study	1, 2, 4, 5, 7, S1
Mito-RFP	*CEN/ARS NcATP9$^{mitochondria\text{-}signal}$-RFP, LEU2*	Westermann and Neupert, 2000	
p1317	[p1079] *GAL1^{prom}- NcATP9$^{mitochondria\text{-}signal}$-3mCHERRY*	This study	3
p1312	[p1079] *GAL1^{prom}-SEC63-3mCHERRY*	This study	3
pGREG503	*CEN/ARS, GAL1^{prom}, HIS3*	Jansen et al., 2005	
MR6474	*CEN, ADH1^{prom}-PRC1^{ss}-mCHERRY-HDEL, LEU2*	Rogers et al., 2014	
p1171	[pGREG503] *GAL1^{prom}-PRC1^{ss}-mCHERRY-HDEL*	This study	4, 6, S2
pGAL-HO	*GAL^{prom}-HO-URA*	Herskowitz and Jensen, 1991	
Addgene #70224	*pHRm-NLS-dCas9-GFP11x7-NLS*	Kamiyama et al., 2016	
Addgene #129416	*pSH-EFIRES-P-GFP(1-10)opti*	Salo et al., 2019	
pRS416-ADH1	*CEN/ARS, ADH1^{prom}, LEU2*	Mumberg et al., 1995	
p2213	[pRS416] *ADH1^{prom}-sfGFP1-10*	This study	8, S3
p2216	[pRS415] *ADH1^{prom}-BFP*	This study	8, S3
p2233	[pRS415] *GAL1^{prom}-ERG6-mCITRINE*	This study	8
P1662	[pRS416] *GAL1^{prom}-PET10-mSCARLET*	This study	S1
pKT175	[pFA6a] *link-yECITRINE, CaURA3*	Sheff and Thorn, 200	



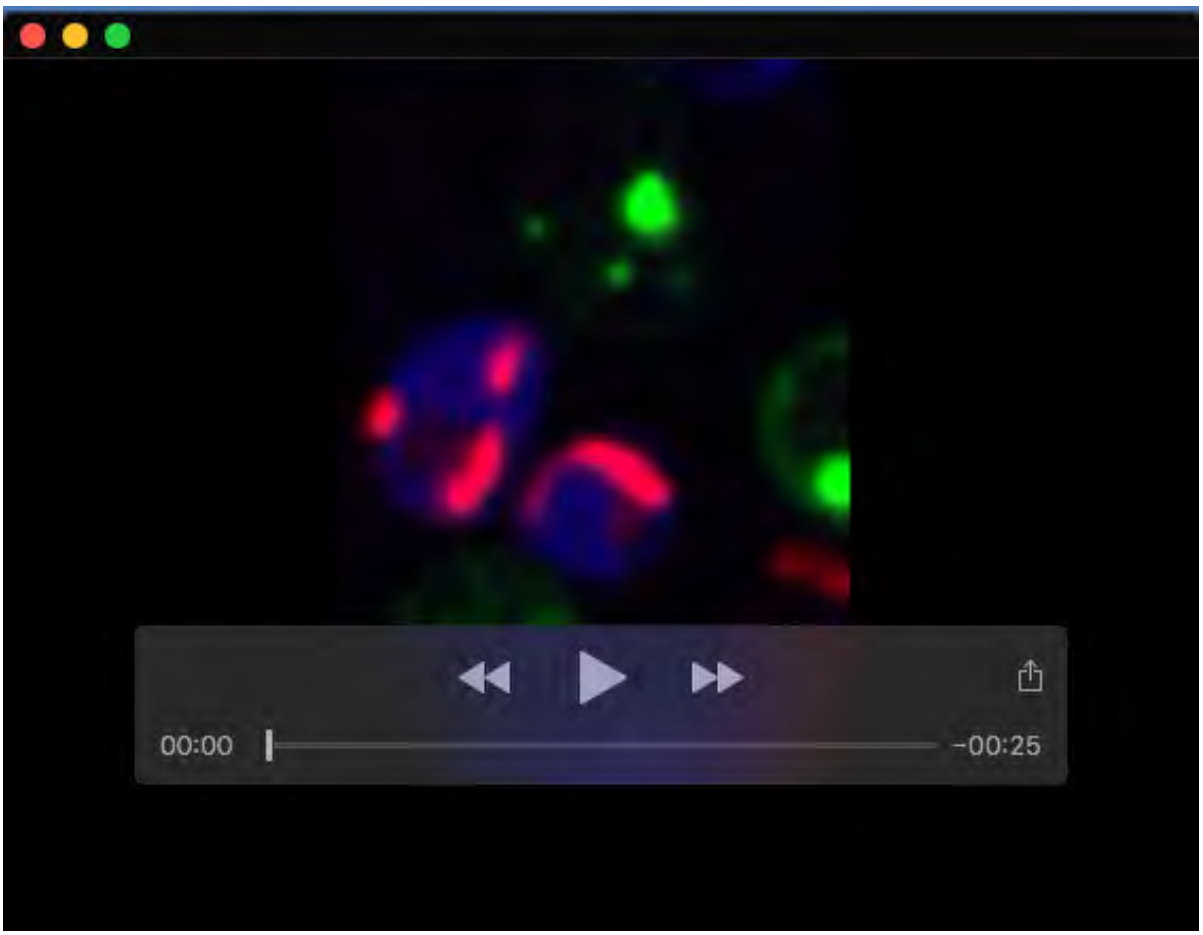
Movie 1. Redistri ution of LD markers upon zygote formation.

Time-lapse images during ygote formation between a *MAT*a cell expressing Dga1- 3mCherry and CFP and a *MAT*α cell expressing Erg6-mCitrine. Expression of the LD marker proteins under control of a galactose inducible promoter was repressed by shifting cells to glucose media 2 h before imaging. Single-plane images were recorded at 1 min intervals.



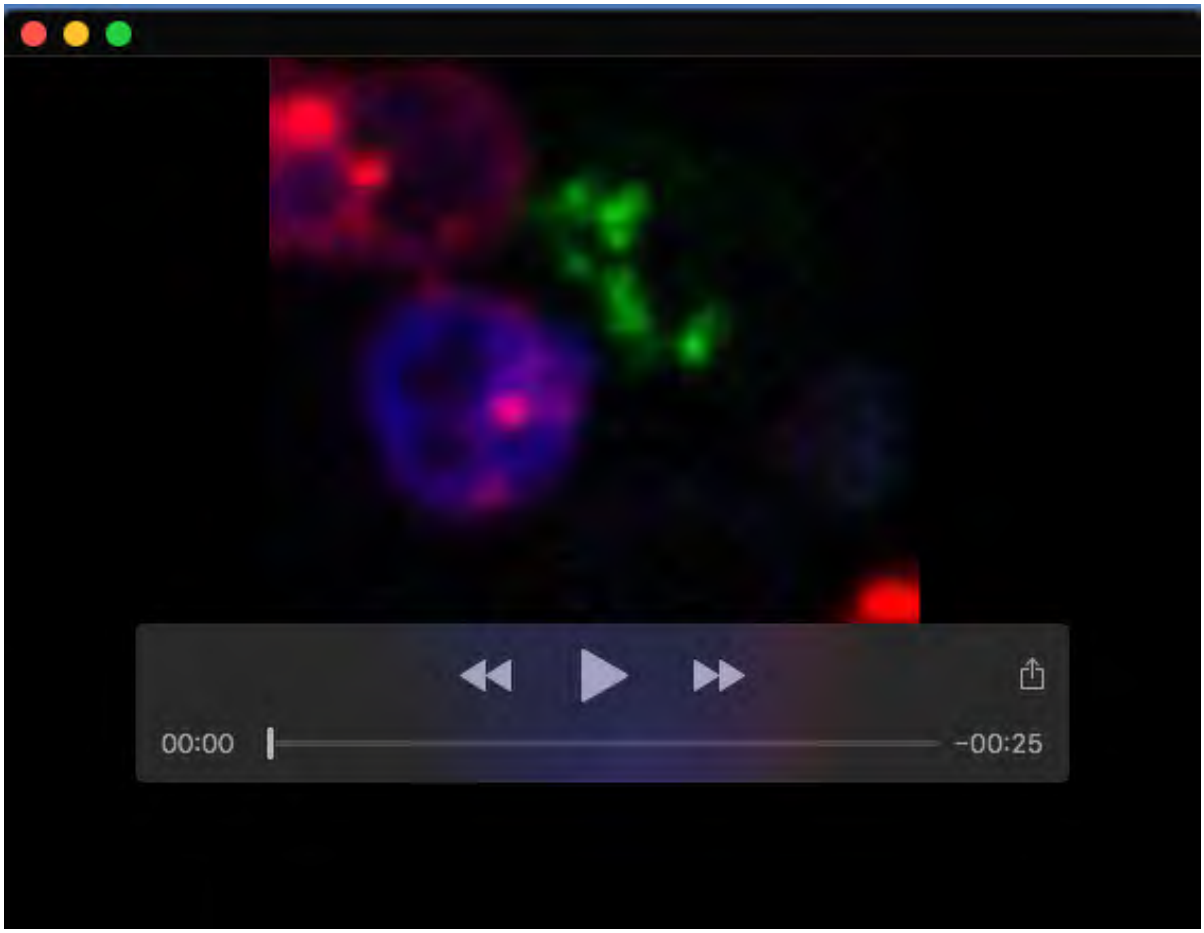
Movie 2. Redistri ution of the ER marker Sec63-mCherry and the LD marker Erg6-mCitrine upon zygote formation.

Time-lapse images of ygote formation between a *MAT*a cell expressing Sec63-mCherry and CFP and *MAT*α cell expressing Erg6-mCitrine. Expression of Sec63-mCherry and Erg6-mCitrine was switched off 2 h before imaging by shifting cells to glucose medium. Single-plane images were recorded at 1 min intervals.



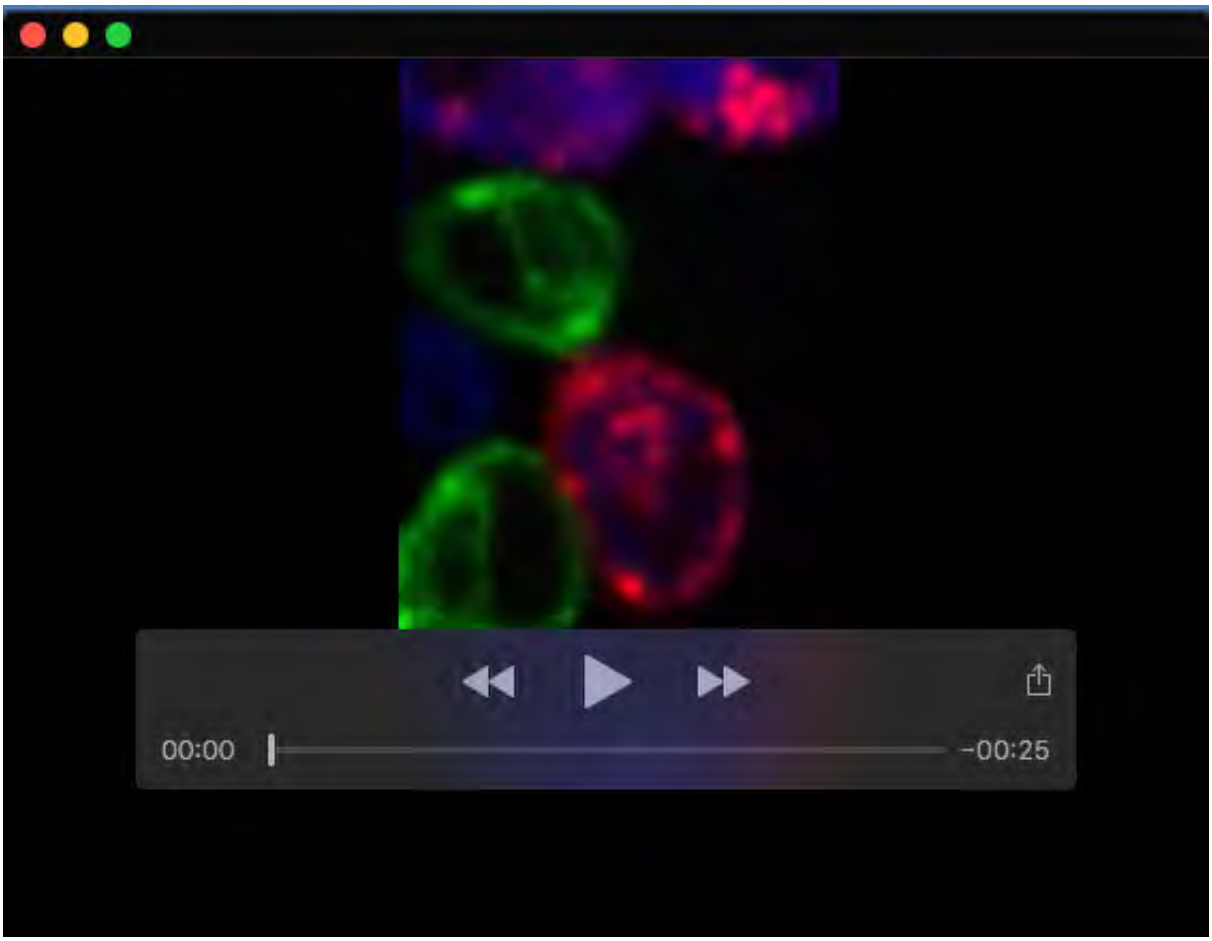
Movie 3. Dynamics of exchange of the LD marker Erg6-mCitrine and the mitochondrial matrix marker MITO-3mCherry upon zygote formation.

Time-lapse images of ygote formation between a *MAT*a cell expressing M T - 3mCherry and CFP and a *MAT*α cell expressing Erg6-mCitrine. Expression of M T - 3mCherry and Erg6-mCitrine was switched off 2 h before imaging by shifting cells to glucose medium. Single-plane images were recorded at 1 min intervals.



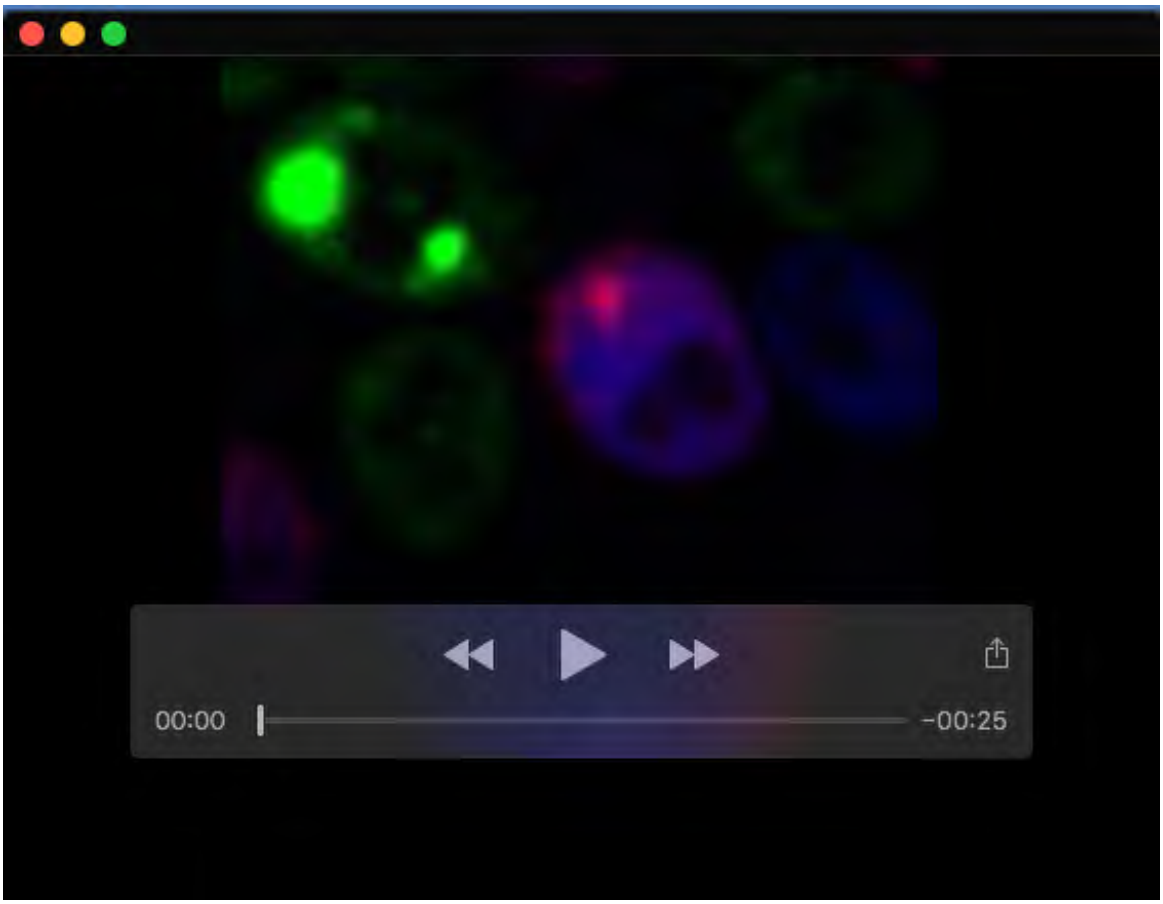
Movie 4. Exchange of Erg6-mCitrine and Dga1-3mCherry in mutants with a delay in ER fusion.

Mating between *sey1 dsl1ΔE* mutant cells expressing Erg6-mCitrine or Dga1-3mCherry and CFP. Note the accumulation of LDs at the fusion neck between the gametes. Expression of the LD marker proteins was repressed 2 h prior to imaging acquisition. Single-plane images were recorded at 1 min intervals.

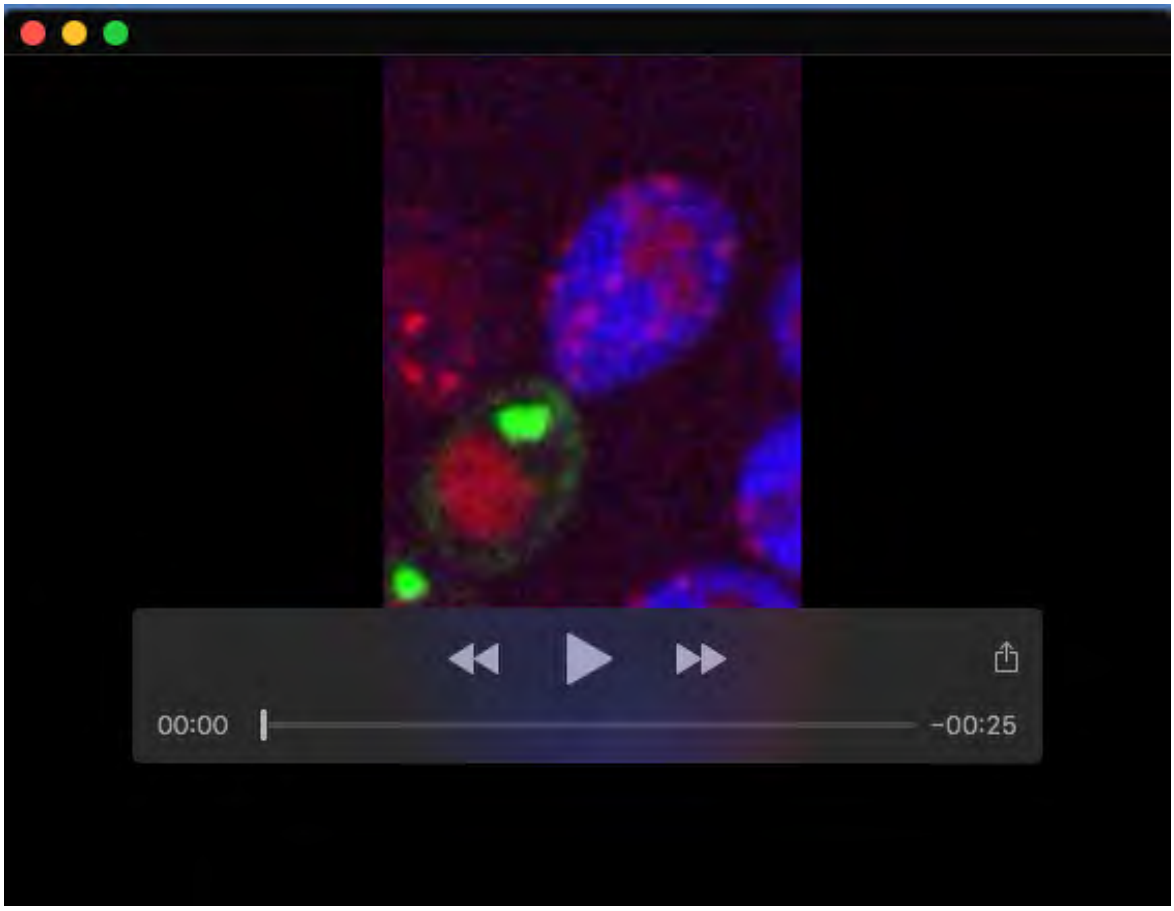


Movie 5. Erg6-mCitrine located in the ER of a quadruple mutant lacking LDs moves to LDs of the mating partner expressing Dga1-3mCherry

Time-lapse images of a *MAT*a cell expressing Dga1-3mCherry and CFP mating with a *MAT*α quadruple mutant *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ*)* lacking LDs and expressing E -locali ed Erg6-mCitrine. Expression of the LD marker proteins was repressed 2 h prior to imaging acquisition. Single-plane images were recorded at 1 min intervals.

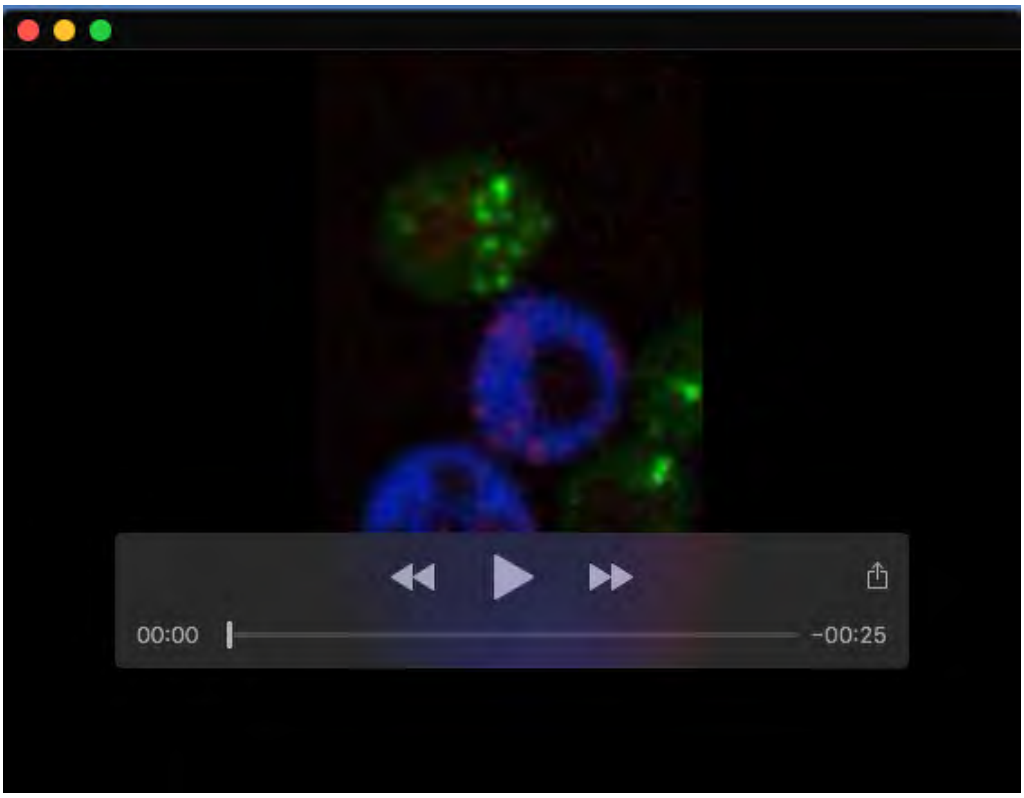


Movie 6. Seipin affects relocalization of LD markers upon zygote formation. Zygote formation between seipin mutant cells *(fld1Δ)* expressing Dga1-3mCherry and CFP or Erg6-mCitrine. Note the large, possibly clustered LDs in both mating partners, a characteristic of seipin mutants. Expression of the LD marker proteins was repressed 2 h prior to imaging acquisition. Single-plane images were recorded at 1 min intervals.



Movie 7. ER sites marked by seipin in wild-type cells acquire the LD marker from the mating partner upon zygote formation.

Mating progression between a cell co-expressing 2mCherry-tagged seipin and CFP and a partner cell expressing Erg6-mCitrine. Erg6-mCitrine expression was repressed 2 h before imaging. Single-plane images were recorded at 2 min intervals.



Movie 8. Seipin domains in the ER are stable and do not mix upon zygote formation.

Zygote formation between mating partners expressing red- and green-fluorescently tagged seipin variants. Time-lapse images of a mating between cells of different mating types expressing either Fld1-2mCherry or a split-GFP Fld1-GFP11$_{x7}$ together with BFP and GFP1-10, respectively. Mating progression was followed by acquisition of single plan images separated by 2 min intervals.

Supplementary References

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