Phospholipids containing ether-bound hydrocarbon-chains are essential for efficient phagocytosis and neutral lipids of the ester-type perturb development in *Dictyostelium*

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**ABSTRACT**

Lipids are the building blocks for cellular membranes; they provide signalling molecules for membrane dynamics and serve as energy stores. One path of their synthesis is initiated by glycerol-3-phosphate acyltransferase (GPAT), which in *Dictyostelium* resides on the endoplasmic reticulum. When an excess of fatty acids is present, it redistributes to storage organelles, the lipid droplets. Mutants, where the GPAT was eliminated by homologous recombination, produce fewer lipid droplets and are almost devoid of triacylglycerols (TAG), rendering them more resistant to cell death and cell loss in the developmental stages preceding fruiting body formation. The enzyme most closely related to GPAT is called FARAT, because it combines a fatty acyl-reductase (FAR) and an acyltransferase (AT) domain in its sequence. The protein is confined to the lumen of the peroxisome, where it transfers a fatty acid to dihydroxyacetone-phosphate initiating the synthesis of ether lipids, later completed at the endoplasmic reticulum. A mutant lacking FARAT produces lipid droplets that are devoid of the storage lipid monoalkyl-diacyl-glycerol (MDG), but the efficiency of spore formation in the developmental cycle is largely unaltered. Instead, these mutants are strongly impaired in phagocytosis of yeast particles, which is attributed to reduced synthesis of membrane phospholipids containing ether-linked chains.

**KEY WORDS:** Endoplasmic reticulum, Peroxisomes, Lipid droplets, GPAT, DHAPAT, Macropinocytosis

**INTRODUCTION**

Membrane phospholipids not only act as a barrier between the cytoplasm of the cell and the extracellular space or the lumen of various organelles, but they also mediate cellular signalling. In particular this involves sphingolipids, the hydrolysis of phosphatidylcholine, and most of all, differential phosphorylation of the inositol head-group followed by local membrane recruitment of specific signalling proteins (Divecha and Irvine, 1995). Ground-breaking work on the last topic was conducted in the field of *Dictyostelium* (Parent et al., 1998; Iijima and Devreotes, 2002), although these lipid modifications were then shown to recruit cytoskeletal proteins to the sites of phagocytosis (Dormann et al., 2004) and turned out to be especially important for macropinocytosis (Hoeller et al., 2013; Kay et al., 2019; Vines and King, 2019) rather than chemotaxis (Hoeller and Kay, 2007).

Similarly, neutral lipids not only serve as a reserve of energy and membrane building blocks but their storage organelle, the lipid droplet, fulfils an increasing number of unexpected physiological roles ranging from protein sequestration (Welte, 2015; Welte and Gould, 2017) to degradation (Moldavski et al., 2015). Across host cell kingdoms even various intracellular pathogens interact with lipid droplets (Barisch et al., 2015; Roingeard and Melo, 2017).

In *Dictyostelium*, lipid droplets (LDs) store neutral lipids mainly in the form of triacylglycerol (TAG) and, to a lesser extent, as monoalkyl-diacyl-glycerol (MDG) and sterol-esters (SE) (Du et al., 2014). In recent work, we have addressed the individual functions of the enzymes mediating the last step in TAG and MDG synthesis, DGAT1 and DGAT2 (Du et al., 2014). The latter enzyme plays a minor role in neutral lipid synthesis, as its knockout does not show a phenotype. On the contrary, DGAT1 is the main enzyme responsible for both TAG and MDG synthesis at the same time. Double knockout cells lacking both enzymes are severely affected in their ability to grow on bacteria as their natural food source (Du et al., 2014). They also become insensitive to the adverse effect of fatty acid addition, which in wild-type cells strongly perturbs the completion of development (Kornke and Maniak, 2017). In order to separate these pathways experimentally, and to be able to attribute individual phenotypes to either ether or ester lipids, we sought to interfere with the enzymes that initiate their pathways of synthesis.

Esters–lipid synthesis takes place in the endoplasmic reticulum and is initiated by the addition of a coenzyme A-activated fatty acid to the sn1-position of a glycerol-3-phosphate backbone. The enzyme forming this ester bond is called G-3-P acyltransferase (GPAT) (Takeuchi and Reue, 2009). The single *Dictyostelium* gene encodes an enzyme that localises to the endoplasmic reticulum (ER) and to LDs. A knockout mutant produces significantly reduced amounts of TAG when a fatty acid is present during their vegetative growth. Under these conditions, the mutants show increased phagocytosis and can successfully complete development, whereas wild-type cells are impaired in both processes.

In contrast, peroxisomes are the organelles where ether–lipid synthesis begins. In mammals, first an enzyme abbreviated DHAPAT adds a fatty acid to the sn1-position of dihydroxyacetone-3-phosphate (Ofman et al., 1998). In a second step, this acyl-chain is then replaced by a fatty alcohol by ADPS (alkyl-dihydroxyacetone-phosphate-synthase) yielding the ether bond. Interestingly, the only crystal structure of this protein, which also allows to delineate the exact reaction mechanism, was derived from the *Dictyostelium* enzyme (Razeto et al., 2007).
We have identified the acyltransferase mediating the first step described above. Because its sequence also bears a fatty acid reductase (FAR) domain N-terminal of the acyltransferase (AT), the protein was called FARAT. We show here, that FARAT indeed localises in peroxisomes, and mutants lacking this enzyme are unable to produce the storage lipid MDG. Probably, because many phosphatidylethanolamines as well as phosphoinositides in Dictyostelium are based on an ether–lipid backbone (Weeks and Herring, 1980; Clark et al., 2014), the mutants are strongly impaired in phagocytosis and show reduced macropinocytosis, as well as a rather mild developmental defect.

RESULTS

The initial step of TAG synthesis is carried out by GPAT on the ER and LDs

In mammalian cells, the majority of glycerolipid syntheses is initiated by GPAT enzymes of which four isoforms exist. GPAT1 and 2 are rather big enzymes of around 800 amino acids located in mitochondria. Their best homologue with only 26% identical residues in the Dictyostelium genome is DDB0305750, the gene that encodes FARAT (see below). A search with GPAT3 and 4, which are only around 450 amino acids in length, also turns up only a single gene, DDB0235400 (named gpat), with a better match of 44% identity to GPAT3 and 36% to GPAT4. The Dictyostelium GPAT protein comprises 488 residues and has a serine-rich region at the N-terminus, with 28 serines occurring within the 40 amino acids after the initial methionine and two predicted transmembrane domains followed by one acyltransferase domain (Fig. 1A). To analyse the subcellular localisation of GPAT, a GFP tag was either fused to the N-terminus of the protein or to its C-terminus. In the clone 1-8, GFP-GPAT shows a strong signal at the expected size of 83 kDa on a western blot, whereas the amount of GPAT-GFP is much lower (Fig. 1B), because in the latter clone (3-23) only about 20% of the cells appeared green by fluorescence microscopy. Nevertheless, both constructs yielded cells where a colocalization could be documented (Fig. 1C,D) between the GFP-tagged GPAT and the protein disulfide isomerase, a known ER-marker (Monnat et al., 1997). However, when the cells were provided with palmitic acid 3 h before fixation, so that they could accumulate lipid droplets, both GFP-GPAT and GPAT-GFP disappeared from the ER and were found to bind to the LD surface instead (Fig. 1E,F). This finding is consistent with our previous proteomic analysis of biochemically enriched LDs (Du et al., 2013).

In order to generate a mutant lacking the GPAT protein, we cloned the genomic gpat locus and inserted a cassette conferring resistance to Blasticidin S into the first of 6 exons (Fig. 2A). After electroporating a linear DNA-fragment into AX2 wild-type cells, we screened individual clones for the occurrence of homologous recombination by PCR analysis and selected two lines (1-21 and 2-6) with independent integration events for further investigation (Fig. 2B,C). gpat mutants fed with a fluorescent fatty acid were found to contain only a few lipid droplets (Fig. 2D). Consistent with this observation, enzymatic tests (Fig. 2E) as well lipid extracts analysed by thin layer chromatography (Fig. 2F) and densitometry of \( n = 3 \) extractions revealed that gpat mutants produced about 70% less TAG but were largely unaffected in the level of MDG. To confirm that this phenotype originates from the disruption of the gpat gene, we transformed knockout strain 1-21 with either GFP-GPAT or GPAT-GFP expressing plasmids and documented the reappearance of wild-type levels of TAG again by enzymatic test (Fig. 2G) as well as by TLC (Fig. 2H).

Peroxisomal FARAT is responsible for MDG synthesis

As mentioned before, the other gene that appeared as a weak homologue of mammalian GPAT sequences was DDB0305750. Because its length of 1279 amino acids exceeded that of the other GPATs, we carried out a domain analysis and found a predicted FAR catalytic domain at the N-terminus, that is followed by the conserved AT centre and two blocks of nine or ten asparagine residues in the backbone (Weeks and Herring, 1980; Clark et al., 2014). It was therefore designated FARAT indeed localises in peroxisomes, and mutants lacking this enzyme are unable to produce the storage lipid MDG.
residues, which is not uncommon to Dictyostelium proteins (Malinovska et al., 2015). At its C-terminus the protein bears the tripeptide SKL, known as a peroxisomal targeting sequence type 1 (PTS1). The name FARAT was chosen for the protein and fatA indicates the corresponding gene (Fig. 3A). For localization studies, GFP was fused to the N-terminus of FARAT and could be detected as a band of 170 kDa on a western blot (Fig. 3B). Because a C-terminal fusion would have blocked the PTS1 signal, we inserted GFP just before the SKL sequence yielding the construct FARAT-GFP-SKL that was less efficiently produced in Dictyostelium cells.

Fig. 2. Lack of GPAT strongly affects TAG production. (A) Diagram of the relevant region from the genomic gpat locus (upper line) with insertion of a Blasticidin S resistance cassette (lower line) by homologous recombination in the coding region of the first exon (E1). Binding sites for diagnostic primers are indicated by arrows and reside upstream or downstream of sequences used for targeting in the noncoding region (775) and in exon three (774) as well as in the BS-resistance gene (456). (B,C) PCR products from genomic DNA isolated from AX2 and two independently derived gpat-mutants (1-21 and 2-6). (B) Combining one primer binding 5’ upstream of the gpat coding region (775) and one primer specific for the resistance cassette (456), the disrupted copy of the gpat gene (*) can only be amplified in the mutants but not in wild type. An unrelated gene, thioredoxin (trx) was included to demonstrate the integrity of the template DNA. (C) Two primers situated in the gpat gene (775 and 774) amplify an 895 bp fragment from wild-type DNA, which increases to 2.5 kbp in the mutant strains, indicating the insertion of the BS’-cassette (gpat*) side by side with the presence of the original gene (gpat). The relevant sizes of the DNA marker (M) are given in base pairs. (D) Wild-type cells (AX2) and gpat-knockout strains (1-21 and 2-6) were cultivated in the presence of palmitic acid together with the lipid tracer C1-BODIPY-C12 for 3 h, fixed, and a single image plane was captured by confocal microscopy to reveal the lipid droplets. Scale bars: 5 µm. (E) Cells were stimulated by palmitic acid addition and allowed to form lipid droplets for 3 h. TAG-levels measured after 3 h through an enzymatic assay (see Materials and Methods). (F) Thin layer chromatography resolving sterol-esters (SE), monoacyl-diacyl-glycerol (MDG) triacylglycerol (TAG), free fatty acids (FFA), cholesterol (CHL), as well as methyl-oleate (MO) as a loading control, from lipid extracts of AX2 and gpat-mutants incubated with palmitic acid aligned with the legend above. (G) Enzymatic assay of triglycerides and (H) TLC of neutral lipids from mutant 1-21 rescued by expression of GFP-GPAT (clone 1-12-6) or GPAT-GFP (clone 2-2) under conditions as used in panels E and F.

Fig. 3. FARAT is a strictly peroxisomal enzyme. (A) Schematic drawing of the FARAT sequence approximately drawn to scale. The fatty acid reductase domain (FAR), indicated by a green box, followed by the acyltransferase (AT, red), the poly N region (blue), and the C-terminal SKL tripeptide representing the type 1 peroxisomal targeting sequence. (B) Western blotting shows that a population of cells expressing N-terminally GFP (AT, red) tagged FARAT (NP) shows a higher overall expression level than the population producing the C-terminally tagged enzyme (CP). In both cases some protein degradation, possibly occurring behind the poly-N region, is visible. AX2 does not show immunoreactivity in the region of 170 kDa as compared to the marker (M), but only to Dictyostelium vacuolin (VAC) used to illustrate equal loading of the gel. (C,D) Single confocal planes through fixed cells expressing FARAT fused to GFP (green channel) at the N-terminal end (C,E) or carrying the GFP-tag including a peroxisomal targeting sequence at the C-terminus (D,F). The peroxisomes were labelled by co-expression of RFP-SKL (red in C and D), whereas lipid droplets are stained by incorporation of the fluorescent fatty acid analogue C1-BODIPY-C12 (red in E and F). The size bar in the overlay images (OL) corresponds to 5 µm.
transformed AX2 wild-type cells to express GFP, while cell survival during development (Kornke and Maniak, 2017), we remained 45% above wild-type levels (Fig. 5B). Were not as dramatically affected, so that their performance was seen in Fig. 6A, Fig. 4E), was subjected to the same type of mixing experiment. As replicates revealed the residual amount of 13% MDG in the mutants previously identified by mass spectrometry (Du et al., 2014), was (Fig. 4D), thin layer chromatography revealed that the MDG band, different from the wild-type level (Fig. 4E); therefore, we refrained (Fig. 4G). Consistent with this observation, distribution did not shift to lipid droplets (Fig. 3E,F), as seen for the labelled peroxisomes is documented in Fig. 3C and D. In this paper, the gene disruption of gpat was indeed the cause for MDG loss (Fig. 4A) yielded two independently isolated strains (I/4 and II/22) that showed the insertion of a BS′-cassette into the fatA gene and correspondingly the absence of the wild-type copy (Fig. 4B,C). Although the mutants’ LDs appeared to be normal by microscopy (Fig. 4D), thin layer chromatography revealed that the MDG band, previously identified by mass spectrometry (Du et al., 2014), was almost absent (Fig. 4E). Careful densitometry of three biological replicates revealed the residual amount of 13% MDG in the mutants (P<0.001). The amount of TAG, however, was not significantly different from the wild-type level (Fig. 4E); therefore, we refrained from conducting enzymatic TAG measurements. The fatA-knockout strain I/4 was chosen for further transformation to express either GFP-FARAT (clone I/17) or FARAT-GFP-SKL (clone I/3). Both constructs rescued the production of MDG to at least the same level as wild type (I/3) or even above AX2 levels (I/17), confirming that the gene disruption of fatA was indeed the cause for MDG loss (Fig. 4F).

Interference with lipid synthesizing enzymes affects phagocytosis and development

Because cells lacking GPAT grew normally in liquid medium, we assumed that their ability to take up fluid by macropinocytosis was normal and resorted to measure phagocytosis. Using yeast particles as prey, both GPAT knockout lines were about 20% more efficient than wild-type cells (Fig. 5A). When the cells were pre-incubated with palmitic acid, so that they could accumulate lipid droplets, the overall efficiency of phagocytosis in wild-type cells decreased by 50%, as described before (Paschke et al., 2012), but gpat knockouts were not as dramatically affected, so that their performance remained 45% above wild-type levels (Fig. 5B). Because the ability to form lipid droplets also strongly impairs cell survival during development (Kornke and Maniak, 2017), we transformed AX2 wild-type cells to express GFP, while gpat–mutants produced RFP, for tracking their efficiency to form spores in mixing experiments. The results were compared to 50/50 mixtures of red and green fluorescent wild-type cells (Fig. 5C). If the cells originated from normal growth medium, the gpat mutant and wild type each contributed 50% of the spores (bar 2 in Fig. 5C), and the same was seen, as expected, from mixtures with only wild-type cells (bar 1 in Fig. 5C). The mutants also formed about 90% of the spores when mixed with palmitic acid-treated wild type (bar 4 in Fig. 5C). However, when the gpat mutants were pre-incubated in palmitic acid-containing medium, they were able to form spores four times more efficiently than wild-type cells (bars 5 and 6 in Fig. 5C). Consistent with this observation, gpat mutants also resisted the adverse effect of fatty acid treatment whereas most of the LD-containing wild-type cells were lost over the 24hoe hours of development (bars 7 and 8 in Fig. 5C).

Overall, the gpat mutants exactly mimic the behaviour of strains bearing fewer lipid droplets because a fatty acid-activating enzyme, FcsA, or one or both DGAT enzymes were mutated (Kornke and Maniak, 2017). Because in these strains TAG and MDG are reduced proportionally, the fatA– mutant, that is specifically affected in MDG synthesis, while producing normal amounts of TAG (see Fig. 4E), was subjected to the same type of mixing experiment. As seen in Fig. 6A, fatA-knockout cells are unable to efficiently contribute to the spore population when they were allowed to form lipid droplet in advance (bar 6') and they also do not dominate mixtures where both strains were pre-treated with palmitic acid (bar 8'). Thus, cells lacking the FARAT enzyme and the neutral lipid MDG almost behave like wild-type cells (compare to Fig. 5C), most likely because they have unaltered TAG-levels.
were incubated for 3 h in growth medium containing (+PA) or lacking palmitic acid (PA) for 3 h prior to measurement. (C) Cells expressing GFP or values. (B) Phagocytosis assays repeated in axenic medium containing from three independent experiments ±s.d. and the curve connects the mean

deed on the phospholipid composition. Therefore, we repeated TLC analysis of our mutants, now focussing on phospholipids. Though the global pattern of the fatA− mutant looked very similar to the wild type, a quantitative reduction was seen in the bands corresponding to PE and PI (Fig. 6F) being lipids that were previously identified to contain ether linked hydrocarbon chains (Weeks and Herring, 1980; Clark et al., 2014). The levels of these two phospholipids returned to normal upon expression of FARAT-GFP-SKL in the fatA− background, and it is notable that the defect in phagocytosis was concomitantly rescued (see Fig. 6D,E). Further inspection of the phospholipid pattern shows that none of the lipids detected in wild-type cells is missing in the gpat− mutant, it rather appears that the bands corresponding to PE, PI and, more clearly lysophospholipids, are becoming more intense, suggesting that from these species more ether variants are produced (Fig. 6F). Interestingly, the increased phospholipid levels in the gpat mutants correlate with increased phagocytosis efficiency (see Fig. 5A,B).

**DISCUSSION**

**Dictyostelium** GPAT initiates TAG synthesis that interferes with the developmental program

Yeast has two enzymes with GPAT activity: Gat1p, which is localised in the ER-membrane and on LDs (Athenstaedt and Daum, 1997; Grillitsch et al., 2011), as well as Gat2p, which is confined to the ER (Athenstaedt and Daum, 1997; Zheng and Zou, 2001). The single gat2 mutant produces 50% less TAG, whereas the gat1 mutant produces about 40% more TAG (Zaremberg and McMaster, 2002). Thus, the single Dictyostelium GPAT localises like Gat1p (Figs 1 and 7) while the knockout resembles the effect of a yeast strain lacking Gat2p (Fig. 2E,F). In mammals, four GPAT activities were discovered. GPAT1 and 2 are related in size and sequence, and both localise to the mitochondrial outer membrane. The other pair of GPATs, isoforms 3 and 4, are ER enzymes (Cao et al., 2006), with GPAT4 able to move on to lipid droplets (Wilfling et al., 2013), similar to what we see for the Dictyostelium GPAT enzyme (Figs 1 and 7).

It is largely assumed that a common pathway exists for making phospholipids and neutral lipids ([Takeuchi and Reue, 2009] and references therein, Fig. 7). Therefore, it may come as a surprise that the single GPAT enzyme detected in Dictyostelium appears to produce only storage lipids, not phospholipids, because the gpat− mutant shows a strong reduction in the TAG level (Fig. 2E,F), but in contrast some increase in phospholipids (Fig. 6F), suggesting that unused building blocks are rerouted into the latter pathway. Similarly, single GPAT−/− mutant mice affecting isoforms 1, 3 and 4 show reduced fat content with some variation for tissues and organs (Hammond et al., 2002; Vergnes et al., 2006; Cao et al., 2014), while overexpression of GPATs 1, 2 and 3 leads to increased fat production (Igal et al., 2001; Cao et al., 2006; Wang et al., 2007). Also, only a few genetic manipulations in mammalian cells show an effect on the phospholipid composition, like the lack of GPAT1

in AX2 cells, but P<0.1 for n=3) can be at least in part attributed to a mild, but non-significant, reduction in the ability to take up fluid by macropinocytosis (Fig. 6B,C), however, a much stronger reduction was seen when phagocytosis was assayed (Fig. 6D). The addition of palmitic acid prior to the measurement neither influenced the relative fluid uptake (Fig. 6C) nor particle internalisation (Fig. 6E) of mutants versus wild type, although it depressed overall phagocytosis efficiency by about 50% (Paschke et al., 2012). Endocytic processes that are accompanied by dramatic morphological changes in the plasma membrane must strongly depend on the phospholipid composition. Therefore, we repeated TLC analysis of our mutants, now focussing on phospholipids. Though the global pattern of the fatA− mutant looked very similar to the wild type, a quantitative reduction was seen in the bands corresponding to PE and PI (Fig. 6F) being lipids that were previously identified to contain ether linked hydrocarbon chains (Weeks and Herring, 1980; Clark et al., 2014). The levels of these two phospholipids returned to normal upon expression of FARAT-GFP-SKL in the fatA− background, and it is notable that the defect in phagocytosis was concomitantly rescued (see Fig. 6D,E). Further inspection of the phospholipid pattern shows that none of the lipids detected in wild-type cells is missing in the gpat− mutant, it rather appears that the bands corresponding to PE, PI and, more clearly lysophospholipids, are becoming more intense, suggesting that from these species more ether variants are produced (Fig. 6F). Interestingly, the increased phospholipid levels in the gpat mutants correlate with increased phagocytosis efficiency (see Fig. 5A,B).

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In contrast to gpat− mutants, cells lacking FARAT grew somewhat more slowly in axenic medium. This consistently observed phenotype (average generation time 13.9 h versus 11.5 h

![Fig. 5. Lack of Gpat stimulates phagocytosis and protects from cell death in development.](image-url)
(Lewin et al., 2008), or GPAT4 overexpression (Chen et al., 2008; Nagle et al., 2008). Whereas one can always infer redundancy between the GPATs to explain this observation, one additional problem is that some of the acyltransferases were initially misassigned. Chen et al. (2008) have clarified that earlier work on AGPAT6 (Beigneux et al., 2006; Vergnes et al., 2006) was really done on mice lacking the enzyme now known as GPAT4, and a similar confusion occurred when GPAT3 (Cao et al., 2006) was found to be identical to AGPAT10 (Sukumaran et al., 2009). Therefore, it is well possible that the mammalian GPAT family may gain new members when other putative AGPATS become analysed more rigorously. Along the same lines, it is also conceivable that one of the several as yet uncharacterised acyltransferases in the Dictyostelium genome is specifically responsible for the synthesis of phospholipids (Fig. 7).

Interestingly, some mammalian GPAT enzymes have cellular functions outside the main fat metabolizing tissues like liver, or fat storage organs like brown or white adipocytes: GPAT4 deficient mice specifically lack the ability to produce milk for feeding their pups (Beigneux et al., 2006), while GPAT2 plays a major role in spermatogenesis (Garcia-Fabiani et al., 2017). Stimulation of macrophages via toll-like receptors shifts metabolism to lipid storage in preparation to fight infection with concomitant increase of a set of lead enzymes, among them GPAT3 (Feingold et al., 2012). Moreover, efficient phagocytosis depends on lipolysis (Chandak et al., 2010). Thus, it is well conceivable that mammalian macrophages lacking GPAT3 or GPAT4 show compromised phagocytosis (Quiroga et al., 2019). In Dictyostelium, however, phagocytosis does not seem to depend on a TAG reservoir, because in gpat” mutants phagocytosis is even increased, whether or not the cells are challenged with palmitic acid (Fig. 5A,B).

Instead, the dominant feature of Dictyostelium cells lacking GPAT is the resistance against fatty acid addition before development and the ability to efficiently produce spores under this condition (Fig. 5C). This supports previous observations on FcsA and the DGAT proteins (Kornke and Maniak, 2017) and means that the ability to make neutral lipids constitutes a risk for development. Because DGAT1 mutants lacked both TAG and MDG (Du et al., 2014; Kornke and Maniak, 2017), whereas in the gpat” mutants only TAG levels are reduced, while MDG is unaffected, we can now ascribe this perturbing effect specifically to excessive TAG storage, or ‘amoeboid obesity’.

**Dictyostelium FARAT produces neutral lipids and phospholipids essential for endocytosis**

In yeast, both Gat enzymes forming the ester bond between glycerol-3-P also accept DHAP as a substrate, meaning that the synthesis of ether and ester lipids are not spatially separated in the Fig. 6.

FatA-knockout cells are unaffected in development, but deficient in endocytosis. (A) Bar diagram showing the percentage of spores formed after palmitic acid pre-incubation in the vegetative phase or untreated controls from GFP-expressing AX2 (dark grey bars) and RFP-expressing fatA” mutant (I/4, open bars). For AX2/AX2 mixtures and further details compare Fig. 5C. (B) Intracellular accumulation of a fluid-phase marker was measured as relative units [r.u.], AX2 cells are represented by filled circles and the fatA” mutants (I/4, II/22) by open symbols. Bars indicate the s.d. of n=3 biological replicates. (C) Determination of macroipinocytosis was performed as in panel B, except that cells were allowed to form LDs from palmitic acid (+PA) 3 h prior to the assay. (D) Uptake of yeast particles is defective in the fatA”-knockout strains (I/4 and II/22) as compared to AX2 (black filled circles). Re-expression of either GFP-FARAT (dark green squares) or FARAT-GFP-SKL (light green squares) in the mutant I/4 fully restores phagocytosis. (E) When cells could form lipid droplets by the induction with palmitic acid (+PA) the differences between AX2 and fatA” mutants is maintained (black lines) but rescues by expression of GFP-tagged FARAT proteins (green lines) are less efficient. For description of symbols see above. (F) TLC analysis of phospholipids in AX2 and in fatA” and gpat” mutants. Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), Lysophosphatidylcholine (LPC), and Phosphatidylserine (PS) were mixed in appropriate amounts to generate the markers (M). Since all marker lipids contain ester-bonded acyl chains, the ether species of PE and PI (red arrowhead) migrate slightly above the standards. The blue arrowhead points to Lysophosphatidylethanolamine as identified by its Rf value and positive reaction to ninhydrine spraying.

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**Fig. 6.** FatA-knockout cells are unaffected in development, but deficient in endocytosis. (A) Bar diagram showing the percentage of spores formed after palmitic acid pre-incubation in the vegetative phase or untreated controls from GFP-expressing AX2 (dark grey bars) and RFP-expressing fatA” mutant (I/4, open bars). For AX2/AX2 mixtures and further details compare Fig. 5C. (B) Intracellular accumulation of a fluid-phase marker was measured as relative units [r.u.], AX2 cells are represented by filled circles and the fatA” mutants (I/4, II/22) by open symbols. Bars indicate the s.d. of n=3 biological replicates. (C) Determination of macroipinocytosis was performed as in panel B, except that cells were allowed to form LDs from palmitic acid (+PA) 3 h prior to the assay. (D) Uptake of yeast particles is defective in the fatA”-knockout strains (I/4 and II/22) as compared to AX2 (black filled circles). Re-expression of either GFP-FARAT (dark green squares) or FARAT-GFP-SKL (light green squares) in the mutant I/4 fully restores phagocytosis. (E) When cells could form lipid droplets by the induction with palmitic acid (+PA) the differences between AX2 and fatA” mutants is maintained (black lines) but rescues by expression of GFP-tagged FARAT proteins (green lines) are less efficient. For description of symbols see above. (F) TLC analysis of phospholipids in AX2 and in fatA” and gpat” mutants. Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), Lysophosphatidylcholine (LPC), and Phosphatidylserine (PS) were mixed in appropriate amounts to generate the markers (M). Since all marker lipids contain ester-bonded acyl chains, the ether species of PE and PI (red arrowhead) migrate slightly above the standards. The blue arrowhead points to Lysophosphatidylethanolamine as identified by its Rf value and positive reaction to ninhydrine spraying.
Fig. 7. Flow of proteins and lipids between organelles. Schematic view of cellular organelles outlined by membrane monolayers (lipid droplets) or bilayers (ER, peroxisomes) in blue. Membrane proteins (red boxes) are inserted into the ER membrane and remain there, if they span the bilayer (DGAT1) or can move to the lipid droplet if only held by the lipid monolayer facing the cytoplasm (GPAT). FARAT (red dot) is a soluble protein that is post-translationally transported into the lumen of the peroxisome by virtue of its C-terminal type1 targeting sequence. Although its N-terminal domain is equivalent to the human FAR1 enzyme, Dictyostelium FARAT does not move from peroxisomes to the lipid droplet surface (broken red arrow). Lipid synthesis (black) is initiated in the ER membrane starting from glycerol-3-phosphate (G-3P) and acyl-coenzyme A (Ac-CoA, left). These substrates are converted by the action of GPAT to monoacylglycerol-3-phosphate (MAcG-3P, aka lysoospholipidic acid). Addition of the second acyl-chain yields diacylglycerol-3-phosphate (DAcG-3P, aka phosphatidic acid) which then is dephosphorylated to diacylglycerol (TAG) before the third acyl-moiety is linked by DGAT1 to generate triacylglycerol (TAG). Following a similar itinerary, ether lipid synthesis begins with the FARAT-catalysed bond formation between dihydroxyacetone-phosphate DHAP and Ac-CoA in the peroxisome (right). The acyl chain on DHAP is subsequently replaced by a long chain alcohol, formed by a reduction reaction on FARAT. This replacement reaction and another subsequent reduction of DHAP to G-3P are not shown, but together they yield monoacylglycerol-3-phosphate (MAcG-3P) that is transferred to the ER. There one more acyl-chain is added, producing monoacyl-monooacylglycerol-3-phosphate (MAIMAcG-3P), which, after removal of the phosphate-group (MAIMAcG), receives another acyl-moiety from DGAT1 to result in monoacyl-diacylglycerol (MDG). Phospholipids carrying the more common ester bonds (PL), as well as ether-bonded variants (Eth-PL) are derived from various intermediates of the pathway. **T. thermophila** encodes the same bifunctional FARAT protein (Dittrich-Domergue et al., 2014) as *Dictyostelium*, where the N-terminal extension encodes a functional fatty acid reductase domain (Dittrich-Domergue et al., 2014) that may provide the fatty alcohol for the subsequent step in ether lipid synthesis (Fig. 7), catalysed by ADPS (Razeto et al., 2007). Whereas functional experiments on the *T. thermophila* enzyme had to be performed in yeast (Dittrich-Domergue et al., 2014), the loss of DHAPAT in *Trypanosoma* appears to be lethal, but in a conditional mutant all ether lipid species decline, with a concomitant increase on ester phospholipids (Zufferey et al., 2017). The *Dictyostelium* *fatA* mutant is almost devoid of the neutral lipid MDG, and residual amounts are assumed to originate from one of the surprisingly many polyketide synthases encoded in the genome (Zucko et al., 2007). Interestingly, DGAT1 accepts long-chain alcohols as substrates in wax-ester production and it is also instrumental in MDG biogenesis (Du et al., 2014). In addition, *fatA* mutants also show a clear reduction in phospholipids (Fig. 6F) but remains viable, albeit growing somewhat more slowly. Its dominant phenotype is a dramatically reduced ability to take up particles by phagocytosis and a mildly affected macropinocytosis (Fig. 6B–E). Phagocytosis and macropinocytosis are similar processes in that a relatively large volume of extracellular space containing a particle or fluid, respectively, is internalised. This depends on the actin cytoskeleton and on associated proteins (e.g. coronin; Maniak et al., 1995; Hacker et al., 1997) in the same fashion. The same plasma membrane proteins are present or selectively excluded from nascent phagosomes and macropinosomes (Mercanti et al., 2006). The sequential appearance of the inositol lipids PI(3,4,5)P3 and PI(3,4,2)P3 has been followed with a time resolution of seconds and was found to be the same (Dormann et al., 2004). In support of this finding, knockout of the Dds54 phosphatase, the enzyme that performs the conversion of the above PIPs, perturbs phagocytosis and macropinocytosis to a similar extent (Loovers et al., 2007). On the other hand, individual knockouts of PI3 kinases (PIK1/2 double and PIK4 single) have been discovered and independently confirmed to affect macropinocytosis and phagocytosis differentially. (Zhou et al., 1995, 1998; Buczynski et al., 1997; Hoeller et al., 2013). Where the differences come from, is not finally established, but it might be due to different vesicle sizes as explained in Bloomfield et al. (2015). In general, it appears that less inositol and ethanolamine type phospholipids lead to reduced endocytosis as seen for the *fatA* mutant (Fig. 6B–E). Of course, this conclusion has to be taken with a grain of salt because for one, we cannot tell to which of the organelles or the plasma membrane these lipids are preferentially delivered, and secondly, within the scope of this paper, we cannot interfere with the production of only one of the PI and PE lipids individually. A conditional inositol mutant has been made very recently (Frej et al., 2016). It shows many changes in PI lipids, but most significant is the decrease in PIP2. Up to now, only slow growth in liquid medium and failure to produce plaques on bacterial lawn were reported, but the cells were not tested for macropinocytosis and phagocytosis with specific assays. Thus, future investigations should go into the function of the ethanolamine head-group in order to dissect the relative roles of phospholipids in endocytosis, especially since PE-ether lipids were recently demonstrated to have a strong impact on *Caenorhabditis* physiology (Shi et al., 2016). Finally, disregarding the head-group properties, ether-based phospholipids have strong impact on membrane structure and dynamics (Dean and Lodhi, 2018), and most notably affect professional phagocytes, where ether lipids are a
truly vital component of neutrophils (Lodhi et al., 2015) and their remodelling specifically accompanies differentiation from the circulating monocyte to the macrophage (Walnner et al., 2014). In contrast, the inverse effect of increased phagocytosis (Fig. 5A,B) as observed in the gpat mutants, may be due to higher levels of lysophospholipids (Fig. 6F), as demonstrated in mammalian macrophages (Ngwenya and Yamamoto, 1985; Balestrieri et al., 2009; Rubio et al., 2015).

**MATERIALS AND METHODS**

**Cell growth, mixing and development**

Cells of the Dictyostelium AX2 strain ‘Gerisch’ (referred to as wild type) and mutants constructed in this genetic background were grown at 21°C in HL5+ medium (Formedium, UK) in shaking suspension and lipid droplet formation was induced by adding palmitic acid to a final concentration of 200 µM as described before (Du et al., 2013). For measuring competition during development, our published protocol and method of data evaluation (Kornke and Maniak, 2017) was followed, where the individual strains were labelled by expression of cytoplasmic GFP or RFP from pDNeo-2a based vectors (Dubin and Nellen, 2010). Each mixing experiment was conducted three times and over 100 spots each were counted. Between 1 and 4% of spots were non-fluorescent and thus omitted from the graphs. Endocytosis of TRITC-labelled yeast or TRITC-dextran was quantified as detailed in Rivero and Maniak (2006).

**Molecular biology**

DNA and protein sequences for Dictyostelium gpat (DDB0235400) encoding GPAT and fatA (DDB0305750) encoding FARAT were obtained from the fully sequenced genome (Eichinger et al., 2005) via http://dictybase.org where they are also linked to studies of expressed sequence tags. Transmembrane regions and targetsignalling sites were identified at http://ch.EMBlenet.org.

To fuse gpat with GFP, reverse transcribed total mRNA from Dictyostelium was used as a template, primers 771 (CCCGATCCAAAAA-TGGGGAAAAAAGTATGATATTACCATCATTGAG) and 772 (CC- GGATCTTATTCGGTGTTGGGTTGCCAATTTTC) further amplified the complete coding sequence of the gpat gene, now flankned by BamHI restriction sites. This fragment was inserted into the unique BamHI site of plasmid 68 pDNeoGFP (Jenne et al., 1998) generating vector 931 that could proceed into the GFP coding region provided by plasmid 48 pDd-GFP-PTS1. To tag the FARAT protein on its C-terminal end, a PTS1 (peroxisomal targeting signal) was used as a template, primers 1023 (ATGGTAGTATGCAATTTTTATGCTGTAAMAC) and 1024 (TAAAGTTTTGAATATGGAATCTCCCAAC) were used. The coding cDNA was ligated into pGEM-T-easy (Promega) resulting in plasmid 1210. This plasmid was taken as a template for a second amplification with primers 1167 (GTCGACAAAAATGGTAGTATTAG-CAGATAATTTTTATG) and 1168 (CTCGAGTTAAAGTTTTGAAGATAATCTGAGATG) resulting in Plasmid 1326.

**Lipid analysis**

The classical method of lipid preparation by Bligh and Dyer (1959) was adapted as described previously (Du et al., 2013). Subsequently, neutral lipids from three independent experiments were separated by thin-layer chromatography (TLC) on silica plates using two solvents. When the first solvent front (hexane:diethyl ether:acetic acid 80:20:1) had reached two-thirds of the separation distance, the plate was air dried and further developed in a second solvent system (hexane:diethyl ether 4:1) to completion. To visualise the lipids, TLC plates were stained for 3 min with copper sulfate (0.6 M in 8.5% phosphoric acid) and heated to 160°C for 15 min to condense the charring reaction. For phospholipid separation, we used high performance TLC (HPTLC) plates with a silicagel S Nano-ADAMANT surface (Machery-Nagel, Ref. 821140). The separation procedure is detailed in (Leary et al., 1987). Briefly, HPTLC plates were impregnated with 2.3% Boric acid dissolved in ethanol allowed to dry under a fume hood for 5 min and further treated for 15 min at 100°C in a hot-air oven. The bottom of the TLC chamber was filled with a solvent system containing chloroform/ethanol/water/triethylamine (30:35/7:35/0.1) and the sides were decorated with filter paper to provide a homogeneous atmosphere. After lipid spotting, the HPTLC plate was placed in the chamber and developed until the solvent reached the top of the plate. After drying, the plate was dipped into a solution containing 0.16 M NaOH, 0.06 M HCl and 1 g/l 8-Anilino-1-naphthalene-sulfonic acid (Sigma-Aldrich, St Louis, MO, USA) in water, and visualised under UV-light. Phospholipids were identified by co-migration with the following standards: Phosphatidylinositol (Sigma-Aldrich, 79403), L-A-Phosphatidylyserine (Sigma-Aldrich, P7769), Lyso-Phosphatidylcholine (Avanti Lipids, 830071P), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (Avanti Lipids, 850375P) and 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (Avanti Lipids, 850725P). In addition, cellular triacylglycerol amounts were measured.
quantified by a coupled enzymatic reaction described previously (Du et al., 2013) in three biological replicates and displayed with mean±s.d. shown in the bar diagram. The values of unstimulated cells were subtracted and the AX2 wild type was used as a reference (for reasons see Du et al., 2014).

**Immunofluorescence experiments, GFP microscopy and western blotting**

Immunofluorescence experiments, GFP microscopy and western blotting were performed as described before (Maniak et al., 1995). The distribution of the endoplasmic reticulum was shown by indirect immunofluorescence using undiluted mouse monoclonal antibody supernatant raised against the protein-disulﬁde isomerase (PDI, MAb 221-64-1) (Monnat et al., 1997). Monoclonal antibodies were detected using CY3-coupled goat-anti-mouse polyclonal secondary antibodies (Dianova). The lipid droplet-speciﬁc dye LD540 (Spandl et al., 2009) was diluted from its stock (0.5 mg ml⁻¹ in ethanol) to a ﬁnal concentration of 0.3 µg ml⁻¹ in phosphate-buffered saline (PBS) and used to stain ﬁxed cells for 30 min instead of using an antibody. Alternatively, LDs were metabolically labelled with the ﬂuorescent fatty acid analogue C1₂-BODIPY-C1₂ (5 µM ﬁnal concentration, Thermo Fisher Scientiﬁc) supplemented together with palmitic acid for 3 h in growth medium. Peroxisomes were labelled by co-expression of RFP-SKL (Schmauch and Maniak, 2008). Images were taken as single confocal planes using a Leica TCS-SP laser scanning microscope. In western blots, mouse monoclonal antibody 264-449-2 (available from Millipore) was used to detect GFP, whereas antibodies directed against Dictyostelium severin (42-65-11; Schleicher et al., 1984) and vacuolin (263-79-2; Wiencek et al., 2006) served as loading controls.

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**Competing interests**

The authors declare no competing or ﬁnancial interests.

**Author contributions**

Conceptualization: M.M.; Methodology: F.K.; Validation: F.K., X.D.M., B.A.H., J.M.K.; Writing - original draft: M.M.; Writing - review & editing: F.K., X.D.M., B.A.H., J.M.K.; Funding: Kay, R. R., Sander, S. P., Veltman, D. M., Pachebat, J. A. (2015). Dicyostelium discoideum Dgat2 can substitute for the essential function of Dgat1 in triglyceride production but not in ether lipid biosynthesis. *J. Biol. Chem.* 289, 21984-21994. doi:10.1074/jbc.M114.579318

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