Drosophila phosphopantothenoylcysteine synthetase is required for tissue morphogenesis during oogenesis

Floris Bosveld¹, Anil Rana¹, Willy Lemstra¹, Harm H. Kampinga¹, Ody C. M. Sibon¹

¹Department of Cell Biology, Section of Radiation & Stress Cell Biology, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands

Submitted for publication
**ABSTRACT**

Coenzyme A (CoA) is an essential metabolite, synthesized from vitamin B₃ by the subsequent action of five enzymes: PANK, PPCS, PPCDC, PPAT and DPCK. Mutations in *Drosophila dPPCS* disrupt female fertility and in this study we analyzed the female sterile phenotype of *dPPCS* mutant females in detail. We demonstrate that *dPPCS* is required for various processes that occur during oogenesis including chorion patterning. Our analysis demonstrates that a mutation in *dPPCS* disrupts the organization of the somatic and germ line cells due to abrogated F-actin remodeling possibly as a result of abnormal PtdIns(4,5)P₂ and Akt/PKB signaling. These defects affect the localization of Grk and Notch, whose activity is required for specification of the follicle cells that pattern the eggshell. Mutations in *dPPCS* also induce alterations in sensory organ patterning and cause wing vein abnormalities. Mutations in *dPANK* and *dPPAT-DPCK* result in similar patterning defects. Together, our results, demonstrate that *de novo* CoA biosynthesis is required for proper tissue morphogenesis.

**CONTENTS**

**INTRODUCTION**
**MATERIAL AND METHODS**
**RESULTS**
- Mutations in *dPPCS* affect egg chamber development, fecundity and eggshell patterning
- Mutation in *dPPCS* affect F-actin dynamics and cytoplasmic dumping
- Grk and Notch localization is disrupted in *dPPCS*¹/¹ follicles
- Membrane localization of PtdIns(4,5)P₂ is impaired in *dPPCS* mutant follicles
- *dPPCS* is required for patterning of various tissues

**DISCUSSION**
- *dPPCS* modulates F-actin remodeling in response to PtdIns(4,5)P₂ signaling
- Mutations in *de novo* CoA synthesis disrupt morphogenesis

**ACKNOWLEDGEMENTS**
**LITERATURE CITED**
INTRODUCTION
Coenzyme A (CoA), the major acyl carrier in all living organisms, constitutes an essential cofactor to support cellular metabolism. Synthesis of CoA occurs in a conserved route in which vitamin B$_5$ is subsequently modified by five enzymes: PANK, PPCS, PPCDC, PPAT, DPCK and the complete biosynthesis pathways in bacteria, archaea, plants, humans and Drosophila have been identified (CHAPTER 4 and ref. 2-5). Although, CoA biosynthesis is well characterized in bacteria and in vitro systems, only recently the impact of abnormal CoA biosynthesis on animals and tissues has been investigated.

CoA is an indispensable cofactor for the synthesis of many lipids and impaired lipid metabolism associated with defects in CoA synthesis has been demonstrated in humans, mice and Drosophila (CHAPTER 4 and ref. 7-11). In addition, altered CoA metabolism has been implicated in a variety of defects ranging from altered growth factor signaling to impaired DNA integrity, mitochondrial dysfunction, delayed growth, reduced lifespan and neurodegeneration (CHAPTER 4 and ref. 8,10-17). Mutations in CoA biosynthesis also cause male and female fertility defects in mice and Drosophila (CHAPTER 4,6 and ref. 18-20). Despite these studies, the exact mechanisms that are responsible for this plethora of phenotypic defects in metazoans remain largely unknown.

Previously, we isolated a dPPCS mutant as a female sterile, neurologically impaired mutant and we demonstrated that CoA metabolism is required to maintain DNA integrity during development of the Drosophila central nervous system (CHAPTER 4). In this report the female sterile phenotype of dPPCS$^{1/1}$ was analyzed in detail. A mutation in dPPCS results in a complex phenotype and several processes required for normal oogenesis are affected. dPPCS mutants show defects in follicle cell organization, migration, specification and abnormal chorion patterning. Our results point to a model in which de novo CoA biosynthesis acts upstream of PtdIns(4,5)P$_2$-Akt/PKB signaling and F-actin remodeling. Disruption of these processes causes loss of tissue/cell morphology which in turn affects normal Notch and Gurken (Grk) localization and may lead to impaired follicle cell fate determination. We not only observed morphogenesis defects in mutant ovaries, but patterning of the sensory organs and venation is also disrupted, indicating that dPPCS function is required for morphogenesis of various tissues. Moreover, mutations in dPANK and dPPAT-DPCK result in similar phenotypes, demonstrating that not only dPPCS but merely de novo CoA synthesis is required for proper morphogenesis. Together our data demonstrate that in addition to a general function of CoA in lipid and energy metabolism, de novo CoA biosynthesis is also required for proper tissue morphogenesis.

MATERIAL AND METHODS
Drosophila stocks and genetics: Fly stocks were maintained at 22 °C according to standard protocols. For wild-type preparations y$^{w}w^{75C}$ was used. dPPCS, dPPCS$^{11}$ and P[dPPCS] are previously described (CHAPTER 4). The UAS-PLCδ-PH-GFP line was a gift from L. Cooley and A. Wodarz and the Act5C-GAL4 line was obtained from the Bloomington Stock Centre (Indiana University, USA).

Immunohistochemistry: Dissection, fixation and immunolabelling of ovaries was performed as described$^{31}$. Primary antibodies used included concentrated supernatants obtained from the Developmental Studies Hybridoma Bank (Iowa, USA); mouse anti-lamin D$_o$ (ADL84.12, 1:5) developed by P.A. Fisher, mouse anti-fasciclin III (7G10, 1:5) developed by C. Goodman, mouse anti-Notch (C17.9C6, 1:5) developed by S. Artavanis-Tsakonas, mouse anti-quail (6B9, 1:5) developed by L. Cooley, mouse anti-DE-cadherin, (DCAD2, 1:5) developed by T. Uemura and mouse anti-gurken (1D12, 1:5) developed by T. Schupbach,. Secondary antibodies included Cy3-conjugated goat anti-mouse (1:200, Jackson ImmunoResearch), FITC-conjugated goat anti-mouse (1:200, Jackson ImmunoResearch) and Cy5-conjugated goat anti-mouse 1:200, Jackson ImmunoResearch). Ovaries were stained with 20 U/ml rhodamin-
dPPCS is required for morphogenesis

phalloidin (Molecular Probes) and 0.2 μg/ml DAPI (Sigma) to visualize F-actin and the DNA, respectively.

Apoptosis was measured in 6-d-old flies that were kept for 3 days in vials containing yeast paste. The TUNEL cell death assay was performed following the ApopTag Fluorescein in Situ Apoptosis Detection Kit (Chemicon). Ovaries were fixed in devitellinizing buffer/heptane21 (1:6 per volume) and pretreated with proteinase K (20 μg/ml in PBS + 0.1% tween-20) for 15 min at room temperature. After labeling and washing ovaries were mounted in citifluor (Agar Scientific) and analyzed by confocal laser scanning microscopy (CLSM) (Leica TCS SP2 DM RXE). Images represent maximal projections (unless otherwise noted) of a z-stack (0.5-1 μm/scan). Images were processed using Leica software and Paint Shop Pro.

For Nile red (Sigma) staining 6-d-old flies that were kept for 3 days in vials containing yeast paste were used. Ovaries were dissected and stained with 100 ng/ml Nile red solution in PBS for 5 min. Ovaries were washed 3 times 10 min with PBS, mounted in citifluor and directly analyzed by CLSM.

Assessment of fecundity and morphological analyses: For studies of fecundity 10 groups of 5 virgin females and 5 males were crossed for 5 days in vials containing yeast paste. Flies were transferred to fresh vials without yeast and transferred to fresh vials every 24 h. The average eggs/24 h deposited was calculated from 3 replicates of each group. To assay embryonic viability embryos were collected (0-6 h) on apple juice plates containing yeast paste, counted, and the hatch rate was determined by visual inspection of the egg cases 2 days after egg laying. Inspection of chorion morphology was carried out using LM. Images were captured with an Olympus BX50 light microscope. To analyze ovary morphology virgin females were placed in vials containing yeast paste and ovaries were dissected 48 h, 72 h and 120 h AE. Ovaries dissected 48 h AE were directly analyzed by light microscopy (LM) (Olympus BX50), while ovaries dissected 72 h and 120 h AE were fixed, labeled and inspected by CLSM. Assessment of wing venation and sensory organ patterning was performed by LM.

Immunoblot analysis: Ovaries from 5-d-old females that were kept for 3 days in vials containing yeast paste were dissected in PBS. Samples were lysed in RIPA buffer supplemented with protease inhibitor cocktail 1697498 (Roche), sonicated and the protein content determined using the Bio-Rad DC protein assay kit (Bio-Rad). Protein extracts were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes (BioRad Mini-Protein electrophoresis system) and the membranes were subsequently incubated with rabbit anti-Akt/PKB pSer473 (1:1000, Cell Signaling) and rabbit anti-Akt/PKB (1:1000, Cell Signaling) antibodies as described by the manufacture. After incubation blots were washed and incubated with HRP-conjugated goat anti-rabbit (Amersham) secondary antibodies (1:2000). The antibody complexes were visualized using an enhanced chemiluminescent (ECL) kit (Amersham). The area x mean intensity of Akt/PKB-P was divided by the area x mean intensity of total Akt/PKB and normalized to wild-type ratios to determine levels of Akt/PKB-P in mutant ovaries. For this analysis, the same blots were first probed for Akt/PKB-P, stripped and then probed for Akt/PKB to normalize for Akt/PKB-P levels (when Akt/PKB-P levels were normalized using actin or tubulin, this resulted in the same outcome). Western blots from 3 experiments were quantified with ImageJ (http://rsb.info.nih.gov/ij/).

RESULTS
Mutations in dPPCS affect egg chamber development, fecundity and eggshell patterning
Initially, dPPCS (Drosophila phosphopantothenoylcysteine synthetase) was identified as a single P element insertion mutant with a female sterile and neurodegenerative phenotype (CHAPTER 4). In this hypomorphic mutant, the P element landed inside the 5'-UTR, which results in the production of a truncated dPPCS mRNA. Removal of the first intron of the dPPCS gene by imprecise P element excision (allele dPPCS1/33) induces lethality during the first instar larval stadium. dPPCS protein levels are reduced in dPPCS1/1 mutants and further reduced in transheterozygous dPPCS1/33 mutants. Likewise transheterozygous mutants generally display a more severe phenotype than dPPCS1/1 mutants alone. In this study we used dPPCS1/1 mutants because only a limited amount of vitellogenic egg chambers could be found in dPPCS1/33 transheterozygous mutants.

The neurodegenerative phenotype of dPPCS mutants has been described in detail (see CHAPTER 4), here the female sterile characteristics are investigated. The female sterile phenotype as described in this manuscript as well as the neurodegenerative phenotype is rescued
by overexpression of a FLAG-tagged dPPCS cDNA ($P[dPPCS]$), (see Table 1 and CHAPTER 4, 6), indicating that these phenotypes are caused by impaired function of dPPCS.

To understand the effect of impaired $dPPCS$ function on female fertility we first investigated the overall morphology of $dPPCS^{+/−}$ ovaries. At 48 h after eclosion (AE), the ovaries from $dPPCS^{+/−}$ females were poorly developed compared to wild-type ovaries (Figs. 1Aa-b). Ovaries from wild-type females were larger in size and contained mature eggs, while $dPPCS^{+/−}$ ovaries were small in size and did not contain mature eggs. In wild-types, the oldest egg chambers (or follicles) found in newly eclosed females are at stage 7 and upon food intake hormones are produced which trigger the follicles to proceed into vitellogenesis, a process whereby the oocyte starts to accumulate nutrients and increases in size\textsuperscript{22}. At 72 h AE, in wild-type females, 100% (n=35) of the ovaries contained vitellogenic egg chambers, while only 11% of the ovaries from $dPPCS^{+/−}$ females (n=36) contained vitellogenic egg chambers (Figs. 1Ac-d). At 120 h AE 80% of the $dPPCS^{+/−}$ ovaries (n=26) contained vitellogenic egg chambers, however the two lobes were frequently different in size and displayed features of degenerating egg chambers (Fig. 1Ae).

At 96-120 h AE, $dPPCS^{+/−}$ females start to deposit eggs and between 144-192 h AE $dPPCS$ mutants produced only $0.03 \pm 0.02$ SEM eggs/24 h that never hatched (n=142 eggs), while wild-type females produced $10.0 \pm 1.4$ SEM eggs/24 h of which 90% hatched (n=1005 eggs). It has been reported that a so called mid-oogenesis checkpoint monitors the integrity of pre-vitellogenic egg chambers, and activation of this checkpoint results in the removal of abnormal egg chambers\textsuperscript{22}. When we performed a TUNEL assay on $dPPCS^{+/−}$ ovaries 144 h AE, we found a 6-fold increase in ovarioles containing apoptotic egg chambers prior to vitellogenesis (32.6%, n=408 ovariules) compared to wild-type ovaries (4.1%, n=245 ovariules). Approximately 32% of $dPPCS^{+/−}$ ovariules (n=222) contained stage 5-7 egg chambers that displayed packaging defects (egg chambers with more than 15 nurse cells) as determined by DAPI staining and lamin D\textsubscript{o} labeling (data not shown). This percentage was enhanced to 79% in $dPPCS^{+/−}$ ovariules (n=132), while only 4% of the wild-type ovariules (n=109) and 11% of the $P[dPPCS]:dPPCS^{+/−}$ ovariules (n=166) contained egg chambers with packaging defects. These results show that early egg chamber development is severely compromised in $dPPCS$ mutants. Within the germaria of $dPPCS^{+/−}$ mutants, aberrant separation of the developing egg chambers by the intercyst cells result in the production of egg chambers that exhibit aberrant interfollicular stalk cell and/or polar follicle cell formation, egg chambers with mispositioned oocytes or egg chambers that display packaging defects (see supplementary Figs. S1-S4). Thus the reduced fecundity of the $dPPCS^{+/−}$ females is likely due to the production of aberrant egg chambers that did not pass the mid-oogenesis checkpoint and were absorbed.

In addition to impaired fecundity, 80% of the eggs deposited by $dPPCS^{+/−}$ females displayed a dumpless phenotype\textsuperscript{23}. The eggs contained reduced amounts of yolk and a wide array of both dorsal-ventral (DV) and anterior-posterior (AP) patterning defects such as four appendages, shortened opercula, fused appendages, abnormal stalks or a complete lack of dorsal appendages were observed (Fig. 1B). These results show that a mutation in $dPPCS$ affects ovary/egg chamber development, disrupts fecundity and causes chorion patterning defects.


dPPCS is required for morphogenesis

Figure 1. Egg chamber development and eggshell patterning is disrupted in dPPCS\textsuperscript{1/1}.

(A) Morphological analyses of wild-type and dPPCS\textsuperscript{1/1} ovaries. (Aa) Wild-type ovaries are well developed 48 h AE and contain mature eggs (arrowheads), (Ab) while dPPCS\textsuperscript{1/1} ovaries were small in size. (Ac-e) Ovaries were labeled with rhodamin-phalloidin to detect the F-actin and stained with DAPI to visualize the DNA. (Ac) At 72 h AE, wild-type ovaries contain vitellogenic egg chambers, as determined by the increased size of the oocyte compartment (asterisk). (Ad) dPPCS\textsuperscript{1/1} ovaries remained small in size (1 entire lobe is shown) and lacked vitellogenic egg chambers. ( Ae) At 120 h AE, dPPCS\textsuperscript{1/1} ovaries contained vitellogenic egg chambers (asterisks) and exhibited features of degenerating egg chambers (arrowheads). The 2 lobes were frequently different in size.

(B) Chorion patterning is disrupted in dPPCS\textsuperscript{1/1}. (Ba, Bd) Wild-type embryos have 2 dorsal appendages. (mp) micropyle; (p) paddle; (s) stalk. (Bb-c, Be-f) Embryos deposited by dPPCS\textsuperscript{1/1} mothers exhibited a dumpless phenotype and had a wide range of patterning defects, which were classified in 5 groups; (Bb) embryos with shortened opercula (bracket, compare with Ba) and 4 appendages, (Bc) abnormal stalks (arrows), (Be) fused appendages (bracket, compare with Bd) or (Bf) lacked dorsal appendages. Percentages are indicated (n=142). The remaining 22.8% had 2 dorsal appendages, but the appendages were of different length, lacked paddles or the bases were shifted posteriorly. Scale bars; 500 µm (Aa-b), 150 µm (Ac-e).
Figure 2. Cytoplasmic F-actin filament assembly and dumping is disrupted in dPPCS\(^{1/1}\) follicles.

(Aa-Ac) Wild-type nurse cells assemble an elaborate network of transverse F-actin filaments prior to cytoplasmic dumping. Quail colocalizes with the F-actin filaments and assist in filament assembly. (Ba-Bc) The cytoplasmic F-actin network is not properly formed inside dPPCS\(^{1/1}\) nurse cells and Quail localization is diffuse inside the cytoplasm. (C) In wild-type ovaries the F-actin bundles anchor the nurse cell nuclei during dumping. (D) dPPCS\(^{1/1}\) nurse cells fail to assemble F-actin filaments and the nurse cell nuclei become trapped inside the ring canals during dumping (arrows in D, F). (E) dPPCS\(^{1/1}\) oocyte nucleus encapsulated by F-actin fibers. (F) In dPPCS mutant follicles nurse cell nuclei were found inside the oocyte compartment (arrow marks a nurse cell nucleus trapped inside a ring canal). Note that the nurse cell nuclei are in close proximity to the anterior of the oocyte and “push” the membrane towards the oocyte compartment (compare with G). (G) During cytoplasmic dumping a tight array of F-actin is present at the subcortical membrane of wild-type oocytes. (H-I) The subcortical F-actin fibers at the membrane of the dPPCS\(^{1/1}\) oocyte compartment were increased in size and thickness (boxed arrowhead), and large clumps of F-actin were found within the oocyte compartment (arrowheads). (J) Centripetal migrating follicle cells express high levels of DE-cadherin. In dPPCS mutants migration of these cells occurred normally, but nurse cells that were pushed inside the oocyte were trapped within the oocyte compartment after these cells finished their migration. The F-actin network was labeled with rhodamin-phalloidin and nuclei were stained with DAPI or antibodies against Lamin D. Asterisks mark the oocyte nuclei. (oo) oocyte compartment. Scale bars; 100 µm (A-B), 20 µm (C-E), 50 µm (F-J).

Table 1. Mutations in dPPCS affect egg chamber development

<table>
<thead>
<tr>
<th>Stage 10-11 F-actin dynamics &amp; cytoplasmic dumping</th>
<th>% of egg chambers</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>dPPCS(^{1/1})</td>
</tr>
<tr>
<td>Nurse cells trapped inside the oocyte</td>
<td>0.0 (n=100)</td>
</tr>
<tr>
<td>F-actin clumps in ooplasm</td>
<td>3.0 (n=100)</td>
</tr>
<tr>
<td>Aberrant nurse cell F-actin</td>
<td>0.0 (n=100)</td>
</tr>
<tr>
<td>Nurse cells plugging ring canals</td>
<td>0.0 (n=100)</td>
</tr>
<tr>
<td>Oocytes with disorganized subcortial F-actin</td>
<td>0.0 (n=100)</td>
</tr>
<tr>
<td>Oocyte nuclei with F-actin fibers</td>
<td>0.0 (n=100)</td>
</tr>
</tbody>
</table>

Nurse cells were stained with DAPI to detect the DNA and labeled with rhodamin-phalloidin to visualize the F-actin network. P[dPPCS] is a FLAG-tagged dPPCS cDNA under control of an ubiquitin promoter.
**Mutation in dPPCS affect F-actin dynamics and cytoplasmic dumping**

Dumpyless embryos with chorion patterning defects can result from defects in actin fiber formation within the nurse cells that in turn affect cytoplasmic transport due to the obstruction of the ring canals by nurse cell nuclei\(^{71}\). We analyzed actin dynamics during cytoplasmic dumping in dPPCS\(^{1/2}\) mutants to investigate the hypothesis that the dumpless phenotype of dPPCS mutants is due to abnormal actin organization. In stage 10 wild-type follicles, an elaborate network of filamentous actin (F-actin) bundles is assembled inside the nurse cells which preludes cytoplasmic dumping. These F-actin bundles anchor the nurse cell nuclei to prevent them from entering the oocyte when the remaining nurse cell material is actively squeezed into the oocyte\(^{26}\). Assembly of the cytoplasmic F-actin network requires the Quail protein which colocalizes with the F-actin fibers (Fig. 2A)\(^{25,27}\). In contrast to wild-type follicles, assembly of the cytoplasmic F-actin fibers was disrupted in dPPCS\(^{1/2}\) follicles and the Quail protein failed to associate with the F-actin bundles and remained diffuse throughout the nurse cell cytoplasm (Fig. 2B). As a result of aberrant F-actin assembly, nurse cell nuclei were indeed found trapped inside ring canals during dumping (Fig. 2D, Table 1). Interestingly, we also found oocyte nuclei that were encapsulated by bundles of F-actin (Fig. 2E, Table 1). Furthermore, large F-actin fibers were assembled at the cortical membrane of the oocyte and the follicular epithelium of the oocytes was frequently disorganized (Figs. 2H-I). This suggests that in mutants the oocyte compartment acquires an abnormal signal to assemble an F-actin network during dumping. Mutant oocytes also contained large clumps of F-actin (Figs. 2H-I, Table 1). Nurse cell nuclei were also found inside the oocyte compartment (Fig. 2F, Table 1), demonstrating that nurse cell nuclei are not anchored properly (Fig. 2F, Table 1). In wild-types, during stage 10 a group of follicle cells that sheet the oocyte will migrate centripetally to cover the anterior of the oocyte\(^{28}\). In numerous cases, we observed in dPPCS\(^{1/2}\) mutants, nurse cell nuclei trapped inside the oocyte compartment, after the centripetal follicle cells enclosed the anterior of the oocyte (as determined by DE-cadherin staining\(^{29,30}\)) (Fig. 2J). This again demonstrates that organization and anchoring of the nurse cells was indeed hampered in dPPCS mutant egg chambers.

We stained freshly dissected (and non-fixed) ovaries with Nile red, which has fluorescent properties in the presence of triacylglycerol and sterol esters\(^{31}\), to determine if neutral lipid synthesis and transport of these lipids to the oocyte was disrupted. In wild-types, synthesis of these neutral lipids increases in the germ line and somatic cells when follicles proceed into late stage oogenesis (Fig. 3A). In dPPCS mutant egg chambers, neutral lipid synthesis was reduced compared to wild-type egg chambers, suggesting that the synthesis of neutral lipids is affected in dPPCS mutants (Fig. 3B). Furthermore, transport towards the oocyte and accumulation inside the oocyte of these lipids appeared abnormal compared to wild-type ovaries (compare Fig. 3A and 3B). Thus the synthesis of neutral lipids and their transport towards the oocyte is disrupted in dPPCS mutant ovaries. The latter is likely the result of aberrant nurse cell anchoring and obstruction of ring canals in dPPCS\(^{1/2}\) follicles.

Next, we investigated whether a mutation in dPPCS\(^{1/2}\) affects cell migration events due to defective F-actin remodeling. During stage 8-10 the border cells, which include the anterior polar cells and part of the main body epithelium, migrate through the nurse cell compartment towards the anterior end of the oocyte\(^{32}\). In wild-types, when the border cells reach the oocyte and the centripetal follicle cells start migrating, Fasciclin III (FasIII) is expressed in the follicle cells of the DA corner (Fig. 4A). After the centripetal follicle cells finished their migration, the FasIII expressing cells form two distinct cell populations at the DA surface of the oocyte that
will initiate production of the dorsal appendages (Fig. 4B)\textsuperscript{23}. In \textit{dPPCS}\textsuperscript{1/1} follicles centripetal migration was finished before the border cells reached the anterior of the oocyte (Fig. 4C), indicating that these two cell migration events are not properly synchronized. Interestingly the border cell cluster was surrounded by an elaborate F-actin network, which may prevent the border cells to make contact with the centripetal follicle cells (Fig. 4Cb). Alternatively, it may be possible that because the nurse cells are not anchored properly, the nurse cells push against the centripetal follicle cells and thereby prevent the centripetal follicle cells from making contact with the border cells in a normal timely manner. This indicates that cell migration an sich is not abrogated in \textit{dPPCS}\textsuperscript{1/1}. Rather, the loss of cell organization, likely due to disrupted structural integrity, impaired proper cell migration. Together, these data demonstrate that \textit{dPPCS} is required for proper F-actin dynamics and cell organization during cytoplasmic dumping.

\textbf{Grk and Notch localization is disrupted in \textit{dPPCS}\textsuperscript{1/1} follicles}

We hypothesized that disorganized tissue integrity may also affect the signaling routes required for specification of the follicle cells that pattern the chorion. To investigate this, we stained ovaries with antibodies against Notch and Grk. Both molecules are required for specification of the follicle cell populations that pattern the eggshell\textsuperscript{23,33-35}. Although we cannot conclude that Grk or Notch signaling was disrupted in \textit{dPPCS}\textsuperscript{1/1} ovaries, the localization of both proteins was frequently impaired compared with wild-type ovaries. In wild-type ovaries when the border cells reach the centripetal follicle cells, Notch is highly expressed at the DA corner, where it is required for the specification of the dorsal appendage producing cells, while Notch expression is restricted to the nurse cell membranes during cytoplasmic dumping (Fig. 4Ba, 5Ac)\textsuperscript{36}. In \textit{dPPCS}\textsuperscript{1/1} stage 11 follicles Notch expression was more diffuse throughout the nurse cells and not restricted to the membranes (Fig. 4Ca). Notch expression was also severely affected during late stage oogenesis (Fig. 5Bc) and FasIII staining revealed that the dorsal appendage/operculum forming follicle cells were not properly organized (compare Fig. 5Ab and 5Bb).

In wild-type stage 9-10 egg chambers Grk is localized at the DA corner of the oocyte compartment. Although in \textit{dPPCS}\textsuperscript{1/1} egg chamber, Grk was present at the DA corner, the residence of the oocyte nucleus, the distribution of the protein was frequently impaired in stage 8-9 follicles (Fig. 5D) and progressively worsened when egg chambers proceeded into later stages of oogenesis (Fig. 5D-F). Because we observed that abnormal Grk localization coincided with the presence of nurse cell nuclei in close proximity to the oocyte nucleus (Figs. 52D-F), it
is likely that the nurse cells, which are not properly anchored, push against the oocyte and the follicle cells of the DA corner, which in turn results in abnormal Grk localization.

Thus it appears that impaired tissue organization due to disruption of F-actin cytoskeletal dynamics affects the localization of Grk and Notch, which in turn may lead to a failure to specify the follicle cell populations required for patterning of the eggshell. Pushing of the nurse cell nuclei against the oocyte compartment could disrupt cell migration events and affect the shape of the oocyte and the oocyte nucleus thereby also affecting signaling routes that specify the follicle cells that produce the operculum and the dorsal appendages. Similarly, a failure to anchor the nurse cell nuclei likely affects the transport of signaling molecules through the ring canals towards the oocyte. Due to their unpredictable nature such defects would give rise to a plethora of chorion defects and thus could explain the variety of eggshell patterning defects in dPPCS mutant embryos. These findings imply that dPPCS is not required for cell specification/signaling *an sich*, but merely cell organization/morphology, which is further supported by the finding that aberrant intercyst cell migration/organization likely underlies the observed packaging and follicle cell specification defects during early oogenesis (see supplementary Figs. S1-S2).

→ Figure 4. A mutation in *dPPCS* affect follicle cell migration and patterning.

(A-Ad) Wild-type stage 10 egg chamber. During stage 8-10 the border cells migrate through the nurse cell compartment towards the anterior end of the oocyte. When the border cells (arrow) reach the oocyte (stage 10) and the centripetal follicle cells start migrating (arrowheads), FasIII is expressed in the follicle cells of the DA corner. At this stage Notch is expressed at the membranes of the follicle cells of the DA corner, where it is required for the specification of the dorsal appendage producing cells.

(B-Bd) During stage 11, after the centripetal cells finished their migration, two patches of follicle cells can be found at the DA corner of the epithelium of wild-type egg chambers (arrowheads). These follicle cells express high levels of FasIII and will initiate the production of the dorsal appendages. At this stage Notch expression is restricted to the nurse cells membranes.

(C-Cd) *dPPCS*<sup>1/1</sup> egg chamber at stage 11. The centripetal follicle cells finished migration (arrowheads), but the border cells (arrow) failed to reach the centripetal follicle cells, while follicle cells of the DA corner were already expressing FasIII. The border cell cluster is surrounded by an elaborate network of F-actin. Notch localization is not restricted to the nurse cell membranes and has a more diffuse character. Boxed arrowheads point to 2 nurse cell nuclei that seem to contact (push against) the centripetal follicle cells. Asterisks mark the position of the oocyte. Scale bars; 50 µm.

→ Figure 5. Notch and Grk are abnormally localized in late stage *dPPCS*<sup>1/1</sup> follicles.

Wild-type and *dPPCS*<sup>1/1</sup> ovaries were labeled with antibodies against Notch and Grk. DAPI was used to visualize the DNA. (Aa-Ac) Wild-type egg chamber at stage 13. The FasIII positive cells produce the dorsal appendages and the operculum. Notch expression is restricted to the anterior follicle cells (arrowhead).

(Ba-Bc) In *dPPCS*<sup>1/1</sup> stage 13 egg chambers FasIII expression was abnormal, dorsal appendage and operculum formation was disrupted and Notch was expressed throughout the entire follicular epithelium. (C-D) Single confocal scans of wild-type (C) and *dPPCS*<sup>1/1</sup> (D-F) follicles. (C) The Grk protein localizes at the DA corner of the oocyte in wild-type stage 8-9 egg chambers. (D-F) Grk expression was present in *dPPCS*<sup>1/1</sup> mutant egg chambers, but the protein was frequently abnormally expressed along the DA corner in stage 8-9 due to a disrupted shape of this corner (D) and progressively worsened when egg chambers proceeded into late stage oogenesis (stage 10-11) (E-F). The nurse cell nuclei are in close proximity to the DA corner, indicating that these nuclei were not properly anchored during dumping (arrowheads). Note that the follicular epithelium appears disorganized. Asterisks mark the position of the oocyte. Scale bars; 150 µm (A-B), 50 µm (C-F).
Membrane localization of PtdIns(4,5)P$_2$ is impaired in dPPCS mutant follicles

Previously, we demonstrated that the levels of the major phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) are reduced in dPPCS mutant flies, indicating a general defect in phospholipid biosynthesis (CHAPTER 4). Moreover, mutations in the CoA biosynthesis enzymes result in reduced levels of phosphorylated Akt/PKB (Akt/PKB-P) during larval brain development (CHAPTER 6). Akt/PKB activity relies on the conversion of PtdIns(4,5)P$_2$ to PtdIns(3,4,5)P$_3$, mediated by PI3K, in response to nutrients and growth factors, suggesting that growth factor/nutrient signaling and/or PtdIns(4,5)P$_2$ levels are affected when CoA biosynthesis is disrupted. Many actin remodeling processes require Akt/PKB signaling and Akt/PKB signaling is also essential for the regulation of neutral lipid droplet synthesis during Drosophila oogenesis. Therefore, aberrant Akt/PKB signaling could underlie the F-actin remodeling and neutral lipid synthesis defects in the dPPCS mutant. In order to test this hypothesis, we determined levels of Ser505 Akt/PKB phosphorylation (equivalent to Ser473 in mammalian Akt/PKB) in mutant and wild-type ovaries (Fig. 6A). Compared with wild-type ovaries, levels of Akt/PKB-P, as determined by the ratio Akt/PKB-P to total Akt/PKB (normalized by using the same blot), were reduced 2-fold (54.3% ± 4.9 SEM, n=3) in dPPCS$^{1/1}$ ovaries (Fig. 6A), indicating that Akt/PKB signaling might be disturbed during oogenesis.

Because a general reduction in phospholipid levels can be found in dPPCS mutant flies it is plausible to assume that phosphatidylinositol production, the precursor for all phosphoinositides, is also reduced. PtdIns signaling is essential for all processes that require actin cytoskeletal remodeling. Therefore altered PtdIns signaling may underlie aberrant F-actin dynamics and reduced PtdIns(4,5)P$_2$ synthesis could also explain low levels of Akt/PKB-P in the dPPCS mutant. Because the PtdIns represent about 5-10% of the total pool of phospholipids, it is difficult to analyze PtdIns levels directly using biochemical assays. To investigate whether PtdIns signaling was disrupted in dPPCS mutant ovaries we expressed a PLCδ-PH-GFP fusion protein, which is able to bind to PtdIns(4,5)P$_2$ (ref. 47). We used an Act5C-GAL4 driver to analyze UAS-PLCδ-PH-GFP expression and thus PtdIns(4,5)P$_2$ localization in all cells. Because PtdIns levels and localization has not been investigated during Drosophila oogenesis, we first investigated the localization pattern of PtdIns(4,5)P$_2$ in wild-types. During wild-type cytoplasmic dumping PtdIns(4,5)P$_2$ is abundant at the cell membranes of the border cells and the apical membranes of the follicle cells that sheet the oocyte, while low levels of PtdIns(4,5)P$_2$ can be detected at the nurse cell membranes (Figs. 6B-C). On the contrary, PtdIns(4,5)P$_2$ localization at the apical membranes of the follicle cells that sheet the oocyte was hardly detectable or absent in dPPCS$^{1/1}$ follicles (Figs. 6D-E,G,I). Moreover, large patches of follicle cells that sheet the oocyte did not accumulate PtdIns(4,5)P$_2$ at their membranes (Figs. 6D-E,G). In addition, these experiments also clearly demonstrate that the follicular epithelium of dPPCS mutant follicles is disrupted (arrows in Figs. 6D, Gb). Finally, in mutants, nurse cell membranes sometimes accumulated distinctive patches of high levels of PtdIns(4,5)P$_2$ and we frequently detected PtdIns(4,5)P$_2$ localization at cell membranes that are reminiscent of the border cells (based on their position within the nurse cell compartment and their small size), again indicating that border cell migration was disrupted in dPPCS$^{1/1}$ follicles (Fig. 6E). Because aberrant apical localization of PtdIns(4,5)P$_2$ at the follicle cell membranes coincides with impaired oocyte cortex integrity and abnormal F$_2$-actin nucleation (Fig. 6I), this suggests that altered PtdIns(4,5)P$_2$ signaling underlies the F-actin remodeling defects in dPPCS mutant egg chambers.
**DISCUSSION**

dPPCS modulates F-actin remodeling in response to PtdIns(4,5)P₂ signaling

We show that a component of the highly conserved CoA biosynthesis pathway is required to ensure morphogenesis during *Drosophila* oogenesis. Interestingly, morphogenesis defects in dPPCS¹/¹ ovaries coincide with abnormal neutral lipid biosynthesis/storage, altered membrane localization of PtdIns(4,5)P₂, decreased levels of Akt/PKB-P and impaired F-actin remodeling. CoA is an essential cofactor for the synthesis of many lipids and mutations in dPPCS disrupt lipid homeostasis including phospholipid biosynthesis (CHAPTER 4). Therefore, it is possible that the abnormal levels of PtdIns(4,5)P₂ at the cell membranes are due to reduced production of PtdIns(4,5)P₂ from the phospholipid phosphatidylinositol. Because Akt/PKB phosphorylation relies on the conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃ mediated by PI3K, levels of Akt/PKB-P would then be reduced in dPPCS mutants as a consequence of the reduced levels of PtdIns(4,5)P₂. Interestingly, many actin remodeling processes require Akt/PKB signaling and Akt/PKB signaling (via LSD2) is also essential for the regulation of neutral lipid synthesis and lipid droplet formation during *Drosophila* oogenesis, suggesting that aberrant neutral lipid biosynthesis and F-actin remodeling within dPPCS¹/¹ ovaries may be due to impaired Akt/PKB activity (Fig. 8). Independently from Akt/PKB signaling, altered levels of PtdIns(4,5)P₂ may influence neutral droplet formation and actin remodeling. This idea comes from the findings that a mutation in the *DGAT/midway* gene, which converts diacylglycerol (DAG) into triacylglycerol (TAG), also cause aberrant F-actin remodeling and affects neutral lipid biosynthesis possibly by modifying PKC signaling via DAG⁵⁹ (Fig. 8).

Although levels and localization of PtdIns has not been investigated in relation with F-actin remodeling within the *Drosophila* ovary, it is generally accepted that actin remodeling processes depend on PtdIns signaling. It has been demonstrated that PtdIns(4,5)P₂ can directly influence actin polymerization by promoting the dissociation of capping proteins and can also act in cooperation with small GTPases such as Cdc42 (and via GEFs) in controlling ARP2/3-mediated nucleation of actin networks. A proper balance between PtdIns(4,5)P₂
Figure 6. PtdIns(4,5)P_2 localization and expression is affected in *dPPCS*^{1/1}.

(A) The same Western blot of ovarian protein extract was subsequently incubated with antibodies against Akt/PKB-P and Akt/PKB to determine levels of phosphorylated Akt/PKB. *dPPCS*^{1/1} ovaries have reduced levels of phosphorylated Akt/PKB (54.3% ± 4.9 SEM, n=3) compared with wild-types.
(B-I) PtdIns(4,5)P_2 expression and localization during wild-type and *dPPCS*^{1/1} late stage oogenesis. We expressed a PLCδ-PH-GFP fusion protein under control of a ubiquitously expressed Act5C-GAL4 driver to analyze PtdIns(4,5)P_2 localization and expression in a wild-type (B-C,F,H) and *dPPCS*^{1/1} (D-E,G,I) background.

(B-C) During wild-type oogenesis the PLCδ-PH-GFP fusion protein is present at the border cells (asterisk) and the apical membranes of the follicle cells that sheet the oocyte compartment, while the nurse cell membranes do not accumulate the fusion protein.

(D,E) In *dPPCS*^{1/1} follicles the PLCδ-PH-GFP fusion protein is not present at the apical membranes of the follicle cells that sheet the oocyte (arrowheads) and large patches of follicle cells are not labeled (dashed lines). The reporter also clearly showed that the follicular epithelium of mutant ovaries is disrupted (arrows in D) and sometimes the nurse cell membranes accumulated distinctive patches of high levels of the PLCδ-PH-GFP fusion protein (arrow id E). The reporter was also frequently detected at membranes of cells that are reminiscent of the border cells (boxed arrowhead in E).

(F) In wild-type follicles the oocyte cortex is in close contact with the apical membranes of the follicle cells, which accumulate the PLCδ-PH-GFP fusion protein.

(G) In *dPPCS*^{1/1} follicles the oocyte cortex is disrupted (arrowheads in Ga) and the follicle cells do not accumulate the PLCδ-PH-GFP fusion protein (arrowheads and dashed lines in Gb). Arrows point to defects in cell organization of the follicular epithelium.

(H) Close-up of a wild-type follicle, showing that the PLCδ-PH-GFP fusion protein accumulates at the apical membranes of follicle cells that are in close contact with the oocyte cortex (boxed arrowheads).

(I) Close-up of a *dPPCS*^{1/1} follicle, showing that the apical membranes of follicle cells that are in close contact with the oocyte cortex do not accumulate the PLCδ-PH-GFP fusion protein (arrowheads in Ia), which coincides with impaired oocyte cortex morphology and aberrant F-actin nucleation (arrow in Hb). DAPI was used to visualize the DNA. Scale bars; 150 µm (B,D), 100 µm (C,E), 50 µm (F-I).
Figure 7. Mutations in de novo CoA biosynthesis affect wing vein and scutellar patterning.
(A) Dorsal wing surface of wild-type. L1-L6: longitudinal veins 1 to 6, acv: anterior (acv) and posterior cross veins (pcv), A-E (intervein sectors). (B-B') dPPCS¹/¹ wings displayed ectopic veins between longitudinal veins L3-L4 and L4-L5 (arrowheads). (C) Thorax of a wild-type fly. The scutellum (dashed) has a pattern of four macrochaetae (bristles, scutellars). (D-F) Thorax of dPPCS mutant flies developed ectopic macrochaetae (arrows). (G) Quantification of wing and scutellar abnormalities. Numbers represent the amount of flies investigated. Scale bars; 250 μm (A-B), 100 μm (B’), 500 μm (C-F).

Figure 8. Defects in CoA synthesis affect neutral lipid homeostasis and F-actin remodeling due to altered PtdIns signaling.
Integrated model in which results from published manuscripts are combined and in which data from this manuscript are incorporated. This model explains the order of events that may lead to defects in F-actin dynamics and neutral lipid storage/synthesis in CoA mutants during oogenesis. Red indicates processes that are disrupted in CoA mutant flies (CHAPTER 4-6). Proteins and solid lines depicted in black are routes known to affect lipid droplet formation and/or F-actin dynamics during cytoplasmic dumping stages in Drosophila. Proteins depicted in white and dashed lines indicate routes known to modify actin dynamics, but have not been investigated during Drosophila oogenesis. Neutral lipid synthesis/storage and F-actin remodeling depend directly or indirectly on PtdIns-Akt/PKB signaling. Both processes are disrupted in the dPPCS mutants pointing to the following model; Low levels of PtdIns(4,5)P² due to reduced phospholipid biosynthesis may affect the production of TAG from DAG and this explains the reduced production of neutral lipids. Similarly, low PtdIns(4,5)P² may affect Akt/PKB activity due to reduced conversion into PtdIns(3,4,5)³ further disrupting neutral lipid synthesis and droplet formation. Likewise, altered PtdIns homeostasis would affect PtdIns-Akt/PKB dependent actin remodeling processes and this explains the observed abnormalities in F-actin organization in the CoA mutants. (references used are: 26,37-41,43-45,47,58-60).
and PtdIns(3,4,5)P₃ at distinct locations within the plasma membrane is required for actin remodeling⁴⁷. Finally, PtdIns(4,5)P₂ is essential to mediate cohesion between the plasma membrane and the underlying cytoskeleton⁶⁰. Based on these studies and in line with our findings, we propose that like in other tissues, F-actin remodeling within the Drosophila ovary depends on PtdIns(4,5)P₂ signaling and that this lipid derived signaling route is disrupted in dPPCS mutants (Fig. 8). Impaired signaling due to reduced Akt/PKB phosphorylation may further impair F-actin dynamics. Abnormal cytoskeletal dynamics in dPPCS¹/² disrupt the overall shape of all membranous structures and the organization of the cells during morphogenesis. Disorganized tissue integrity likely affects Notch and Grk localization and possibly signaling, which is required for specification of the follicle cells that pattern the eggshell, and causes severe chorion patterning defects. Similar defects in F-actin remodeling due to changes in PtdIns(4,5)P₂ signaling may also underlie the observed defects in patterning of the sensory cells and the wing vein cells or the follicle cells during early oogenesis.

**Mutations in de novo CoA synthesis disrupt morphogenesis**

If disrupted lipid/PtdIns homeostasis underlies the F-actin remodeling defects in dPPCS¹/², one would predict that mutations in other CoA biosynthesis enzymes give rise to similar defects. Indeed, mutations in the first enzyme (dPANK/fumble) and the final enzyme (dPPAT-DPCK) in the CoA biosynthesis route result in similar characteristics compared to the dPPCS mutant phenotype. dPANK/fumble and dPPAT-DPCK mutant females have poorly developed ovaries, have fecundity defects, produce eggs that exhibit polarity defects, synthesize abnormal neutral lipids (droplets), had reduced levels of Akt/PKB-P (45.2% ± 10.3 SEM and 42.9% ± 4.4 SEM of wild-type levels for dPANK/fumble and dPPAT-DPCK mutants, respectively) and flies displayed macrochaetae and wing vein patterning defects (see supplementary Fig. S5). Like in dPPCS¹/², a mutation in dPPAT-DPCK disrupts actin remodeling and results in plugging of the ring canals by nurse cell nuclei during dumping (see supplementary Fig. S5H). dPANK/fumble mutants produce small ball-shaped eggs, which are typically due to a loss of actin regulatory elements that control the polarized arrangement of F-actin fibers at the basal cortex of follicle cells required to establish planar cell polarity²⁸,⁶¹. It has been shown that dPANK/fumble physically associates with the actin depolymerization factor TSR⁶² and the phenotypic overlap between the tsr and dPANK/fumble mutants, argue that dPANK/fumble is required for F-actin remodeling. Mutations in both genes affect chromosome segregation, cytokinesis, aster separation and actin-contractile ring formation²⁰,⁶³. Furthermore, recently it was shown that tsr is required for planar cell polarity patterning in various Drosophila epithelia⁶⁴. These findings imply that impaired CoA synthesis in general disrupts morphogenesis due to aberrant F-actin remodeling and this is not specific for the dPPCS mutant.

In summary, although the molecular function of dPPCS suggest a general housekeeping role for this enzyme, our findings provide the first physiological evidence that dPPCS and thus CoA biosynthesis is required for PtdIns(4,5)P₂ homeostasis, Akt/PKB signaling and F-actin remodeling during morphogenesis (Fig. 8). Because the biosynthesis route towards the production of CoA is conserved amongst species it would be interesting to explore the significance of CoA during processes that involve actin/PtdIns dynamics such as chemotaxis, axon growth cone guidance, endocytosis/exocytosis, cell division or actin dependent chromatin remodeling.
ACKNOWLEDGEMENTS
We thank L. Cooley and A. Wodarz for the \textit{UAS-PLC\textdelta-PH-GFP} line and S. Wasserman for the \textit{P[dPANK]} line. This work was supported by a VIDI grant from the Netherlands Organization for Scientific Research (NWO; 971-36-400) to O.C.M.S and by a Topmaster grant from the Graduate School GUIDE to A.R.
LITERATURE CITED
CHAPTER 5

dPPCS is required for morphogenesis