Physiological Implications of impaired de novo Coenzyme A Biosynthesis in Drosophila melanogaster
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APPENDIX.
Figure S1. Establishment of embryonic cell fate depends on maternally supplied Mediator components.

We used array and in situ data from the BDGP expression database (www.fruitfly.org and ref. 1) to investigate Mediator expression during early development. Microarray data were downloaded from http://www.fruitfly.org/cgi-bin/ex/insitu.pl (complete dataset). Expression data of the individual Mediator genes was averaged\(^1\), clustered and visualized with ArrayAssist (Statagene). Expression profiles of the Mediator genes, the maternal morphogenes, the gap genes, the pair-rule genes and the Hox genes were generated with Sigmaplot using Excel spreadsheets by averaging the expression profiles of 4 or more genes.

(A) The heat map represents a dendrogram of principal component analysis of the average gene expression (each square represents the average expression of 3 arrays). The color code represents the relative expression of the genes compared to each other. Genes were clustered into three distinct groups (red, blue, green). Gene identifiers are depicted at the left and their respective names at the right. The colors represent the 3 clustered groups of Mediator genes. At the far right is the location within the Mediator complex depicted (Srb8-11, head, middle, tail). Submodule localization is largely based on predictions from yeast\(^2,3\). BDGP in situ analysis revealed that dMED26, dMED24, dMED8, dMED31, dMED16, dMED27, dMED17, dMED25, dMED1, dMED9 and dMED30 (CG17183, bold, not on the array) are ubiquitously expressed in st.1-3 embryos, indicating a maternal origin (mat. ubq.). The maternal origin of the Mediator is not surprising as approximately 70% of all genes have maternal contributions\(^4\). Similarly, Boube et al. (ref. 5) suggested that dMED17 and dMED13 were maternally contributed and Northern blot analysis of some Mediator components revealed high expression upon egg deposition\(^6\).

(B) Graph of the Mediator gene profiles during embryonic development adopted from the diagram in A. The colors represent the 3 clustered groups of Mediator genes. The black line marks the profile of the entire gene collection. Expression of the genes depicted in green show high expression in the first hours AED, while the expression of the Mediator genes in blue show a distinct expression peak during gastrulation (3-5 h AED). Expression of the Mediator genes is high upon embryo deposition and gradually declines after gastrulation has initiated. Only the Mediator genes CDK8, dMED12, dMED18 and dMED19 show an increase in expression 5 hours after egg deposition.

(C) Graphs represent the average profile measured over several genes and were used to visualize global changes in gene expression during embryogenesis. Therefore the graphs do not tell anything about the actual expression levels of the genes. The expression profile of the dMED31 gene displays a distinct peak in expression prior to gastrulation. Expression profiling of the entire Mediator complex revealed a gradual loss in expression of the Mediator genes during embryogenesis. The only outsider is the CDK8 gene. Expression of this gene is relatively high compared with the other Mediator components and its expression increases during development (see B.). Expression of dMED12, dMED18 and dMED19 also increases approximately 5 hour AED. Upon embryo deposition the maternal mRNA pool is high. During embryonic development the first genes transcribed are the gap genes, followed by the pair rule genes, which in turn are followed by the segment polarity and Hox genes. Expression of the Mediator genes is highest 2-4 hours AED, while expression of the maternal morphogenes is highest 0-2 h AED. Thus the Mediator and the maternal genes might act together and prelude expression of the gap and the pair-rule genes whose expression peaks when the expression of most Mediator genes starts to decrease. The Mediator profile opposes the Hox gene profile. Expression of the Mediator genes is high when the expression of the Hox genes is low, while the expression of the Mediator genes is low when the Hox genes are highly expressed during late stage embryogenesis (6-12 h AED). These results demonstrate that the Mediator genes have a maternal origin and are highly expressed during early embryogenesis.

Gene profiles represent average profiles of maternal morphogenes: bcd (CG1034), hh (CG9786), nos (CG5637), cad (CG1759). Gap genes: Kr (CG3340), kni (CG4717), gf (CG7952), tll (CG1378). Pair-rule genes: lkb (CG9768), ftz (CG2047), eve (CG2328), h (CG6494). Hox genes: lab (CG1264), Dfd (CG2189), Scr (CG1030), Antp (CG1028), Ubx (CG10388), abd-A (CG10325), Abd-B (CG11648).
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SUPPLEMENTARY METHODS CHAPTER 4

Bioinformatics analysis: Protein sequences were aligned with CLUSTAL W\(^1\), manually edited and used to create a structural alignment with the program DEEP-VIEW and modeled by the SWISS-MODEL server\(^2\). Between D251 and L252 of dPANK/Fbl 3 insertions and between S122 and V123 of dPPCS 2 insertions were allowed in the initial model making process and were religated prior to structure comparison and validation. Structures were not refined and represent crude models. Monomers were individually reconstructed during remodelling and analysed independently. Differences between monomers reflect slight differences in the template monomers used during modelling. Dalilite\(^3\), PROCHECK\(^4\) and WHATCHECK\(^5\) were used for pair wise structure comparison and validation. Figures were prepared with ESPript\(^6\) and YASARA\(^7\).

Physiological assays: Larval crawling was analysed by placing third instar larvae in the centre of a petri dish containing non-nutritive agar (0.8%) and the path length that larvae crawled within 5 m was recorded\(^8\). The trail traversed by each larva was drawn on the dish, scanned, and the tract length for at least 25 larvae was measured using UDruler (AVPSof). Paralysis was assayed by analysing the absolute climbing ability of 7-d-old males, prior, directly after heat exposure (2 h 37 \(^\circ\)C) and 15 m after recovery from heat exposure. The absolute climbing ability was determined by tapping 10-20 flies to the bottom of a standard food vial and counting the flies that were attached to the side after 30 s. 10 trails were performed for each cohort and the average climbing ability was calculated from at least 7 cohorts.

Immunostaining and apoptosis: Dissection, fixation and immunolabelling of ovaries was performed as described in ref. 11. Rabbit anti-Histone H2AvD pS137 (1:100, Rockland) was used as primary antibody and Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch) was used as the secondary antibody. After immunolabelling ovaries were stained with 0.2 μg/ml DAPI and mounted in citifluor (Agar Scientific). Apoptosis was measured in 6 day old flies that were kept for 3 days in vials containing yeast paste. The TUNEL cell death assay was performed following the ApopTag Fluorescien in Situ Apoptosis Detection Kit (Chemicon). Ovaries were fixed in devitellizing buffer/heptane\(^9\) (1:6 per volume) and pretreated with proteinase K (20 μg/ml in PBS + 0.1% tween-20 ) for 15 m at RT. Ovaries were analysed by CLSM. CLSM images represent maximal projections of a z-stack (0.5-1 μm/scan) and were obtained with a 63X/1.32 oil lens (Leica TCS SP2 DM RXE).

Statistical analysis: P-values were calculated using the Student’s t-test (two-tailed and where appropriate with equal or unequal variance). P-values < 0.05 were considered significant.

Figure S1. Drosophila Coenzyme A biosynthesis is conserved.

The Drosophila melanogaster de novo CoA biosynthesis route was reconstructed by bioinformatics analysis. (A-E) Multiple sequence alignments of pantothenate kinase (PANK), 4'-phosphopantothenoylcysteine synthetase (PPCS), (R)-4'-phospho-N-pantothenoylcysteine decarboxylase (PPCDC), 4'-phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK). Higher eukaryotes have a bifunctional PPAT-DPCK, while bacteria have a bifunctional PPCS-PPCDC. The Drosophila genome encodes a single copy of PANK (CG5725), PPCS (CG5629), PPCDC (CG30290), a bifunctional PPAT-DPCK (CG10575) and a DPCK (CG1939). Humans have multiple copies of the CoA biosynthesis enzymes. Here the enzymes that displayed the strongest sequence homology with Drosophila are shown. The % identity and similarity between the human and the Drosophila enzymes is:

- PANK2 (46%, 61%), PPCS (39%, 59%), PPCDC (48%, 66%), PPAT-DPCK (31%, 47%) and DPCK (38%, 60%).
  - Multiple sequence alignment of PANK; D. melanogaster (gi17864532), H. sapiens (gi24430171, PANK2), M. musculus (gi23943834), C. elegans (gi32565377), S. cerevisiae (gi6320740) and A. thaliana (gi30696417).
  - Multiple sequence alignment of PPCDC; D. melanogaster (gi24657262), H. sapiens (gi15680133), M. musculus (gi28849879), C. elegans (gi17560194), S. cerevisiae (gi6322762, aa 267-571), A. thaliana (gi13124313), and E. coli (gi16131510, aa 177-430).
  - Multiple sequence alignment of PPCS; D. melanogaster (gi4972686), H. sapiens (gi13375919), M. musculus (gi18255582), C. elegans (gi17560194), S. cerevisiae (gi577131), A. thaliana (gi26451224) and E. coli (gi16131510, aa 177-430).

- Multiple sequence alignment of PPCDC; D. melanogaster (gi24657262), H. sapiens (gi15680133), M. musculus (gi28849879), C. elegans (gi17560194), S. cerevisiae (gi6322762, aa 267-571), A. thaliana (gi13124313), and E. coli (gi16131510, aa 1-218).
- Multiple sequence alignment of bi-functional PPCS-DPCK; D. melanogaster (gi10728128), H. sapiens (gi17981025), M. musculus (gi27229125) and C. elegans (gi25143409).
- Multiple sequence alignment of monofunctional DPCK; D. melanogaster (gi24644728) and H. sapiens (gi19923601).

For sequence alignments of S. cerevisiae and E. coli amino acids (aa) that correspond with their PPCS and PPCDC portions were used. Strictly conserved residues are shaded in black, while similar amino acids are boxed and in the bold type. Sequences were aligned with CLUSTAL W\(^1\) and the figures were prepared with ESPript\(^6\). GenBank identifiers (gi) are denoted between brackets.
Figure S2. *Drosophila melanogaster* dPANK/Fumble, CG5629 and CG30290 encode the structural homologs of human PANK, PPCS and PPCDC.

To explore functional conservation of the CoA biosynthesis route 3D models of dPANK, dPPCS and dPPCDC were created by modelling. Models were created with known x-ray structures of human PANK3 (2I7P), PPCS (1P9O) and PPCDC (1QZU), to explore functional conservation and were not refined for in depth structural analysis. Despite the percentages of residues within the most favoured region of the Ramachandran plot are below 90% (83%–88%) and the confidence factors (B-factor) are >50 (52–55), which likely reflect their sequence identities (39–48%), we conclude from this *in silico* approach that dPANK/Fbl, dPPCS and dPPCDC represent the structural/functional homologs of their human relatives. These are the only genes present in the *Drosophila* genome whose ORFs produce significant hits with a pantothenate kinase, a phosphopantothenoylcysteine synthetase and phosphopantothenoylcysteine decarboxylase and the analysis of bond lengths, angles, and Φ-Ψ properties from the models show that they resemble acceptable drafts of their human relatives (*Table S1*).

(A) dPANK/Fbl and hPANK3 display sequence homology and topology.

(B) Superimposition of the hPANK3 homodimer (yellow) and the dPANK/Fbl model (blue) (rmsd = 0.8 Å; 354 Ca atoms aligned per monomer).

(C) dPPCS and hPPCS display sequence homology and topology. Conserved residues involved in phosphopantothenate and ATP binding, based on structural data obtained from the hPPCS, are highlighted in blue (and black from the adjacent monomer) and pink, respectively.

(D) Superimposition of the hPPCS homodimer (yellow) and the dPPCS model (blue) (rmsd = 0.5 Å; 264 Ca atoms aligned per monomer).

(E) The dPPCDC protein signature follows the universal signature of PPCDC.

(F) dPPCDC and hPPCDC display sequence homology and topology.

(G) Superimposition of the hPPCDC homotrimer (yellow) and the dPPCDC model (blue) (rmsd = 0.9 Å; 154 Ca atoms aligned per monomer).

(A, C and F) Amino acids are marked according to their physico-chemical properties. Strictly conserved residues are highlighted in red boxes and similar residues with red letters. Amino acids considered similar are: HKR (polar positive), DE (polar negative), STNQ (polar neutral), AVLIM (non-polar aliphatic), FYW (non-polar aromatic), PG, C. Secondary structure conformations are denoted at the top and underneath the sequence alignment. (α) α-helix; (β) β-strand; (η) 3_10 helix; (TT) turn.

**Table S1.** dPANK, dPPCS and dPPCDC model statistics.

<table>
<thead>
<tr>
<th></th>
<th>dPANK/Fbl</th>
<th>dPPCS</th>
<th>dPPCDC</th>
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<tbody>
<tr>
<td>RMS deviations from ideality(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.014</td>
<td>0.016</td>
<td>0.015</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.938</td>
<td>2.307</td>
<td>2.562</td>
</tr>
<tr>
<td>Ramachandran plot(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favored (%)</td>
<td>87.4</td>
<td>84.3</td>
<td>83.5</td>
</tr>
<tr>
<td>Allowed (%)</td>
<td>11.9</td>
<td>13.3</td>
<td>13.7</td>
</tr>
<tr>
<td>Generously allowed (%)</td>
<td>0.3</td>
<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Disallowed (%)</td>
<td>0.3</td>
<td>0.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Average B factor(^c)</td>
<td>53.2</td>
<td>52.8</td>
<td>54.9</td>
</tr>
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</table>

Monomers were individually reconstructed and analyzed independently. Representative statistics of 1 monomer are indicated.

\(^a\) WHATCHECK

\(^b\) PROCHECK

\(^c\) YASARA
Supplementary material CHAPTER 4

A universal PPC decarboxylase signature (Kupke, 2001)


H*-X

P*-X

G*-X

P-A-M-N-X

M-X

P-X

C-X

G-X-G

S S L A S H T G A


H--X

P--X

G--X

P-A-M-N-X

M-X

P-X

C-X

G-X-G

PPC decarboxylase signature D. melanogaster (CG30290)

Human PPCDC

Fly PPCDC model

Human PPCS

Fly PPCS model

Human PANK3

Fly PANK model

D. melanogaster

Human PPCS

Fly PPCS model

D. melanogaster

Human PPCDC

Fly PPCDC model

D. melanogaster

Human PANK3

Fly PANK model

D. melanogaster

Human PPCDC

Fly PPCDC model

D. melanogaster

Human PANK3

Fly PANK model
Figure S3. Mutations in CoA biosynthesis impairs larval crawling and cause paralysis after heat exposure.

(A) Quantification of locomotor activity of wild-type and mutant third instar larvae. Larval locomotor activity of dPANK/fbl<sup>1/1</sup> larvae is severely impaired, while dPPCS<sup>1/1</sup> show no decrease in activity and the activity of the dPPPAT-DPCK<sup>43/43</sup> larvae is slightly decreased. Impaired larval motility has been implicated in impaired CNS function<sup>13</sup>. (B) Absolute ability to climb after heat exposure of 7-d-old wild-type and mutant males. dPPCS<sup>1/1</sup> and dPPPAT-DPCK<sup>43/43</sup> mutants become paralyzed after heat exposure (2 h 37 °C) and recover to near normal activity 15’ min after heat exposure as determined by analysing the absolute ability to climb. A paralytic phenotype after heat exposure is frequently associated with defects in the CNS<sup>14</sup>. (*p < 0.05, **p < 0.005, ***p < 0.001 as determined by t-test)

Figure S4. dPPCS<sup>1/1</sup> follicle cells display increased DNA damage and apoptosis.

γ-H2AvD staining of wild-type and mutant ovaries. DAPI was used to label the DNA.

(A) The follicular epithelium of dPPCS<sup>1/1</sup> stage 3-7 follicles contained cells that stained positive for γ-H2AvD (arrows).

(B) Quantification of stage 3-7 follicles that contained γ-H2AvD positive cells. Numbers depicted at the top of each histogram represent the amount of follicles investigated.

(C) A TUNEL assay was performed to detect apoptotic cells. Follicle cells of the follicular epithelium of dPPCS<sup>1/1</sup> stage 3-7 follicles were frequently positive for TUNEL staining, indicating that these follicle cells were apoptotic.

(D) Quantification of the amount of stage 3-7 TUNEL positive follicles. Numbers depicted at the top of each histogram represent the amount of follicles investigated. Scale bars; 20 µm
Table S2. Summary of phenotypes caused by mutations in the Drosophila CoA biosynthesis enzymes.

<table>
<thead>
<tr>
<th>Physiologic abnormalities</th>
<th>dpANK/bl</th>
<th>dPPAT-DPCK</th>
<th>dPPCS</th>
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<tr>
<td>Developmental growth delay</td>
<td>++ (*)</td>
<td>+ (*)</td>
<td>+ (*)</td>
</tr>
<tr>
<td>Reduced larval motility</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Reduced flight performance</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Abnormal muscle contraction</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Impaired geotaxis</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Progressive loss of locomotor function</td>
<td>ND</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Paralytic upon heat-shock</td>
<td>ND</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Decreased lifespan</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<table>
<thead>
<tr>
<th>Cellular/nuclear abnormalities</th>
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<tr>
<td>Aberrant mitosis</td>
<td>++ (1)</td>
</tr>
<tr>
<td>Enhanced apoptosis</td>
<td>++</td>
</tr>
<tr>
<td>Enhanced DNA damage</td>
<td>+</td>
</tr>
<tr>
<td>Cytokinesis defects</td>
<td>+++ (1)</td>
</tr>
<tr>
<td>Abnormal chromatin</td>
<td>++</td>
</tr>
<tr>
<td>Aberrant F-actin dynamics</td>
<td>+++ (1)</td>
</tr>
<tr>
<td>Reduced Akt phosphorylation</td>
<td>++ (*)</td>
</tr>
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<table>
<thead>
<tr>
<th>Other</th>
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<tbody>
<tr>
<td>Sensitivity to 20 Gy IR</td>
<td>++</td>
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<tr>
<td>Sensitivity to 100 mM cysteine</td>
<td>+</td>
</tr>
<tr>
<td>Sensitive to ROS</td>
<td>++</td>
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<tr>
<td>Reduced triglycerides</td>
<td>++</td>
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<tr>
<td>Reduced neutral lipids</td>
<td>+ (*)</td>
</tr>
<tr>
<td>Reduced phospholipids</td>
<td>ND</td>
</tr>
<tr>
<td>Normal PtdIns(4,5)P2</td>
<td>ND</td>
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<tr>
<td>Reduced pericerebral fat body</td>
<td>ND</td>
</tr>
<tr>
<td>Neurodegeneration</td>
<td>ND</td>
</tr>
<tr>
<td>Retinal degeneration</td>
<td>ND</td>
</tr>
</tbody>
</table>

(ND) not determined, (-) not present/affected, (+) present/affected, (+++) present/clearly affected, (+++) present/ extremely affected, (1) ref. 15, (*) communicated elsewhere

LITERATURE CITED


**Table S1.** *dPPCS*<sup>1/1</sup> affect nurse cell chromatin morphology

<table>
<thead>
<tr>
<th>Stage 6-8 nurse cell chromatin morphology</th>
<th>% of total (egg chambers)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type (n=407)</td>
</tr>
<tr>
<td>normal chromatin</td>
<td>97.1</td>
</tr>
<tr>
<td>aberrant chromatin</td>
<td>0.5</td>
</tr>
<tr>
<td>degenerative chromatin</td>
<td>0.7</td>
</tr>
<tr>
<td>&gt; 15 nurse cell nuclei</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Nurse cells were stained with DAPI to detect the DNA. P[dPPCS] is a FLAG-tagged dPPCS cDNA under control of an ubiquitin promotor.

Figure S1. *dPPCS* is required for nurse cell chromatin condensation, egg chamber packaging and polarity.

(A) In wild-type egg chambers the Orb protein accumulates in the posterior located oocytes (arrowheads). (B) *dPPCS*<sup>1/1</sup> ovarioles contain egg chambers with multiple (arrows) or mispositioned (arrowhead) oocytes. (C) Overexpression of a FLAG-tagged dPPCS cDNA (P[dPPCS]) construct suppressed the occurrence of multiple oocytes and supernumerary follicles (see also Table S1). (D) During wild-type oogenesis follicle cells are mitotically (pH3Ser10 staining) active until stage 6 and subsequently proceed into endocycling<sup>2</sup>. At stage 5 the nurse cell chromatin has a 5-lobed appearance and after the mitotic-to-endocycle switch this chromatin becomes dispersed<sup>3,4</sup>. (E) The mitotic-to-endocycle switch was intact in *dPPCS*<sup>1/1</sup> follicles, (E-G) but the chromosomes failed to disperse properly and some nuclei remained 5-lobed (arrowheads). (H) Stage 10 *dPPCS*<sup>1/1</sup> follicle showing 2 nurse cell nuclei that are poorly replicated and small in size. (I) Supernumerary *dPPCS*<sup>1/1</sup> egg chamber with nurse cell nuclei that are heterogenous in size. (J) Some *dPPCS* mutant egg chambers were undergoing premature apoptosis as indicated by the presence of fragmented nuclei (arrowhead). (K) *dPPCS*<sup>1/1</sup> follicles frequently exhibited a degenerative appearance. Scale bars; 150 µm (a-c), 50 µm (d-h).

Figure S2. Follicle cell migration and organization is disrupted in *dPPCS*<sup>1/1</sup> germaria.

(A) Wild-type cysts in region 2b adopt a lens-shape (asterisk, arrow marks the stalk cells). (B) In *dPPCS*<sup>1/1</sup> germaria the cysts do not adopt the characteristic lens-shape (asterisk). Formation of the interfollicular stalk (arrow) is severely disrupted and newly formed follicles display features of fusion (arrowhead). (C) Wild-type follicle cells that migrate between the cysts express FasIII. When the egg chambers bud from the gerarium only the polar follicle cells (asterisk) express FasIII<sup>5-7</sup>. (oo) oocyte. (D) In *dPPCS*<sup>1/1</sup> germaria migration of the follicle cells is disrupted (arrow) and results in packaging defects (7-8, 10-11), mispositioning of the oocytes (9,10,12) and egg chambers without stalks (arrowhead). Numbers mark the oocytes. (E-E') In wild-type follicles Arm and DE-cad are highly expressed in the migrating follicle cells and later the stalk cells (arrowheads)<sup>9-10</sup>. Asterisks mark the position of the oocytes. (F-F') Arm and DE-cad were abnormally expressed in *dPPCS*<sup>1/1</sup> stalk cells (arrowheads). (E'') Wild-type germ line cells express the Vasa protein. The migrating follicle cells in region 2b adopt a convex lens-shape <sup>11</sup> (arrows). (F'') *dPPCS*<sup>1/1</sup> germ line cells accumulated normal amounts of Vasa, demonstrating that specification of the germ line cell was not affected. Migrating follicle cells do not exhibit a convex lens-shape (arrows). Scale bars; 20 µm.

Figure S3. *dPPCS*<sup>1/1</sup> follicles exhibit features of aberrant polar follicle cell and stalk cell specification.

(A) Wild-type egg chambers contain 2 groups of polar follicle cells, one at the anterior and one at the posterior. (B) *dPPCS*<sup>1/1</sup> follicle with 3 groups of polar follicle cells (arrows) and a mispositioned oocyte (asterisk). (C) *dPPCS*<sup>1/1</sup> egg chambers where the cuboidal follicle cells that sheet the egg chambers are maintained in an undifferentiated state (high FasIII expression). The follicular epithelium displays a discontinuous character and the nurse cell chromatin is heterogenous in size (arrows indicate polar follicle cells). (D) Wild-type egg chambers are connected by an interfollicular stalk (arrow). (E) Single confocal scan showing a *dPPCS*<sup>1/1</sup> follicle where the interfollicular stalk is missing (arrow). Note that the follicular epithelium appears to be a bilayer (arrowheads). (F) Single confocal scan showing a *dPPCS*<sup>1/1</sup> follicle where the follicle cells accumulated in a bilayer at the posterior of the oocyte (arrowheads). (G) In *dPPCS*<sup>1/1</sup> ovaries the interfollicular stalk were sometimes elongated and composed of undifferentiated follicle cells (arrowhead). Arrows mark 2 groups of polar follicle cells in the adjacent egg chamber. Scale bars; 50 µm (c), 20 µm (a,b, d-g).
Supplementary Methods Chapter 5

Dissection, fixation and immunolabelling of ovaries was performed as described (ref. 1). Primary antibodies used included concentrated supernatants obtained from the Developmental Studies Hybridoma Bank (Iowa, USA); mouse anti-fasciclin III (7G10, 1:5) developed by C. Goodman, mouse anti-quail (6B9, 1:5) developed by L. Cooley, mouse anti-Notch (C17.9C6, 1:5) developed by S. Artavanis-Tsakonas, mouse anti-DE-cadherin, (DCAD2, 1:5) developed by T. Uemura, mouse anti-gurken (1D12, 1:5) developed by T. Schupbach, mouse anti-orb (6H4, IgG2a, 1:5) developed by P. Schedl, mouse anti-armadillo (N27A1, IgG2a, 1:5) developed by E. Wieschaus and mouse anti-Histone H3 pS10 (1:100, Cell Signaling). Rabbit anti-Vasa (1:50) was a kind gift of P. Lasko. Secondary antibodies included Cy3-conjugated goat anti-mouse (1:200, Jackson ImmunoResearch), FITC-conjugated goat anti-mouse (1:200, Jackson ImmunoResearch), Cy5-conjugated goat anti-mouse 1:200, Jackson ImmunoResearch) and Alexa 647 goat anti-mouse IgG2a (1:200, Molecular probes). Ovaries were stained with 20 U/ml rhodamin-phalloidin (Molecular Probes) and 0.2 μg/ml DAPI (Sigma) to visualize F-actin and the DNA, respectively. 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.

Antibodies against pH3Ser10 were used to detect mitotic chromatin. FasIII stains all undifferentiated follicle cells in the germarium and marks the polar follicle cells after the follicles bud from the germarium. Armadillo and DE-cadherin are expressed in the adhesive junctions of the migrating follicle cells that assist in the rearrangement of the germ line cells. Orb marks the oocytes, while Vasa specifically accumulates inside the germ line cells.

A mutation in dPPCS affects early oogenesis

In summary (supplementary Fig. S1 to S4), dPPCS1/1 germaria displayed incomplete follicle cell migration, fusion of cysts, aberrant formation of the interfollicular stalk that separate neighbouring egg chambers, and cysts in region 2b of the germarium did not show the characteristic lens-shape, indicative of aberrant encapsulation of cysts. Furthermore, stage 3-6 dPPCS1/1 follicles with mispositioned oocytes exhibited ectopic polar follicle cells and we frequently observed egg chambers that were not separated by interfollicular stalk cells or egg chambers that were separated by long elongated stalks composed of undifferentiated follicle cells. Proper formation of these stalk cells is essential to ensure packaging of the egg chambers prior to budding from the germarium. Because the polar follicle and the stalk cell populations are derived from the intercyst cells16-18, it is possible that packaging defects in dPPCS1/1 were due to aberrant intercysts cell specification/organization6,7,12,19-21. Aberrant follicle cell specification/organization is supported by the finding that in dPPCS1/1 mutants FasIII expression was also detected in stage 7 egg chambers, indicating that differentiation of the cuboidal follicle cells that sheet the follicles was disrupted in some egg chambers. Moreover, we found dPPCS1/1 follicles where the follicle cells accumulated in a bilayer at the posterior of the oocyte and Notch was frequently abnormally expressed/localized in mutant germaria.

The ratio nurse cells to oocytes did not remain 15:1 and the mispositioned oocyte(s) in dPPCS1/1 follicles did not have more than the normal amount of 4 ring canals22, suggesting that supernumerary cells were not due to extra cell divisions with incomplete cytokinesis23. Similarly, supernumerary cells were also not the result from extra cell division with complete cytokinesis, since extra germ cells were accompanied with extra ring canals24. Because dPPCS1/1 oocytes accumulated normal amounts of the Orb protein25, while the germ line cells accumulated normal amounts of the Vasa protein26, the observed supernumerary cells were not due to disrupted germ cell identity or oocyte specification. Finally, no defects in follicle cell proliferation were observed (no major gaps in the follicular epithelia we detected) that also can lead to the formation of egg chambers with supernumerary cells27-30.
Taken together these analyses indicate that packaging defects in dPPCS^{1/2} females may result from abnormal cyst encapsulation and budding due to aberrant intercyst cell behavior and organization. Because we observed abnormal cyst development from region 2b and onwards, we believe that dPPCS is required for normal encapsulation of the cyst by the intercyst cells and not the follicle cell precursors\textsuperscript{15}. Aberrant migratory behaviour or organization of the intercyst cells likely induces mislocalization of the Arm, DE-cad and Notch expressing follicle cells, which in turn disrupts differentiation of the stalk cells, the polar follicle cells and the cuboidal follicle cells. Concomitantly cysts encapsulation, AP axis formation (germ line cell rearrangement) and budding of follicles will be disrupted. Because the correct positioning of the germ line cells and the follicle cells in the germarium is largely driven by cytoskeletal rearrangements and changes in cell adhesion\textsuperscript{31}, it is also possible that a mutation in dPPCS affects, like during late stage oogenesis, cell organization/migration by disrupting cell adhesion and/or cytoskeletal remodeling due to changes in PtdIns homeostasis within the germarium.
APPENDIX

Figure S4. Notch expression is disrupted in dPPCS<sup>1/1</sup> germaria.

Formation of the stalk and the polar cells depends on a Delta-Notch signaling route that specifies the anterior polar follicle cells, which in turn induce stalk cell formation<sup>12,13</sup> and abnormal follicle cell differentiation, multiple layering and aberrant packaging have been observed in Notch mutant females<sup>6,7,12,14,15</sup>. (A-A”) In wild-type germaria Notch is highly expressed in region 2b, while Notch localizes cortically in newly produced egg chambers<sup>14</sup>. (B-B”) In dPPCS<sup>1/1</sup> germaria expression of Notch was frequently lower/abnormal (40%, n=20 germaria) compared with wild-type germaria (arrowheads). In a fused egg chamber Notch localization at the cortical membrane was not disrupted. The posterior half of this egg chamber expressed low levels of FasIII, while the anterior half expressed high levels of FasIII, indicating that differentiation of the cuboidal follicle cells was not affected (arrows indicate polar follicle cells) Scale bars; 20 μm.

→ Figure S5. Mutations in the de novo CoA biosynthesis route affect morphogenesis.

Like dPPCS<sup>1/1</sup> females, dPANK and dPPAT-DPCK mutant females have fertility defects. The dPANK<sup>1</sup>, P[dPANK] and dPPAT-DPCK<sup>43</sup> lines are previously described<sup>12</sup>. Females that carry a mutation in the dPANK gene did not deposit eggs, while the dPPAT-DPCK<sup>43/43</sup> females deposited 0.36 ± 0.04 eggs/24 h of which 20.1% (n=232) was able to hatch.

(A) dPANK<sup>1/1</sup> ovaries dissected 48 h AE, were poorly developed and did not contain eggs. (B) 5-d-old dPANK<sup>1/1</sup> ovaries contained eggs, which were all small/ball-shaped and contained short dorsal appendages. This phenotype is identical to the eggs found in myospheroid<sup>9</sup>, Dlar<sup>34,35</sup>, Kugelei<sup>96</sup>, Dystroglycan<sup>37,38</sup> and Quail<sup>98</sup>. All these genes encode factors that are required for proper F-actin dynamics and small ball-shaped eggs are typically due to a loss of actin regulatory elements that control the polarized arrangement of F-actin fibers at the basal cortex of all follicle cells. During stage 5-8 these F-actin arrays are arranged such that the fibers run perpendicular to the AP axis of the egg chamber and give the egg chamber a planar polarity that is required to create elongated eggs<sup>31,40,41</sup>. (C-C”) In dPANK<sup>1/1</sup> wings acv and pcv formation was incomplete (arrowheads) and mispositioned cross veins between L2 and L3 were found (boxed arrowhead).

(D) dPPAT-DPCK<sup>43/43</sup> ovaries dissected 48 h AE, were poorly developed and did not contain eggs. (E) 17% of the eggs from 5-d-old dPPAT-DPCK<sup>43/43</sup> females were elongated along the AP axis and exhibited a collapsed phenotype. (F-F”) In dPPAT-DPCK<sup>43/43</sup> wings, ectopic vein formation initiated from the posterior cross vein (arrowhead).

(G) Quantification of wing and scutellar abnormalities in dPANK<sup>1/1</sup> and dPPAT-DPCK<sup>43/43</sup> flies. Numbers represent the amount of flies investigated. dPANK<sup>1/1</sup> flies did not develop ectopic macrochaetae, however in flies that carried a FLAG-tagged dPANK cDNA under the control of an ubiquitin promoter (P[dPANK])<sup>15</sup> an increase in the formation of macrochaetae was found, suggesting that dPANK overexpression induced the formation of ectopic scutellars.

(H) Wild-type (Ha) and dPPAT-DPCK<sup>43/43</sup> (Hb-Hd) ovaries were stained with rhodamin-phalloidin to visualize the F-actin network during cytoplasmic dumping. DAPI was used to visualize the DNA. (Hb-Hc) Cytoplasmic dumping and centripetal migration of the follicle cells (arrows), was frequently severely disrupted in dPPAT-DPCK<sup>43/43</sup> egg chambers. (Hd) Likely as a result of aberrant F-actin assembly we frequently found ring canals plugged with nurse cell nuclei in dPPAT-DPCK mutant follicles, suggesting that aberrant dumping underlies the production of long elongated eggs as observed in E.

(I) Production of neutral lipids (Nile red staining) was hardly detected in dPANK<sup>1/1</sup> follicles.

(J) Production of neutral lipids (Nile red staining) and transport of lipid droplets to the oocyte was disrupted during oogenesis in dPPAT-DPCK<sup>43/43</sup> mutants. Abnormal large lipid droplets were observed, indicating that lipid droplet formation was impaired<sup>43</sup> (compare Fig. 3A). Scale bars: 500 μm (A,D), 250 μm (C,F), 50 μm (C’,F’), 100 μm (Ha-Hc, IJ), 20 μm (Hd).
APPENDIX

LITERATURE CITED


