Cct1, a phosphatidylcholine biosynthesis enzyme, is required for Drosophila oogenesis and ovarian morphogenesis

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Summary

Patterning of the Drosophila egg requires cooperation between the germline cells and surrounding somatic follicle cells. In order to identify genes involved in follicle cell patterning, we analyzed enhancer trap lines expressed in specific subsets of follicle cells. Through this analysis, we have identified tandem Drosophila genes homologous to CTP: phosphocholine cytidylyltransferase (CCT), the second of three enzymes in the CDP-choline pathway, which is used to synthesize phosphatidylcholine. Drosophila Cct1 is expressed at high levels in three specific subsets of follicle cells, and this expression is regulated, at least in part, by the TGF-β and Egfr signaling pathways. Mutations in Cct1 result in a number of defects, including a loss of germline stem cell maintenance, mispositioning of the oocyte, and a shortened operculum, suggesting that Cct1 plays multiple roles during oogenesis. In addition, Cct1 mutants display a novel branched ovariole phenotype, demonstrating a requirement for this gene during ovarian morphogenesis. These data provide the first evidence for a specific role for CCT, and thus for phosphatidylcholine, in patterning during development.

Key words: CTP: phosphocholine cytidylyltransferase, Cct1, Drosophila, Oogenesis, Follicle cells

Introduction

The follicle cell epithelium of the Drosophila egg chamber plays essential roles in the development of the egg and embryo. During oogenesis, the follicle cells signal extensively to each other and to the germline cells to pattern the egg along its anterior-posterior and dorsoventral axes (reviewed by Nilson and Schüpbach, 1999). The follicle cells also undergo dramatic migrations and cell shape changes, making the follicle cell epithelium an excellent system for studying such processes as morphogenesis and cell adhesion.

Oogenesis begins at the anterior tip of the ovary in the region called the gerarium when a germline stem cell divides to form another stem cell and a daughter cystoblast [for a review of oogenesis, see Spradling (Spradling, 1993)]. The maintenance and division of the germline stem cells require both intracellular mechanisms and intercellular communication between the germline cells and three somatic cell types at the anterior of the gerarium: terminal filament cells, cap cells and inner germarial sheath cells (reviewed by Xie and Spradling, 2001). After division of a germline stem cell, the daughter cystoblast undergoes four rounds of division with incomplete cytokinesis to produce a cyst of 16 interconnected cells. One of the germline cells differentiates into the oocyte and the other 15 cells become nurse cells. As the germline cyst moves through the gerarium, it becomes surrounded by a layer of somatically derived follicle cells. During this time, in a process mediated by cadherin- and β-catenin-dependent adhesion between the oocyte and follicle cells, the oocyte becomes positioned at the posterior of the germline cyst (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998; Peifer et al., 1993), thus establishing the anterior-posterior axis of the egg chamber.

The anteroposterior polarity of the oocyte itself is not established until mid-oogenesis when Gurken (Grk), a TGFα-like ligand, is localized to the posterior of the oocyte and signals to the overlying follicle cells via torpedo, the Drosophila EGF receptor, to specify a posterior follicle cell fate (González-Reyes et al., 1995; Roth et al., 1995). These follicle cells then signal back to the oocyte, resulting in a reorganization of the microtubule network within the oocyte and establishment of its anteroposterior polarity (Ruohola et al., 1991; Theurkauf et al., 1992). Those follicle cells at the opposite end of the egg chamber that do not receive a signal from the oocyte acquire an anterior cell fate. Two signaling pathways appear to play important roles in anterior patterning. The Janus kinase (JAK-STAT) pathway is required for the specification of the anterior follicle cell fate (Xi et al., 2003), while the TGFβ pathway is required slightly later in oogenesis for correct patterning of the anterior region of the chorion (Twombly et al., 1996).

After signaling to the posterior follicle cells, Grk protein becomes localized to the future dorsoanterior of the oocyte where it again signals to the overlying follicle cells via the EGF receptor, this time to establish a dorsal cell fate (Neuman-Silberberg and Schüpbach, 1993). Activation of the EGF receptor in the dorsoanterior follicle cells leads to expression of a number of genes including broad-complex, rhomboid and argos (Deng and Bownes, 1997; Ruohola-Baker et al., 1993; Wasserman and Freeman, 1998), which are required for the differentiation of the dorsoanterior follicle cells in the complex...
pattern that gives rise to the two dorsal appendages and operculum.

Although the follicle cells are essential for many processes throughout oogenesis, it has been difficult to identify genes acting in these cells through traditional female sterile screens. Many of the genes required in the follicle cells are also required in other populations of somatic cells early in development, and as a result, mutations in these genes are often lethal. An alternative approach to the identification of genes important for patterning of the egg is to screen for gene expression in particular subsets of follicle cells. We used enhancer traps to screen for genes that are specifically expressed in the anterior follicle cells. Through this analysis, we identified a gene, Drosophila Cct1, that is required in the follicle cells for anterior patterning, as well as a number of other processes during oogenesis and ovarian morphogenesis. CTP: phosphocholine cytidylyltransferase (CCT) is the second of three enzymes in the CDP-choline pathway, through which phosphatidylcholine is synthesized. CCT catalyzes the conversion of phosphocholine to CDP-choline, which is a rate-limiting step in the pathway. Phosphatidylcholine is a major lipid component of all eukaryotic cell membranes and is the second most abundant phospholipid in Drosophila cell membranes (Jones et al., 1992). We have identified two Drosophila homologs of CCT (Drosophila Cct1 and Cct2) and found that Cct1 plays unexpected roles in a number of developmental processes. We describe the effects of mutations in Cct1 on oogenesis and ovarian morphogenesis.

Materials and methods
Fly stocks
Stocks were obtained from the Bloomington stock center unless otherwise noted. Wild-type controls were Ore R. The insertion Cct1B081 was generated by mobilization of a P[w+; lacZ element (Bier et al., 1989); insertion Cct1B0919 was a gift from Ursi Weber and Marek Modzik. Deletions in Cct1 were generated by excision of BN81 using Δ2-3 transposase (Robertson et al., 1988). Excision chromosomes were recombined with P[ry1172=neoFRT]80B ry506 and P[w+mF3=Fr]12A for generation of clones. Other strains used were: fsl1K10 (Wieschaus et al., 1978), 4P-X grk, which has four extra copies of the grk gene (Neuman-Silberberg and Schüpbach, 1994), UAS dpp, T155-GAL4 (gift from N. Perrimon), DWnt4c1/CyO (Cohen et al., 2002) and Mad1F, FRT00b/SM6a (gift from Rick Padgett).

Plasmid rescue and cloning of rescue construct
A ~0.7kb genomic DNA fragment flanking the P-element BN81 was isolated by plasmid rescue and sequenced. Blast searches of the Drosophila genome revealed that BN81 is inserted in the 5′UTR of Cct1, which is predicted to map to 62A5 based on molecular mapping. Mutations were generated by excision of the P-element and mapped by PCR analysis. The genomic rescue construct contains an 11.194 kb BamHI-Xhol fragment isolated from BDGP P1 clone DS05969 (nucleotides 13718–24912) (Kimmerly et al., 1996). The genomic fragment was cloned into pCasper4 (Thummel and Pirotta, 1992).

After the completion of our experiments, a new gene CG32313 was predicted to map 5′ to Cct1 by the BDGP (Fig. 1A). The presence of CG32313, however, should not affect our results because the phenotypes that we described have been observed using the null allele Cct1L79, which disrupts only Cct1 (Fig. 1A), and in transheterozygous combinations of this allele with homozygous viable Cct1 alleles.

Clonal analysis
Follicle cell clones were induced using the directed mosaic GAL4/UAS-FLP technique of Duffy et al. (Duffy et al., 1998). Follicle cell clones mutant for Mad were generated on 2L using a GR1-GAL4, UAS-FLP line (made in our lab), which is expressed in all the follicle cells including the follicle cell stem cells, and w1118; P[w+; pM]21C, P[w+; pM]36F, P[ry1172=neoFRT]40A (Xu and Rubin, 1993). Follicle cell clones mutant for Cct1 were generated on 3L using a c22c-GAL4, UAS-FLP line provided by N. Perrimon (Yoffe et al., 1995) and w1118, P[w+; pM]75C P[ry1172=neoFRT]80B (Xu and Rubin, 1993). Germline clones mutant for Cct1 were made using the hsFlp; ovoD1 method of Chou and Perrimon (Chou and Perrimon, 1996) P[w+;mC=ovoD1-18]; P[neoFRT]2A was obtained from N. Perrimon. Simultaneous germine and follicle cell clones mutant for Cct1 were made using hsFlp; ruPrIca/TM3, Ser and w1118; P[ubi-GFP(S65T)nl3];3L P[neoFRT]80B/TM3. Positively marked clones mutant for Cct1 were generated using the MARCM method of Lee and Luo (Lee and Luo, 1999). The stock hsFlp; tubGAL4/CyO; GAL80, FRT008 was provided by S. Cohen.

In situ hybridizations, X-gal staining, and immunocytochemistry
BDGP EST clone LD34141 (Rubin et al., 2000) was used to make the Cct1 probes. LD34141 is a 2.49kb full-length cDNA that contains the entire open reading frame and should detect all Cct1 transcripts. BDGP EST clone GH25855 is a full-length cDNA that was used to make the Cct2 probes. Digoxigenin-labeled probes were made using the DIG RNA labeling kit (Boehringer Mannheim). Ovaries for in situ hybridizations were dissected in PBS and fixed in 4% paraformaldehyde, 10% DMSO and three volumes of heptane for 20 minutes at room temperature. Subsequent steps were carried out according to a modified version of Tautz and Peifer (Tautz and Peifer, 1989), hybridizing at 55°C.

Adult ovaries with GFP-marked clones and ovaries for Arm antibody staining were dissected in PBS and fixed by incubation in 3.7% formaldehyde for 10 minutes. Monolocal Arm antibody N2-7A1 was used at 1:5 (gift from E. Wieschaus) (Peifer et al., 1994). Adult ovaries used for all other antibody staining were dissected in PBS and fixed in 4% paraformaldehyde and three volumes of heptane for 20 minutes at room temperature. Pupal ovaries were dissected in PBS and fixed in 4% paraformaldehyde for 1 hour at room temperature. Rat anti-Enc antibody was used at 1:1000 (Van Buskirk et al., 2000). Fas3 monoclonal 7G10 was used at 1:4 (Developmental Studies Hybridoma Bank). Monolocal Orb antibodies 4H8 and 6H4 were each diluted 1:60 and mixed together (gift from P. Schedl) (Lantz et al., 1994). α-Spectrin monoclonal antibody 3A9 was used at a dilution of 1:40 (Developmental Studies Hybridoma Bank). Rabbit anti-β-Galactosidase antibody was used at 1:2000 (Chemicon). Rabbit anti-phosphorylated SMAD (PS1) was used at 1:650 (gift from T. Tabata) (Tanimoto et al., 2000). Rabbit anti-Vasa was used at 1:2000 (gift from P. Lasko). Myc expression was induced by heat shocking adult flies at 37°C for 45-60 minutes (Xu and Rubin, 1993). Mosaic ovaries were stained with the monoclonal anti-Myc antibody 9E10 (Oncogene) diluted 1:50. All secondary antibodies were obtained from Molecular Probes and used at 1:1000. Oregon Green and Alexa Fluor 546 phallolidin were used at 1:500 (Molecular Probes). Hoescht was used at 1 μg/ml (Molecular Probes).

Ovaries stained for β-galactosidase activity were fixed in 2.5% glutaraldehyde for 10-12 minutes and stained as described previously (Ashburner, 1989).

Results
Identification of Cct1 and generation of mutations
In our enhancer trap analysis, one insertion line called BN81 showed very specific expression during mid-oogenesis in the

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anterior follicle cells (see below). Genomic DNA flanking the P-element was isolated by plasmid rescue and the sequence was used to identify the corresponding cDNA from the BDGP collection of EST clones. BN81 was found to be inserted in the 5'UTR of a gene homologous to the phosphatidylcholine biosynthesis enzyme CCT. We refer to this gene as Drosophila Cct1. Subsequent sequence analysis revealed the presence of a second Cct transcript, Drosophila Cct2, located just 3' to Cct1 (Fig. 1A). CCT has been cloned from organisms as diverse as yeast, plants and mammals and is highly conserved, particularly in its catalytic domain (Kent, 1997). A comparison between the predicted protein sequences of the Drosophila Ccts and those of the yeast and rat orthologs show that the Drosophila proteins are also very highly conserved (Fig. 1B).

Mutations in Cct1 and Cct2 were generated by imprecise excision of the P-element insertion BN81. The breakpoints of the deletions were then mapped by PCR revealing two classes of mutation: deletions 5' to the P-element, which are homozygous viable, and deletions 3' to the P-element, which are homozygous lethal (Fig. 1A). The lethal excision Cct1179 is a 5.02 kb deletion that includes the translation start codon, and therefore, is a molecular null allele (Fig. 1A). The lethality of this allele, which disrupts only Cct1, can be rescued with a genomic rescue construct that includes all of Cct1 and part of Cct2 (Fig. 1A). Cct1299 is a large deletion (>12.9 kb) that includes both Cct1 and Cct2 (Fig. 1A). The viability of the Cct199 and Cct1124 excisions, which presumably delete the entire promoter region of Cct1, is most probably due to the presence of alternate promoters in the first intron of the gene, based on the existence of EST clones with transcription start sites in this intron (Fig. 1A).

Expression from these promoters appears to be sufficient to rescue the larval lethality of mutations in Cct1, but not sufficient to fully rescue the ovarian phenotypes. All phenotypes described in this paper were seen in ovaries from Cct1BN81 and Cct199 homozygous flies as well as in all transheteroallelic combinations tested.

Cct1 mRNA is expressed in a spatially restricted manner during oogenesis

The enhancer trap BN81 is expressed in the border cells, the squamous nurse cell associated follicle cells and the centripetally migrating follicle cells (Fig. 2A). However, RNA in situ hybridization using a Cct1 antisense probe revealed that the BN81 insertion only partially reflects the expression of Cct1 in the ovary. High levels of Cct1 mRNA expression can first be detected in region 2B of the germarium in the follicle cells that migrate to surround the flattened germline cyst and separate it from the preceding cyst (Fig. 2B). Expression of Cct1 is next observed in a group of anterior follicle cells beginning at stage 7. During mid-oogenesis, the follicle cells migrate towards the posterior of the egg chamber so that by stage 10, all but ~50 of the anterior-most follicle cells are surrounding the oocyte. During stage 10B, Cct1 expression is detected in those remaining 50 follicle cells that are now stretched over the nurse cells and in a group of follicle cells that migrate centripetally between the nurse cells and the oocyte (Fig. 2C). This expression pattern is the same as that of the BN81 enhancer trap, with the exception of expression in the border cells, which we cannot detect by in situ hybridization. Finally, Cct1 is expressed during stages 12-13 in the two groups of dorsal anterior follicle cells that secrete the dorsal appendages and in the follicle cells at the posterior of the egg chamber that secrete the aeropyle (Fig. 2D). Expression of Cct1 is also detectable in the germline throughout oogenesis. Although it is difficult to determine expression levels in the germline cells by in situ hybridization, Cct1 expression in the oocyte and nurse cells appears to be high early in oogenesis and low during mid- and late oogenesis.
compared with the expression of Cct1 in the follicle cells (Fig. 2B-D). Cct2 expression could not be detected in the ovary by either Northern or in situ hybridization analysis (data not shown).

**Cct1 is required for maintenance of germline stem cells**

Germaaria from females mutant for Cct1 display a range of phenotypes, including a general disorganization. There are, however, many morphologically normal looking germaaria, which at a variable frequency, contain few or no germline cysts. These germaaria are generally part of ovarioles with only a few egg chambers (Fig. 3A), rather than the normal six or seven egg chambers, a phenotype that is usually indicative of a loss of germline stem cell maintenance (King and Lin, 1999). To test whether this is the case in Cct1 mutants, we looked for the presence of spectrosomes, structures rich in membrane cytoskeletal proteins, which are found in the germline stem cells and cystoblasts. In wild-type germaaria, there are generally two to three germline stem cells, which can be identified by their position and the presence of round spectrosomes, and seven or eight dividing germline cysts with fusomes. (B) Wild-type germaaria contain two or three germline cells, marked by the presence of spectrosomes, and seven or eight dividing germline cysts with fusomes. Arrowhead indicates a spectrosome. (C) Cct1 mutants contain few or no germline cells with spectrosomes. Scale bars: 20 μm.

Fig. 2. Expression pattern of Cct1 in wild-type ovaries. (A) X-gal staining showing BN81 enhancer trap expression in the anterior follicle cells from stage 7 to stage 10. At stage 10, expression is seen in the border cells, nurse cell associated follicle cells and centripetally migrating follicle cells. (B-D) Cct1, expression detected by whole-mount in situ hybridization. (B) Cct1 is first expressed in the follicle cells in region 2B of the germaarium. (C) During mid-oogenesis, Cct1 expression is detected in the anterior follicle cells. Expression begins during stage 7 in the follicle cells at the anterior-most end of the egg chamber. At stage 10B, Cct1 expression is seen in the nurse cell associated and centripetally migrating follicle cells. (D) From stages 12-13, Cct1 is expressed in the dorsoanterior follicle cells and the posterior follicle cells. Cct1 expression can also be detected in the germline cells at various levels throughout oogenesis.

Fig. 3. Defect in germline stem cell maintenance in Cct1 mutants. (A) Wild-type ovariole labeled with an antibody to Vasa protein, which marks the germline cells (green) and phalloidin to visualize the actin cytoskeleton (red). Ovaries from Cct1 mutants contain ovarioles with only one or two egg chambers. (B,C) Germaaria stained with antibodies to Vasa (green) and α-Spectrin (red), which marks the spectrosomes and fusomes. (B) Wild-type germaaria contain two or three germline cells, marked by the presence of spectrosomes, and seven or eight dividing germline cysts with fusomes. Arrowhead indicates a spectrosome. (C) Cct1 mutant germaaria sometimes contain few or no germline cells with spectrosomes. Scale bars: 20 μm.
Mutations in Cct1 result in packaging defects and mispositioned oocytes

Females mutant for Cct1 produce egg chambers with mispositioned oocytes and packaging defects. Packaging defects occur when more or less than 16 germline cells are encapsulated into one cyst by the follicle cells in the germarium. In Cct1 mutant ovaries, packaging defects occur with variable frequency and include egg chambers with fewer than 16 germline cells as well as egg chambers that contain multiple germline cysts (Fig. 4B). A more consistently seen defect is in the positioning of the oocyte. In wild-type ovaries, the oocyte is always positioned at the posterior of the egg chamber (Fig. 4A). This positioning occurs in the germarium, as the egg chamber moves from region 2B to region 3. During this time, the disk-shaped germline cyst becomes rounded, and the oocyte contacts and protrudes into the invaginating follicle cell layer in region 2B, taking its position at the posterior of the cyst (Fig. 4D). Ovaries from Cct1 mutant females contain egg chambers in which the oocyte is found in the middle or at the anterior of the egg chamber (Fig. 4C). Mispositioning of the oocyte in Cct1 mutant ovaries can already be seen in the germarium, suggesting a requirement for Cct1 in the initial positioning of the oocyte, not just in its maintenance at the posterior of the egg chamber (Fig. 4E). The mispositioned oocyte phenotype occurs in ~10% of egg chambers from 3- to 4-day-old Cct1BN81 homozygous females (n=264). The penetrance of this and other phenotypes seen in ovaries from Cct1 mutants appears to increase with the age of the females, but they are difficult to quantify at later ages because of increasing disorganization and degeneration within the ovary.

A number of genes have been shown to be required for positioning of the oocyte at the posterior of the germine cyst. Mutations in some of these genes, such as those in the spindle class, appear to affect oocyte positioning by causing a delay in
the oocyte was always positioned at the posterior of the egg. The cadherin.

obtained using an antibody to DE-cadherin. Similar results were seen in the oocyte.

posterior follicle cells. (C,D) In ovaries from Cct1BN81 mutant females with mispositioned oocytes, accumulation of Arm protein is still seen in the oocyte and anterior and posterior follicle cells. Similar results were obtained using an antibody to DE-cadherin.

and Arm (Fig. 5A-D and data not shown). One possibility is that Cct1 is required for proper determination of the anterior and posterior follicle cell populations. Oocyte mispositioning has been shown to result from misspecification of the polar follicle cells, specialized pairs of follicle cells that are determined very early in oogenesis and that lie at the anterior and posterior poles of each egg chamber (Grammont and Irvine, 2002). We therefore examined egg chambers from Cct1 mutants using several oocyte-specific markers and have not observed any defects in the timing of oocyte determination, suggesting that mutations in Cct1 most likely have a more direct effect on oocyte positioning (data not shown). One possibility is that Cct1 is required for proper determination of the anterior and posterior follicle cell populations. Oocyte mispositioning has been shown to result from misspecification of the polar follicle cells, specialized pairs of follicle cells that are determined very early in oogenesis and that lie at the anterior and posterior poles of each egg chamber (Grammont and Irvine, 2002). We therefore examined egg chambers from Cct1 mutants using an antibody to the polar follicle cell marker, Fascin 3 (Fas3). Expression of Fas3 in the polar follicle cells was normal in egg chambers with mispositioned oocytes, indicating that mutations in Cct1 do not cause a general defect in the determination of the anterior and posterior follicle cells (Fig. 4F,G).

In order to determine whether Cct1 mutations cause defects in oocyte positioning by affecting shg/arm mediated adhesion, antibodies to each protein were used to look at their expression levels in mutant egg chambers. In wild-type Cct1 mutant ovaries with mispositioned oocytes have an accumulation of both DE-cadherin and Arm proteins in the anterior and posterior follicle cells, suggesting that mutations in Cct1 are not causing mispositioning by significantly reducing levels of DE-cadherin or Arm (Fig. 5A-D and data not shown).

Based on the expression of Cct1 in the follicle cells of region 2B (Fig. 1B), we expected that Cct1 mutant clones in the posterior follicle cells would result in mispositioning. To test this, the Gal4/USAFilp system was used to generate clones specifically in the follicle cells. Surprisingly, using both the Cct1 null allele Cct1179 and the Cct1/Cct2 double null Cct1259, the oocyte was always positioned at the posterior of the egg chamber, even when there were very large posterior follicle cell clones (n=56) (Fig. 4H). Germline clones mutant for Cct1 were then made using the ovoD13 method to determine whether Cct1 is required in the oocyte. These germline clones did not result in a significant number of mispositioned oocytes (n=619).

GFP-marked clones were then induced during the larval stages using heat-shock-flipase to allow us to examine egg chambers with completely mutant follicle cell epithelia as well as egg chambers in which both the germline and entire follicle cell layer were mutant. When clones were generated in this manner, no mispositioned oocytes were observed when the germline was completely mutant (n=101). By contrast, 1.5% of egg chambers with completely mutant follicle cell layers contained mispositioned oocytes (n=264; Fig. 4I) Two egg chambers with wild-type germline cells and mosaic follicle cell epithelia also contained mispositioned oocytes. Interestingly, when both the germline and follicle cell layer were completely mutant, egg chamber development appeared to arrest during mid-oogenesis, although it was still possible to determine the position of the oocyte. We found that when the germline and follicle cell epithelium were both mutant, 11% of oocytes were mispositioned (n=54; Fig. 4J), which is similar to the frequency of mispositioned oocytes seen in egg chambers from Cct1BN81 homozygous mutant females (see above). These data indicate that Cct1 is expressed in both the germline and follicle cells but that expression in either population of cells is generally sufficient to ensure correct positioning of the oocyte. At a very low frequency, however, partial or complete follicle cell clones can also result in mispositioning, suggesting that the Cct1 acts primarily in the follicle cells.

Mutations in Cct1 affect ovarian morphogenesis

Ovaries from wild-type females consist of ovarioles that are attached near the oviduct but are otherwise fully separated from each other by epithelial sheaths that are formed during pupation. These sheaths are composed of muscle tissue and epithelial cells that secrete a thick basement membrane (Mahowald and Kambsellis, 1980). In ovaries from females homozygous for mutations in Cct1, we have observed a novel ‘branched ovariole’ phenotype, in which multiple ovarioles are attached to...
Ovarian morphogenesis has been previously described (King, 1970). Two hours after puparium formation (AFP), four populations of cells in the developing ovary can be distinguished by morphological criteria. These populations consist of an apical group of somatically derived cells, short flattened stacks of terminal filament cells, a group of germ cells intermingled with somatic cells believed to be precursors of the follicle cells, and a population of basal cells that will form the basal stalks and the calyx of the oviduct. At 24 hours APF, the apical cells have migrated between the terminal filament stacks and secreted basement membrane, separating the ovarioles from each other, and basal stalks have started to form. By 36 hours APF, the apical cells have become squamous, and the now fully formed basal stalks have become thin and elongated (Fig. 7A-C).

In order to examine whether mutations in Cct1 cause defects during ovarian morphogenesis, pupal ovaries were stained with phalloidin to visualize the actin cytoskeleton and Fas3 antibody to mark the basal cells. At 20-22.5 hours APF, the ovarioles in ovaries from wild-type pupae are clearly separated into distinct structures, and basal stalks have started to form (Fig. 7D). In 100% of ovaries from pupae mutant for Cct1, the apical cells do not appear to have migrated through the cluster of basal cells and basal stalks have not yet started to form, although some separation between the ovarioles is seen on the apical side of the ovary (n=10) (Fig. 7E). This suggested a possible defect in the migration of the apical cells. However, because adult ovaries with the branched ovariole phenotype typically also contain normal ovarioles, at least some of the apical cells must eventually migrate fully during pupation. Therefore, ovaries were also examined at 39-40 hours APF. At this stage, ovaries from wild-type pupae have developed long, thin basal stalks that are fully separated except at their most basal point (Fig. 7F). By contrast, ovaries from mutant pupae have started to form basal stalks, but they are shorter and thicker, and most significantly, some of the basal stalks show incomplete separation (n=12) (Fig. 7G). These data are consistent with a defect in the migration of the apical cells, which is required for the separation of the basal stalk cells. To determine whether mutations in Cct1 cause an overall disorganization of the structure of the ovary, pupal ovaries 20-21 hours APF were stained with an antibody to Vasa to stain the germ cells and an antibody to α-Spectrin, which associates with cell membranes and marks the spectrosomes and fusomes. Although the apical cells do not migrate properly in Cct1 mutant ovaries, the clustering and division of the germline cells and the overall organization of the ovary appears to be normal (data not shown).

To test whether Cct1 is required in the apical cells for their migration, positively marked apical cell clones mutant for Cct1 were generated, and their position was examined in ovaries 20 hours APF. Mutant cells appeared to migrate as in wild type, separating the ovarioles and allowing formation of the basal stalks (Fig. 7H). As with the follicle cell clones, this result suggests a non-autonomous effect of Cct1 in the apical cells or a requirement in another population of cells.
**Cct1 is required for formation of the operculum**

Although females mutant for Cct1 have reduced fecundity, a sufficient number of eggs are laid to enable us to examine the requirement for Cct1 in eggshell patterning. One eggshell defect observed is a reduction in the average length of the operculum. This reduction in length was quantified by measuring the angle between a line drawn horizontally through each egg and a line drawn from the anterior-most tip to the posterior-most end of the operculum. The angles for all the eggs were then averaged. Larger angles indicate shorter opercula. Eggs from OreR females had an average angle of 26.58° (±0.82°, n=24) (Fig. 8A). By contrast, eggs from females transheterozygous for Cct1 alleles 124 and 299 had an average angle of 40.22° (±0.60°, n=89) (Fig. 8B). Thus, a reduction in the level of Cct1 results in a significant reduction in operculum length.

Consistent with a defect in anterior chorion patterning, Cct1 is expressed in anterior populations of follicle cells (Fig. 8C). The expression pattern of Cct1 during mid-oogenesis is identical to that of the decapentaplegic (dpp) gene. dpp, the Drosophila homolog of vertebrate BMP2/4, is expressed in the anterior follicle cells during mid-oogenesis and has been shown to be required for correct patterning of the anterior region of the chorion (Twombly et al., 1996). As with mutations in Cct1, mutations in dpp result in a reduction in operculum length (Twombly et al., 1996). In order to determine whether Cct1 and the dpp signaling pathway interact, we first examined the expression of Cct1 mRNA in a background in which dpp was misexpressed in all of the follicle cells using UAS dpp and the GAL4 line, T155B. When dpp was ectopically expressed in all follicle cells, Cct1 was also expressed in all of the follicle cells, suggesting that Cct1 expression is regulated by dpp signaling (Fig. 8D). We then tested whether a loss of dpp signaling would result in a loss of Cct1 expression by making Myc-marked follicle cell clones mutant for Mad, a downstream transducer of the dpp signaling pathway. To detect Cct1 expression in this background, the enhancer trap BN81, which is expressed in the anterior follicle cells and is responsive to dpp signaling, was used. Surprisingly, expression of BN81 was still detected even in large Mad− clones (Fig. 8E). This suggests the presence of an additional unknown factor that acts in a redundant or partially redundant manner with dpp to regulate Cct1 expression in the anterior follicle cells.

In order to determine whether Cct1 affects operculum length by affecting dpp signaling directly, we examined the expression of an activated form of Mad, using an antibody to the phosphorylated form of the protein (pMAD). In wild-type ovaries, pMAD expression is seen in the centripetal follicle cells during stage 10B. In follicle cell clones mutant for Cct1 (Cct1179 or Cct1299) pMAD expression is still seen in the centripetal follicle cells, indicating that Cct1 most probably does not directly affect the dpp signal to the anterior follicle cells (Fig. 8F). Instead, it probably acts farther downstream and partially independently of dpp to affect operculum formation.

**Cct1 expression is regulated by Egfr signaling**

In wild-type ovaries, Cct1 is expressed during stages 12 and 13 in the two groups of dorsoanterior follicle cells that secrete the dorsal appendages and in the follicle cells at the posterior of the egg chamber that secrete the aeropyle (Fig. 9A). Follicle cells respond to grk signaling through the EGF receptor in a dose-dependent manner, so that a dorsal appendage cell fate is repressed in the cells at the dorsal midline, which initially receive the highest level of grk signal, and activated in the more lateral follicle cells, which initially receive less grk signal (reviewed by Van Buskirk and Schüpbach, 1999). To determine whether the expression of Cct1 during stages 12 and 13 is downstream of and regulated by the Egfr pathway, Cct1 expression was examined in two genetic backgrounds in which the normal pattern of EGF receptor activation in the
dorsoanterior follicle cells is disrupted. In \textit{fs(1)K10} mutants, \textit{grk} mRNA and protein are expressed in a ring around the anterior of the oocyte, rather than tightly localized at the future dorsoanterior (Roth and Schüpbach, 1994). This results in activation of the EGF receptor around the entire anterior circumference of the oocyte. In a \textit{fs(1)K10} mutant background, \textit{Cct1} is expressed in a ring of follicle cells around the anterior of the oocyte, and the gap between the two domains of \textit{Cct1} expression increases, consistent with the ectopic activation of the EGF receptor in the anterior follicle cells (Fig. 9B).

\textit{Cct1} expression was also examined in ovaries from flies in which there are four extra copies of the \textit{grk} gene (Neuman-Silberberg and Schüpbach, 1994). In this background, \textit{Cct1} expression is expanded ventrally, and the width of the gap between the two domains of dorsoanterior \textit{Cct1} expression is increased, again reflecting a \textit{grk}-dependent expansion of EGF receptor activation in the follicle cells (Fig. 9C). These data confirm that

\textit{Cct1} expression during late oogenesis is regulated by the Egfr signaling pathway.

The phenotype of eggs laid by females mutant for \textit{Cct1} is also consistent with its expression in the dorsal-anterior follicle cells. Approximately 10-35\% of eggs from flies homozygous for \textit{Cct1}\textit{BN81} exhibit a weakly ventralized phenotype in which the dorsal appendages are closer together or fused (Fig. 9E). In addition to weakly ventralized eggs, females homozygous for \textit{Cct1}\textit{16919}, an insertion in the first intron of the gene, lay between 37\% and 77\% collapsed eggs, which are also more strongly ventralized (Fig. 9F), consistent with a role for \textit{Cct1} in dorsoventral patterning of the egg.

\textbf{Discussion}

In recent years, increasing numbers of genes involved in lipid biosynthesis and metabolism have been found to play key roles in developmental processes. In \textit{Drosophila}, for example, \textit{wunen}, a phosphatidic acid phosphatase, and \textit{columbus}, a HMGCoA reductase, have been shown to be required for migration of the germ cells to the somatic gonad (Zhang et
Requirement for Cct1 in germline stem cell maintenance

We have found that Cct1 is required during oogenesis for the maintenance of the germline stem cell. As germline clones do not result in this phenotype, Cct1 appears to be required in the somatic cells of the ovary. The somatic cell types associated with germline stem cell maintenance are the terminal filament, cap, and inner sheath cells. These three cell types act as a 'stem cell niche', which regulates germline stem cell behavior primarily through the dpp signaling pathway. dpp is expressed in the cap cells and inner sheath cells and signals to the germline stem cells to regulate their proliferation (Xie and Spradling, 2000; Xie and Spradling, 1998). Another signaling molecule that appears to play a more minor role in germline stem cell maintenance is hedgehog (hh), which is expressed in the terminal filament and cap cells (King et al., 2001). In addition to these signal transduction factors, the cell adhesion molecules DE-cadherin and Arm are important for germline stem cell maintenance. Both DE-cadherin and Arm proteins accumulate at high levels in the junctions between cap cells and germline stem cells and are required for anchoring the germline stem cells in the niche (Song et al., 2002).

Although we have not been able to detect Cct1 mRNA expression in the terminal filament, cap, or inner sheath cells, the effect of Cct1 mutations on germline stem cell maintenance suggests that it is likely to be required in one or more of these three cell types. It is interesting to note that the dpp pathway is required for germline stem cell maintenance, as Cct1 is expressed in response to dpp signaling later in oogenesis and could also be regulated by the same signaling pathway in the gerarium. Another intriguing possibility is that Cct1 is required in the cap cells for their adhesion to the germline stem cells. As discussed below, a function in adhesion could also explain the role of Cct1 in oocyte positioning.

Cct1 function in oocyte positioning

The expression of Cct1 in the follicle cells in region 2B suggested a direct role for Cct1 in oocyte positioning, similar to that seen with the adhesion molecules DE-cadherin and Arm (Drosophila β-catenin). In wild-type ovaries, both DE-cadherin and Arm proteins are expressed in all follicle and germline cells but become transiently enriched in the oocyte and the anterior and posterior follicle cells in the germarium (Godd and Tepass, 1998; González-Reyes and St Johnston, 1998). As the germarium cyst moves through the germarium and becomes rounded, the oocyte contacts the posterior follicle cells and adheres to them more strongly than to the more lateral follicle cells. Although mutations in Cct1 may be causing defects in oocyte positioning by affecting adhesion of the posterior follicle cells to the oocyte, they do not appear to significantly affect shg- or arm-mediated adhesion. First, the accumulation of DE-cadherin and Arm proteins in Cct1 mutant ovaries appears to be similar to wild-type as judged by immunofluorescence. Second, removal of one copy of shg or arm does not appear to significantly enhance the mispositioned oocyte phenotype seen in Cct1 homozygotes (data not shown). Third, Cct1 mutant follicle cell clones do not behave like shg mutant clones in that mutant and wild-type cells do not sort out and form a straight boundary between the two cell populations (González-Reyes and St Johnston, 1998).

In addition to an effect on adhesion, it is possible to imagine a number of other mechanisms by which mutations in Cct1 may be causing the mispositioning of the oocyte. One model is that Cct1 is required for the migration of the follicle cells in region 2B to surround the developing germarium cyst. If there was a delay in the migration of these follicle cells, they would not be in contact with the oocyte when the germarium cyst becomes rounded, and the oocyte would end up randomly positioned within the egg chamber. A defect in the migration of these cells could also explain the packaging defects seen in Cct1 mutants.

Our clonal analysis results indicate that Cct1 is expressed in both the follicle cells and the germline, and that expression in either tissue is sufficient to ensure proper positioning of the oocyte. Because the mispositioned oocyte phenotype is seen in only 11% of egg chambers mutant for the null allele Cct1, it appears that Cct1 is not absolutely required for oocyte positioning. Instead, Cct1 probably plays a secondary role in the process to ensure that the oocyte is correctly positioned even when there is slight variability in the primary positioning mechanisms. The low frequency of mispositioned oocytes in egg chambers that have a wild-type germ line but mosaic (partially mutant) follicle cell epithelium suggests that Cct1 is primarily required in the follicle cells and not the germline for oocyte positioning, but that Cct1 from the germline can generally 'rescue' the mispositioned oocyte phenotype when all of the follicle cells are mutant.

Surprisingly, analysis of Cct1 mutant clones also suggested that the gene may be functioning non-autonomously. It is unlikely that Cct1 or phosphatidylcholine itself is being secreted; instead, Cct1 is likely to be required for the production of a specific species of phosphatidylcholine that is then converted to a secreted signaling factor.

Requirement for Cct1 during ovarian morphogenesis

We have described a novel phenotype of 'branched' ovarioles resulting from mutations in Cct1, a phenotype that appears to be caused by a delay in apical cell migration during ovarian morphogenesis. Defects in apical cell migration have also...
been described for mutations in *Drosophila Wnt4* and the *Drosophila Wnt4* cell motility pathway (Cohen et al., 2002). Mutations in these genes result in ovarioles in which the ovariolar sheath is shorter than in wild-type, and the egg chambers are curled up within the sheath rather than stretched out, a defect that is also seen in *Cct1* mutant ovaries. *Drosophila Wnt4* signals through *Drosophila Fz2*, Dsh and PKC resulting in an accumulation of focal adhesion kinase in the apical cells, which is required for their migration. It is unclear how *Cct1* is affecting the migration of the apical cells, as our clonal analysis suggests that *Cct1* is either having a non-autonomous effect in the apical cells, e.g. through the production or secretion of a signaling molecule, or that *Cct1* is required in a different cell population. Interestingly, we have observed a loss of germline stem cell maintenance, packaging defects and occasional oocyte mispositioning in *Drosophila Wnt4* mutant ovaries (T.G., unpublished; R. Wallace and E. Wilder, personal communication). In addition, mutations in *Cct1* can suppress the effects of Fz and Dsh overexpression on planar cell polarity during eye development (U. Weber and M. Mlodzik, personal communication). These data also suggest a possible link between a requirement for phosphatidylcholine and Wnt signaling, raising the intriguing possibility that *Cct1* could be required for the secretion of *Drosophila Wnt4*.

**Mutations in *Cct1*** affect anterior eggshell patterning

*Cct1* is the only gene other than *dpp* for which a shortened operculum phenotype has been described. We found that although *dpp* signaling is sufficient to drive *Cct1* expression in the follicle cells, it appears to be acting in a redundant or partially redundant manner with another factor to regulate *Cct1* expression. A similar result was obtained looking at *Cct1* expression in *Mad* clones in eye imaginal disks (Weber et al., 2003). *Cct1* was expressed in the absence of *dpp* signaling in eye disks, although it was strongly reduced in *Mad* clones that were within the antennal disk. It has been suggested that the expression of *Cct1* in *Mad* clones in eye disks may be due to redundancy in the *dpp* and *hh* signaling pathways during morphogenetic furrow progression (Weber et al., 2003). In the ovary, it is not clear what other factors are regulating *Cct1* expression; however, one possible candidate is the JAK/STAT signaling pathway, which has recently been shown to be involved in anterior follicle cell patterning (Xi et al., 2003).

Although we do not know the mechanism by which *Cct1* is affecting operculum formation, it is likely that additional downstream factors are involved. *Cct1* is expressed in the nurse cell associated follicle cells and centripetal follicle cells during mid-oogenesis, and these cells must communicate with the follicle cells over the anterior of the oocyte, which actually secrete the operculum. As *Cct1* itself is probably not secreted, it is most probably required for the production of or secretion of another molecule.

In addition to shortened opercula, eggs from *Cct1* mutants exhibit weak ventralization. Although the ventralization phenotype is consistent with a requirement for *Cct1* in the dorsal anterior follicle cells in late oogenesis, it could also reflect the requirement for *Cct1* in the establishment of anterior cell fates during mid-oogenesis (see above). It has been shown that *dpp* signaling is required for the specification of anterior cell fates and positioning of the dorsal appendages along the dorsoventral axis (Peri and Roth, 2000). As *Cct1* is required for anterior patterning, it is possible that this requirement also affects proper positioning of the dorsal appendages. Because it is technically difficult to eliminate *Cct1* activity in the dorsoanterior follicle cells without affecting the earlier activity, we have not been able to determine whether or not this is the case.

**Possible roles for *Cct1*** in intercellular signaling

Recent studies on the role of *Cct1* during *Drosophila* eye development have suggested that *Cct1* has a specific function in the regulation of signaling pathways through membrane trafficking (Weber et al., 2003). Specifically, Weber et al. argue that during eye development, levels of phosphatidylcholine affect endocytic pathway components, resulting in an alteration in the subcellular localization of the EGR and Notch receptors. Although we have no specific evidence that *Cct1* is acting in the same way during oogenesis, a similar role for *Cct1* in signaling in the ovary could potentially explain the phenotypes that we see. It seems unlikely, however, that *Cct1* would be affecting receptor localization in the ovary, as many of its effects appear to be non-cell autonomous. Alternatively, the function of *Cct1* in the ovary could be more similar to what has been suggested by studies carried out in yeast and rat liver cells, which indicate a role for phosphatidylcholine in secretion through the Golgi apparatus (reviewed by Kent and Carman, 1999). In yeast, for example, it has been found that mutations in *sec14*, a phospholipid transfer protein that is required for budding of vesicles from the Golgi, is suppressed by mutations in CDP-choline pathway enzymes, including yeast *Cct1* (Fang et al., 1998). A model in which *Drosophila Cct1* is involved in the secretion of various ligands would be consistent with phenotypes that we see during oogenesis. For example, a role for *Cct1* in the secretion of signaling molecules such as *dpp* or *hh* in the gerarium could explain the loss of germline stem cell maintenance, and involvement of *Cct1* in secretion of *Drosophila Wnt4* would be consistent with the function of *Cct1* during ovarian morphogenesis and oogenesis.

A second possible function for *Cct1* is in the production of a signaling molecule. As mentioned above, phosphatidylcholine is the source of a number of bioactive lipids. Some of these molecules, such as diacylglycerol, function in intracellular signal transduction, whereas others, such as lysophosphatidic acid, are secreted (Pelech and VANCE, 1989). Work in zebrafish has shown for the first time that lysolipid phosphates have essential functions during development. During heart development, for example, sphingosine-1-phosphate, which is closely related to lysophosphatic acid, binds to the lysosphingolipid receptor Miles Apart to regulate heart morphogenesis (Kupperman et al., 2000). A role for *Cct1* in signaling could explain all of the phenotypes that we see in the ovary, and conversion of phosphatidylcholine to a secreted signaling molecule would also explain the non-autonomous function suggested by our clonal analysis.

Future studies examining the intracellular localization of *Cct1* and the identification of downstream factors will be important in determining the molecular basis for the role of this gene during development.

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