Ack kinase regulates CTP synthase filaments during Drosophila oogenesis

Todd I Strochlic1,4,*, Kevin P Stavrides1,2, Sam V Thomas1, Emmanuelle Nicolas3, Alana M O’Reilly1,2,** & Jeffrey R Peterson1,***

Abstract

The enzyme CTP synthase (CTPS) dynamically assembles into macromolecular filaments in bacteria, yeast, Drosophila, and mammalian cells, but the role of this morphological reorganization in regulating CTPS activity is controversial. During Drosophila oogenesis, CTPS filaments are transiently apparent in ovarian germ line cells during a period of intense genomic endoreplication and stockpiling of ribosomal RNA. Here, we demonstrate that CTPS filaments are catalytically active and that their assembly is regulated by the non-receptor tyrosine kinase DAck, the Drosophila homologue of mammalian Ack1 (activated cdc42-associated kinase 1), which we find also localizes to CTPS filaments. Egg chambers from flies deficient in DAck or lacking DAck catalytic activity exhibit disrupted CTPS filament architecture and morphological defects that correlate with reduced fertility. Furthermore, ovaries from these flies exhibit reduced levels of total RNA, suggesting that DAck may regulate CTPS synthase activity. These findings highlight an unexpected function for DAck and provide insight into a novel metabolic pathway governing nucleotide biosynthesis.

Keywords Ack; CTP synthase; cytoophidia; Drosophila oogenesis

Subject Categories Development & Differentiation

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Introduction

Metabolic enzymes can be dynamically regulated in response to nutrient availability and growth-promoting signals. Enzyme activity may be altered by transcriptional or post-transcriptional mechanisms such as covalent modifications (e.g. phosphorylation) or assembly into regulatory complexes. Recently, examples have emerged of transient assembly of metabolic enzymes into macromolecular structures [1,2], although, in general, the architecture and regulation of these assemblies is poorly understood.

One assembly that has been described in organisms from bacteria to mammals is comprised of the nucleotide biosynthetic enzyme CTP synthase (CTPS) [3,4]. CTPS assembles into filaments dynamically in response to nutrient deprivation in yeast [5] although it has been unclear whether CTPS filaments are catalytically active. Consequently, the role of these structures in CTP biosynthesis has remained mysterious. CTPS filaments also occur in germline cells of the Drosophila ovary [5,6] where their function is also unknown. Here, we demonstrate that CTPS filaments form transiently during oogenesis and are catalytically active and that their assembly is regulated by a novel filament component, the non-receptor tyrosine kinase DAck. Our results establish a framework for understanding how the assembly of CTPS filaments provides temporal control over the production of CTP, an essential nucleotide and precursor for phospholipid biosynthesis, which is required during specific stages of oogenesis.

Results and Discussion

DAck-deficient female flies exhibit reduced fertility

Drosophila oogenesis depends on the production of “egg chambers” composed of 16-cell germline cysts (one oocyte and 15 supportive nurse cells) surrounded by a follicular epithelium. Egg chambers proceed through 14 morphologically defined stages of development over the course of 8 days to produce a mature egg [7]. While the kinase DArk is important for Drosophila spermatogenesis [8], its role in oogenesis is unknown. We observed that female flies homozygous for a loss-of-function allele of DArk, DArk6 [9], deposited eggs at a substantially slower rate than wild-type flies (Fig 1A), and expression of a wild-type DACK transgene in the DArk6 genetic background rescued this phenotype (Fig 1A and B). In contrast, transgenic expression of a kinase-dead DAck mutant (DAck-K156A)
failed to rescue (Fig 1A and B), demonstrating that DAck kinase activity is critical for oogenesis.

Egg chambers from DAck86 flies exhibited an apparent disruption in the continuity of the plasma membrane between adjacent nurse cells resulting in nurse cell fusion (arrowheads in Fig 1C). No plasma membrane defects were observed in the follicular epithelium. Transgenic re-expression of wild-type DAck but not DAck-K156A restored normal egg chamber morphology (Fig 1C and D), further demonstrating a key role for DAck activity in regulating germline cell membrane integrity.

Ack localizes to CTP synthase filaments

Kinases can localize to structural components within germ cells to regulate key developmental events. For example, Tec kinase localizes to ring canals between nurse cells during Drosophila oogenesis and failure of Tec recruitment leads to plasma membrane disruption and reduced fertility [10]. We observed that DAck localized to single approximately 20-μm-long filamentous structures within the cytoplasm of each nurse cell in wild-type egg chambers (Fig 2A and Supplementary Movie S1). As expected, filamentous DAck staining

Figure 1. DAck kinase activity is required for normal oogenesis in Drosophila.

A Egg laying rates of w1118, DAck86, UAS-DACK/pCOG-Gal4:VP16; DAck86, and UAS-DACK-K156A/pCOG-Gal4:VP16; DAck86 flies. Error bars indicate standard deviation from three independent experiments. * denotes P-value < 0.01 as calculated by an unpaired, two-tailed Student's t-test, and n.s. denotes not significantly different than w1118.


C Stage 10 egg chambers from flies of the indicated genotypes stained with FITC-phalloidin to label the subcortical actin cytoskeleton (green) and Draq5 to label nuclei (blue). Single 0.2-μm confocal sections are shown. White arrowheads denote discontinuities in nurse cell plasma membranes. Scale bar, 20 μm.

D Quantitation of the membrane defect phenotype from stage 10 egg chambers (n = 50) of the indicated genotypes.

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was absent from DAck86 flies (Supplementary Fig S1A) and localization to filaments was restored upon re-expression of transgenic DAck, demonstrating specificity of the antibody. FLAG-DAck expressed in the female germline also localized to filaments (Supplementary Fig S1B), confirming this localization.

DAck filaments were reminiscent in appearance of cytoplasmic assemblies recently described in bacteria [11], yeast [5], flies [4,6], and mammalian cells [12] that are composed of the enzyme CTPS, which catalyzes the rate-limiting step in the production of cytidine nucleotides [13]. Strikingly, DAck co-localized with GFP-tagged CTPS [14] filaments in germline cells of Drosophila egg chambers (Fig 2B). Interestingly, however, no filamentous DAck staining was observed in cells of the surrounding follicular epithelium, despite the presence of short CTPS filaments there (arrowheads in Fig 2B).

Pharmacological depletion of CTP by treatment with the CTPS inhibitors 6-diazo-5-oxo-L-norleucine (DON) or azaserine induces filament formation in metazoan cells and tissues [5,15]. To determine whether filamentous localization of Ack is conserved in mammals, we immunostained DON-treated MCF7 cells with anti-CTPS1 and anti-phospho-Y284-Ack1 antibodies, revealing that co-localization of Ack and CTPS to filaments is conserved (Fig 2C).

Membrane defects in DAck mutants are linked to reduced CTPS activity

In addition to its role as an essential nucleotide, CTP plays a critical role as a precursor for the biosynthesis of phospholipids including phosphatidylcholine and phosphatidylinositol [16]. Inhibition of CTPS decreases cellular CTP pools with a concomitant reduction in phospholipid biosynthesis [17,18]. Furthermore, Cct1, the enzyme downstream of CTPS in the phosphatidylcholine biosynthetic pathway, is critical for Drosophila oogenesis [19]. To determine whether the apparent membrane defects in DAck86 egg chambers reflect reduced phospholipid levels, we examined the distribution of the plasma membrane-enriched lipid phosphatidylinositol 4,5-bisphosphate.

Figure 2. DAck/Ack1 localizes to CTPS filaments.
A Three-dimensional confocal projection of Drosophila egg chambers stained with an anti-DAck antibody. Spherical structures are non-specific staining of germ cell nuclear membranes. Scale bar, 25 μm.
B Egg chamber from CTPS-GFP protein trap fly line (CA06746) stained with an anti-DAck antibody and Draq5. A single 0.2-μm section is shown. White arrowheads denote filaments in follicle cells labeled with CTP synthase, but not DAck. Scale bar, 20 μm.
C MCF7 cells treated with 200 μM DON were fixed and stained with anti-CTPS1 and anti-phospho-Y284-Ack1 antibodies. Scale bar, 10 μm.
(PIP₂) using GFP fused to the pleckstrin homology domain of phospholipase Cβ (GFP-PH-PLCβ), a selective binder of PIP₂ [20]. GFP-PPH-PLCβ was expressed in the female germline of either wild-type or DAck⁸⁶ flies, and isolated egg chambers from the two genotypes were pooled and analyzed by confocal microscopy. DAck immunostaining was used to differentiate wild-type from DAck⁸⁶ mutant egg chambers within the same field in order to allow direct comparison of GFP signal intensities. We observed strikingly reduced intensity in DAck⁸⁶ egg chambers compared to wild-type egg chambers (Fig 3A). These results demonstrate reduced phospholipid membrane composition in DAck⁸⁶ flies perhaps due to reduced CTPS catalytic activity in this genotype.

If reduced phospholipid levels in DAck⁸⁶ egg chambers are indeed due to reduced CTPS activity, then flies expressing reduced CTPS should exhibit similar phenotypes. We generated heteroallelic CTPS mutant flies (hereafter referred to as CTPS⁻⁰⁻), in which CTPS gene expression is reduced by 62% (Fig 3B) and CTPS protein levels are similarly reduced (Fig 3C). As expected, CTPS immunostaining is markedly decreased in CTPS⁻⁰⁻ egg chambers (Fig 3D) as is GFP-PH-PLCβ staining at germ cell membranes (Supplementary Fig S2). These CTPS hypomorphic female flies are viable but exhibit reduced egg production (see Fig 4C). Moreover, discontinuities in the plasma membranes of germline cells and dramatic nurse cell fusion were observed, phenotypes highly similar to DAck⁸⁶ (compare Fig 3D, right panel with Fig 1C, top right panel). These results support the idea that maintenance of adequate CTP levels is promoted by DAck and is required for germ cell membrane integrity and oogenesis.

**DAck regulates CTP synthase filament morphology**

Other macromolecular assemblies exhibit highly regulated assembly that promotes catalytic activity [1]. CTPS filaments were more numerous and appeared fragmented in DAck⁸⁶ nurse cells compared to wild-type nurse cells (Fig 3E). Quantitation of three-dimensional image stacks confirmed that total filament length per egg chamber is indeed less in DAck⁸⁶ flies (Fig 3F). However, due to the increased number of smaller, thinner filaments, total filament volume in wild-type and DAck⁸⁶ egg chambers is comparable (Fig 3G), indicating that the morphological organization of the filaments is the most significant difference. Importantly, the morphology of CTPS filaments was dependent on DAck kinase activity, as expression of wild-type but not kinase-dead DAckK156A rescued the altered CTPS filament morphology in the DAck⁸⁶ genetic background (Fig 3H).

An analysis of filament number across the stages of oogenesis also revealed striking differences between the genotypes. Short CTPS foci are observed beginning in stage 2 egg chambers of wild-type flies that grow increasingly longer and proportionately wider through subsequent stages. At stage 11 and beyond, CTPS filaments began to disappear (Fig 3I), consistent with published results [6]. However, CTPS protein was still present at equal levels in these late-stage egg chambers (Supplementary Fig S3), suggesting that the filaments are disassembled rather than degraded. By contrast, filaments were already observed in the gerarium in DAck⁸⁶ flies, earlier in the developmental timecourse than for wild-type flies (Fig 3I). Filaments in DAck⁸⁶ flies also persisted inappropriately into late-stage egg chambers. These observations demonstrate a role for DAck in regulating the temporal assembly of filaments during oogenesis, perhaps linking the timing of filament assembly and disassembly to the morphological defects observed in DAck⁸⁶ egg chambers.

**Catalytically active CTP synthase localizes to filaments**

During mid-oogenesis, a massive supply of nucleotides is required for the synthesis of cellular DNA and RNA. Nurse cells in egg chambers of stages 2–10 undergo 10–12 cycles of endoreplication of nuclear DNA [21] producing an approximately 1,000-fold increase in their DNA content. Endoreplication is essential to support increased transcription and protein production for deposition into developing oocytes [22]. Likewise, RNA production, particularly ribosomal RNA, is strikingly upregulated in nurse cells from these same stages in parallel with increases in ribosomal gene copy...
number [23]. The presence of CTPS filaments in Drosophila ovarian germ cells at stages with a high demand for CTP, together with the observation that reduced CTPS expression is associated with defects in oogenesis, suggests that CTPS filaments are assembled to promote CTP biosynthesis and support endoreduplication. Consistent with this model, the diameters of DAck86 nurse cell nuclei were significantly smaller than wild-type (Fig 4A), most likely as a result of defects in endoreduplication due to reduced CTP production.

The catalytic activity of CTPS is controlled by both transcriptional and post-transcriptional mechanisms including phosphorylation [24–27]. Indeed, filaments can be labeled with a phospho-specific antibody against CTPS phosphorylated on serine 36 [6], a post-translational modification associated with increased enzymatic activity [25]. An alternate method of enzyme regulation occurs via end product inhibition, when CTP inhibits the activity of CTPS itself [16]. An evolutionarily conserved glutamate residue at position 161 (Supplementary Fig S4A) is critical for end product inhibition, as expression of a mutant form of CTP synthase (E161K) that is insensitive to feedback inhibition causes an increase in cellular CTP [17]. We generated the E161K mutation in human CTPS1 (CTP synthase isoform 1) and expressed it in HEK293 cells as a GFP fusion protein. Remarkably, while wild-type GFP-tagged CTPS1 was primarily cytosolic, CTPS1-E161K localized to filaments (Fig 4B) that were morphologically similar to those in Drosophila egg chambers [5,6] or DON-treated mammalian cells [12,15]. Thus, unlike the wild-type form, the constitutively active mutant human protein is prone to assemble into filaments.

The observation that a constitutively active mutant of CTPS spontaneously assembles into filaments supports the hypothesis that filaments are assembled for the purpose of nucleotide generation and that CTPS is catalytically active within them. We tested whether GFP-CTPS1-E161K could rescue the sterility of CTPS−/− flies.
female flies when expressed in the germline at endogenous levels (Supplementary Fig S4B). Remarkably, the active human enzyme assembled into filaments in the *Drosophila* ovary (Fig 4C) and rescued both the membrane integrity defects (Fig 4C) and the sterility of CTPS<sup>−/−</sup> female flies (Fig 4D). Based on these results, we conclude that *Drosophila* CTPS is likely catalytically active within filaments and that filament assembly promotes CTP synthesis.

Although transgenic expression of human GFP-CTPS1-E161K rescued the fertility defect of CTPS<sup>−/−</sup> mutant flies, it did not rescue the fertility of *DAck<sup>86</sup>* mutant flies (Fig 4D). While this could be explained by additional, CTPS-independent roles for D Ack during oogenesis, morphological analysis of transgenic GFP-CTPS1-E161K in both genetic backgrounds suggested an alternative possibility. CTPS1-E161K filaments in CTPS<sup>−/−</sup> mutant egg chambers appeared morphologically normal, while in the *DAck<sup>86</sup>* mutant background, CTPS1-E161K filaments were more numerous and fragmented (Fig 4E) with a similar appearance as endogenous CTPS filaments in *DAck<sup>86</sup>* egg chambers (Fig 3E). This suggests that CTPS1-E161K filaments remain dependent on D Ack for normal assembly and function. Furthermore, it implies that filament assembly and enhancement of CTPS activity are separable events.

To more directly assess a role for D Ack in regulating CTPS activity, we measured total RNA from wild-type and *DAck<sup>86</sup>* mutant ovaries (Fig 4F). While *DAck<sup>86</sup>* flies lay fewer eggs (Fig 1A), the proportion of early- to late-stage egg chambers from these flies is similar to wild-type flies, allowing for a direct comparison of RNA levels using equal volumes of tissue. Total ovarian RNA levels were decreased in *DAck<sup>86</sup>* mutant flies compared to wild-type flies, consistent with an important role of D Ack in RNA production. Importantly, CTPS<sup>−/−</sup> flies also exhibited reduced ovarian RNA levels, as expected, and this phenotype was rescued by transgenic expression of GFP-CTPS1-E161K (Fig 4F). Taken together, these data suggest that D Ack plays a role in nucleotide biosynthesis by modulating CTPS filament organization which ultimately promotes CTPS enzymatic activity.

The regulation of metabolic enzyme activity by the reversible formation of macromolecular assemblies is an emerging paradigm in biology with only a few other examples [1,2]. Here, we provide new evidence that CTPS filaments are catalytically active based on the constitutive activity and dramatic filament-forming ability of the E161K mutant both in mammalian cells and when expressed in germ cells of the *Drosophila* ovary. Active CTPS filaments form in a stage-specific manner during oogenesis to support the high demand for CTP for rapid membrane expansion and RNA production in developing germ cells. Our data suggest that a mechanism utilized by single-celled organisms during nucleotide deprivation [5] has been integrated with tyrosine kinase signaling pathways in a tissue that requires transiently high levels of CTPS activity during development. Our results support a model in which the catalytic activity of D Ack regulates the morphology and subsequently the enzymatic activity of CTP synthase filaments, suggesting that the assembly of a proper filamentous structure is key for promoting CTPS catalytic activity. As we have not been able to detect phosphorylation of CTPS by Ack (Supplementary Fig S5), D Ack likely phosphorylates an as yet unidentified substrate that is essential for linking individual CTPS filaments into large, bundled, catalytically active assemblies. Thus, elucidation of the upstream signaling events that control Ack function and identification of Ack substrates in germline cells of the ovary will be informative for understanding how dynamic CTPS assembly is controlled.

**Materials and Methods**

**Antibodies**

Anti-D Ack antibody (JCD2) was provided by J. Dixon. Anti-CTPS synthetase 1/2 (y-88), anti-CTPS1 (C-13), anti-Vasa (d-260), anti-myc (9E10), anti-β-tubulin (H-235), and anti-phospho-tyrosine (PY20) antibodies were from Santa Cruz Biotechnology. Anti-GFP antibody (JL-8) was from Clontech. Anti-FLAG antibody (M2) was from Sigma-Aldrich. Anti-phospho-Y284-Ack1 antibody (ab74091) was from Abcam.

**Fly stocks**

Stocks were maintained and all crosses performed at 25°C. *w<sup>1118</sup>* flies were used as a control in all experiments. *DAck<sup>86</sup>* flies were provided by N. Harden. The CTP synthase GFP protein trap line, CA06746, was provided by A. Spradling. CTPsyn<sup>01207/TM6b</sup> and CTPsyn<sup>01942/TM6b</sup> were generated by Exelixis and maintained by the Harvard stock center. CTPS<sup>−/−</sup> flies were generated by crossing CTPsyn<sup>01207/TM6b</sup> and CTPsyn<sup>01942/TM6b</sup>. UAS-GFP-PH-PLC<sub>6</sub> flies were provided by L. Cooley. Expression of UAS-DAck, UAS-FLAG-D Ack, and UAS-GFP-CTPS1-E161K transgenes in germline cells of the ovary was driven with pC0G-Gal4:VP16.

Fertility analysis was performed essentially as described [28]. Briefly, equal numbers of 3- to 4-day-old virgin females of the indicated genotype were crossed to an equal number of *w<sup>1118</sup>* males at 25°C. Eggs were collected on grape juice agar plates and counted at 24 h.

**Supplementary information** for this article is available online: http://embor.embopress.org

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**Author contributions**

TIS, AMO, and JRP designed experiments, TIS, KPS, SVT, and EN performed experiments and analyzed data, and TIS, AMO, and JRP wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


