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The Ovhts polyprotein is cleaved to produce fusome and ring canal proteins required for *Drosophila* oogenesis

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An essential component of normal development is controlling the transition from cell proliferation to differentiation. One such transition occurs during *Drosophila* oogenesis. In early oogenesis, germ cells undergo mitotic proliferation and contain a specialized organelle called a fusome, whereas later post-mitotic cells differentiate and lose the fusome as F-actin-rich ring canals form. The *hts* gene encodes the only *Drosophila* Adducin, and is a female-sterile mutant that affects both the fusome and ring canals. We show that one Hts protein, Ovhts, is a polyprotein that is cleaved to produce two products, Ovhts-Fus and Ovhts-RC. Whereas Ovhts-Fus localizes to the fusome in mitotic cells, Ovhts-RC localizes to ring canals throughout later oogenesis. We demonstrate that an uncleavable version of Ovhts delays the transition from fusome-containing cells to those that have ring canals. Ovhts is the first polyprotein shown to produce proteins that function in separate structures.

KEY WORDS: Fusome, Ring canals, *Drosophila* oogenesis, Polyprotein

INTRODUCTION

Germline development, like many other developmental programs, involves a preliminary stage of mitotic expansion, followed by differentiation into mature cell types. One hallmark of germline development is that the proliferative phase occurs in clusters of cells interconnected by cytoplasmic bridges (Matova and Cooley, 2001). In some systems, such as oogenesis in mammals, cytoplasmic bridges disappear early and cells differentiate separately. However, in most known forms of spermatogenesis and in insect oogenesis, cytoplasmic bridges persist and contribute to differentiation. In insect oogenesis, one cell in each cluster becomes the oocyte and is nourished by the nurse cells through cytoplasmic bridges. In all cases, germline cells must undergo specific changes in gene expression, morphology and function when making the transition from proliferation to differentiation.

Drosophila oogenesis is an attractive model to study the transition from germline cell expansion to differentiation and the formation of intercellular bridges called ring canals (RCs). In the most anterior compartment of the ovary, the germarium, germline stem cells (GSCs) undergo division to form another GSC and a daughter cystoblast (Huynh and St Johnston, 2004). The cystoblast undergoes exactly four mitoses with incomplete cytokinesis forming a 16-cell cyst in which cells are connected by arrested cleavage furrows. Once mitosis is complete the cells differentiate into 15 nurse cells and one oocyte. The proliferating cells and differentiated cells can be easily identified through subcellular structures. During proliferation, an organelle called the fusome is present. After mitosis is complete, the fusome begins to disappear and RCs form at the arrested cleavage furrows.

The fusome is derived from a precursor structure called a spectrosome (Lin et al., 1994), which is present in GSCs. A portion of the spectrosome is inherited by the daughter cystoblast, after which it grows and branches during cystoblast divisions to form the fusome (de Cuevas and Spradling, 1998). Spectrosome- and/or fusome-like structures have been described in the germline of insects, *Xenopus* and mouse (Pepling et al., 1999). A potentially analogous structure is in mammalian lymphocytes (Dubielecka et al., 2003). The spectrosome and fusome in *Drosophila* are areas of highly condensed vesicles including modified endoplasmic reticulum (de Cuevas et al., 1997; Snapp et al., 2004). Fusome membranes are associated with a cytoskeleton that includes the Adducin homolog Hu-li tai shao (Hts), α-Spectrin, β-Spectrin, Filamin (Cheerio – Flybase), a Spectraplakin homolog named Short Stop (Shot), and Ankyrin (de Cuevas et al., 1996; Lin et al., 1994; Roper and Brown, 2004; Sokol and Cooley, 2003). Hts and α-Spectrin are necessary for fusome structure because in mutants the fusome is absent (de Cuevas et al., 1996; Lin et al., 1994) causing dramatic defects in nurse cell number and oocyte specification.

RCs allow the movement of essential proteins and RNA from nurse cells into the oocyte; thus, mutations that result in their disruption are female sterile. Formation of RCs at arrested cleavage furrows follows an organized pattern of RC protein accumulation. F-actin, Filamin and Ovhts-RC, a novel product of the *hts* locus, begin to accumulate on RCs starting immediately after mitosis ends (Robinson et al., 1994). Mutations in the gene for Filamin (*cheerio*) or *hts* result in a failure of F-actin accumulation and arrested RC development (Robinson et al., 1997).

hts was isolated as a female sterile mutant (Ding et al., 1993; Yue and Spradling, 1992), and products of the *hts* gene are present on both the fusome and RCs, making Hts an attractive candidate for participating in the transition from fusome-containing proliferating cells to RC-containing differentiating cells. Subsequent work showed that there are four distinct proteins made from splice variants of the *hts* gene in the ovary (Whittaker et al., 1999) (this article). All predicted Hts proteins share identical N-terminal Head and Neck domains homologous to mammalian Adducin proteins (Fig. 1), but have unique C-termini. Add1 and Add2 contain the Adducin Tail and MARCKS domains. ShAdd protein has 23 novel C-terminal amino acids and no MARCKS domain. Ovhts contains 80% of the Adducin Tail domain, and a large C-terminal domain unique to *Drosophila* Adducins that we call the Ring Canal domain.

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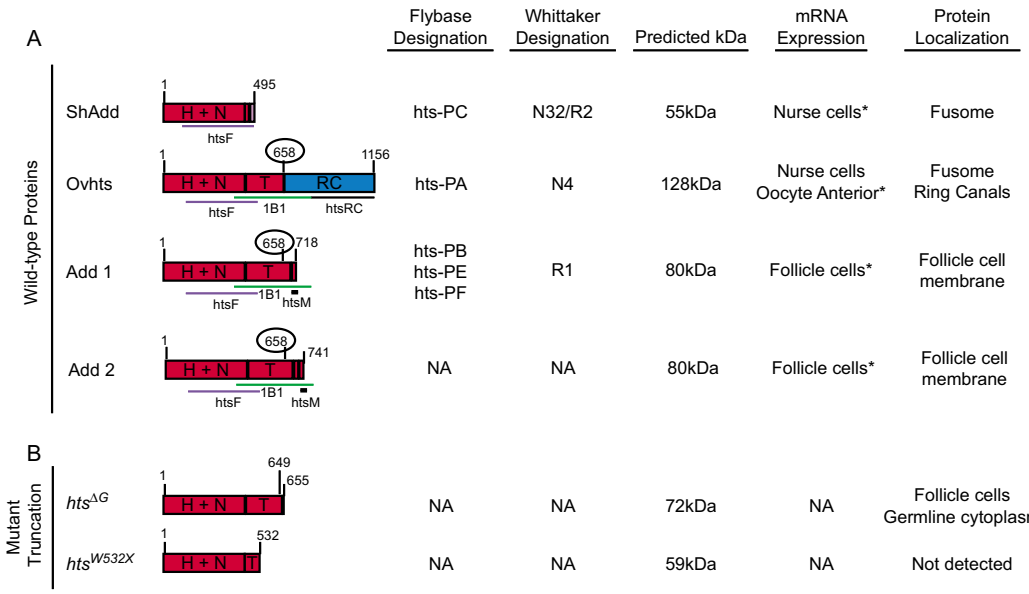

Fig. 1. Wild-type and mutant Hts protein domains and expression. (**A**,**B**) For each wild-type and mutant protein, the Flybase designation, Whittaker mRNA designation (Whittaker et al., 1999), predicted size, RNA expression and protein localization are given. (A) All wild-type proteins contain the Head (H) and Neck (N) domains of Adducin (homology shown in red). ShAdd contains a truncated Tail (T) domain and then 23 novel amino acids at its C-terminus (purple). Ovhts, Add1 and Add2 share a common Tail. Alternative splicing at the codon for amino acid 658 (circled) results in three transcripts with different C-terminal domains. Ovhts has a novel domain designated the RC domain (blue). Add1 and Add2 maintain homology to mammalian Add, with Add2 splicing into an exon encoding 23 amino acids and both encoding the C-terminal MARCKS domain. The regions used as antigens for antibody production are underlined. (B) Two truncation mutants lack the wild-type C-terminal domains. *hts*$^{\Delta G}$ has a single base pair deletion of G2346 resulting in a frameshift, six novel amino acids and a premature stop codon. In *hts*^{W532X} Trp532 of both Ovhts and Add1/2 is mutated to a stop codon in the Tail domain.

Antibodies made against Hts proteins label the fusome and RCs in the germline as well as the follicle cell membranes (Fig. 1) (Robinson et al., 1994; Zaccai and Lipshitz, 1996). Mutant phenotypes are consistent with expression data and include loss of oocyte specification, too few nurse cells, and RC deformities (Yue and Spradling, 1992). Protein localization and mutant phenotype analyses were done prior to knowing that the *hts* gene produces multiple proteins; therefore, further characterization of *hts* is needed to understand its roles during oogenesis.

We carried out detailed characterization of the localization of Hts proteins, focusing on the germline-specific proteins Ovhts and ShAdd. Remarkably, Ovhts is a polyprotein that is cleaved to form two functional products we call Ovhts-Fus and Ovhts-RC. Each Ovhts protein is stable during different developmental stages, with Ovhts-Fus present in proliferating cells and Ovhts-RC in post-mitotic differentiating cells. Although other polyproteins have been described, this is the first example where the products are maintained in different developmental time points and subcellular locations. Further experiments with both wild-type and uncleavable Ovhts proteins demonstrate that there is a developmental link between the degradation of the fusome and RC establishment. Finally, we show that Ovhts-RC is functionally required in RCs throughout oogenesis.

MATERIALS AND METHODS

Genetics and fly strains

Drosophila cultures were maintained using standard procedures (Ashburner, 1989). The wild-type control was w^{1118} (Lindsely and Zimm, 1992). *hts*$^{\Delta G}$ was from the Zuker collection (Koundakjian et al., 2004) and *hts*^{W532X} was from Trudi Schüpbach (Princeton University, Princeton, NJ). Expression of *P{UASH}* transgenes was done by crossing to one of the following lines: *P{bamP-bam5-Gal4:VP16}* (Chen and McKearin, 2003), *P{tub-Gal4}LL7* (Lee and Luo, 1999), *P{nos-Gal4-VP16}* (Van Doren et al., 1998) or a maternal triple driver, *MTD-Gal4*, containing the *P{Gal4-nos.NGT}40* (Tracey et al., 2000), *P{COG-GAL4:VP16}* (Rorth, 1998) and *P{nos-Gal4-VP16}* (Van Doren et al., 1998) germline drivers.

Sequencing of mutant alleles

The *hts* gene was amplified from homozygous mutants by PCR, and the products were sequenced (W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University).

Generation of transgenes and transgenic animals

The pCOH vector was made by removing the K10 3′ UTR fragment from pCOG (Robinson and Cooley, 1997) and replacing it with the first 301 nucleotides of the *ovhts* 3′ UTR (representing base pairs 3868 to 4168) (Whittaker et al., 1999). The pUASH vector was made using the same methodology to replace the K10 3′ UTR of pUASP (Rorth, 1998) with that of *ovhts*.

The coding region of Ovhts from the cDNA (bp 397-3864) was subcloned into the pCOH or pUASH vectors. The GFP (S65T) gene was modified from the pCS2*mt-GFP (gift of Michael Klymkowsky, University of Colorado, Boulder) vector by PCR to add an 8 Ala linker on the N-terminus of GFP and cloned into Bluescript KS. GFP (S65T) was then subcloned into *P{COH-Ovhts}* and *P{UASH-Ovhts}* to make *P{COH-Ovhts::GFP}* and *P{UASH-Ovhts::GFP}*. *P{COH-Ovhts-Δ3::GFP}* was made by subcloning the *Stu*I to *Bgl*II restriction fragment from pMT-Ovhts-Δ3 (see below) into *P{COH-Ovhts::GFP}*. To make *P{COH-Cer::Ovhts}*, the Cerulean gene was modified using PCR from the mCerulean-C1 vector (Rizzo et al., 2004) to add flanking EcoRI sites, and then subcloned in-frame directly upstream of Ovhts in *P{COH-Ovhts}*.

To make *P{COH-ShAdd::Ven}*, ShAdd was cloned into pCOH. Venus (Nagai et al., 2002) was modified using PCR to add a 5′ *Xho*I site and 3′ 7×His tag followed by a stop the another *Xho*I site. Venus was then subcloned in-frame directly downstream of ShAdd in *P{COH-ShAdd}*.

Embryo injections to generate transgenic animals were either performed as previously described (Robinson and Cooley, 1997), or were done at Duke University, Model System Genomics.

Antibody production and purification

htsM antibody was modeled after the peptide antibody raised against the MARCKS domain of human β-Adducin (Matsuoka et al., 1998) using a peptide representing amino acids 689-718 of Add1, GSPKKDKKK-KKGLRTPSFLKKKKEKKKAEA (W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University). Peptide conjugation, injection and antibody production was done at Cocalico Biological, Inc. (Cocalico Biological, Inc., Reamtown, PA). htsM antibody purification was done using the SulfoLink Kit (Pierce #44895).

S2 cell vectors and transfection

All S2 cell vectors were made in the pMT V5 His copper-inducible system (Invitrogen). Full-length pMT-Ovhts contained the entirety of the Ovhts coding region (bp 397-3864). Nucleotides were removed by two-step PCR in each deletion construct resulting in the loss of the following amino acids from Ovhts: Ovhts-Δ1 amino acids 632 to 651, Ovhts-Δ2 amino acids 639-658, Ovhts-Δ3 amino acids 650-669 and Ovhts-Δ4 amino acids 785 to 794.

S2 cells were cultured, transfected with 1 μg of DNA using Lipofectin (Invitrogen #50503) and induced with 0.7 mM $CuSO_4$.

Immunoblots

Protein samples were prepared as previously described (Robinson et al., 1997). Primary antibodies were diluted in PBT with 5% milk as follows: polyclonal rat htsF at 1:5000 (Lin et al., 1994; Robinson et al., 1997), monoclonal htsRC at 1:10 (Robinson et al., 1994) (Developmental Studies Hybridoma Bank), monoclonal 1B1 at 1:10 (Zaccai and Lipshitz, 1996) (Developmental Studies Hybridoma Bank), polyclonal rabbit anti-GFP antibody 1:1000 (Torrey Pines TP401). Horseradish-peroxidase-conjugated secondary antibodies were used at 1:30,000 (Pierce #31430, #31460 and #31470). Proteins were detected using SuperSignal West Dura ECL Substrate (Pierce #34076).

Immunofluorescence and image collection

Ovaries were dissected and fixed as described previously (Robinson and Cooley, 1997). To visualize actin, egg chambers were incubated with 2 U rhodamine-conjugated phalloidin in PBT. For antibody labeling, ovaries were incubated either overnight at 4°C or 2 hours at room temperature with htsRC antibody at 1:10, 1B1 antibody at 1:7, htsF antibody at 1:500, purified htsM antibody at 1:250 or anti-GFP antibody at 1:750 (Molecular Probes A11122). Secondary antibodies conjugated to Alexafluor 488 or 568 (Molecular Probes) were used at 1:500 and were incubated with ovaries for 2 hours at room temperature. Ovaries were stored in Antifade (0.23% DABCO in 0.1 M Tris-HCL 90% glycerol) overnight at 4°C and then mounted. All images were taken on either a ZEISS LSM-510 or a ZEISS LSM-510 META microscope (Center for Cell and Molecular Imaging, Yale University School of Medicine), and images were processed using Adobe Photoshop 7.

RESULTS

Localization of *hts* proteins in the ovary

Because previous examination of Hts protein localization was done before there was a complete understanding of the *hts* locus, we characterized Hts antibody localization in more detail. We used four antibodies directed against different Hts protein domains (see Fig. 1), three of which were published previously. htsF (Lin et al., 1994; Robinson et al., 1997) recognizes ShAdd, Add1 and Add2 (Add1/2) and Ovhts. In germaria, htsF antibody labeled the fusome in the germline (Fig. 2A, arrow) and plasma membranes in follicle cells (Fig. 2A, arrowhead). 1B1 antibody (Zaccai and Lipshitz, 1996), which recognizes Ovhts and Add1/2, had an identical germarium-labeling pattern to htsF (Fig. 2B). htsM antibody, which recognizes only Add1/2, labeled follicle cell membranes (Fig. 2C) and showed no labeling of the germline. In later-stage egg chambers, htsF, 1B1 and htsM antibodies continued to show specific labeling of lateral follicle cell membranes but no germline labeling (Fig. 2E-G).

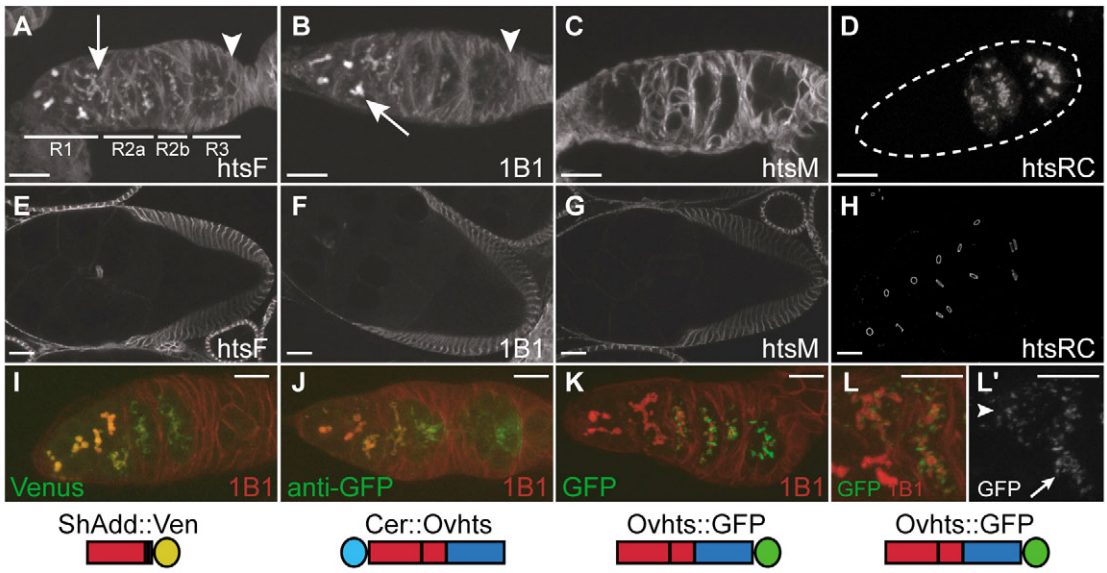

Fig. 2. *hts* antibodies and tagged proteins show differential localization of Hts proteins. (**A-D**) Germaria and (**E-H**) stage 9 egg chambers labeled with the designated antibody. (A) Regions of the germaria are underlined (King, 1970). Region 1 (R1), all dividing cells; Region 2, 16-cell cysts not surrounded by follicle cells (R2a, cysts are spherical; R2b cysts are lens shaped and span the width of the germarium); Region 3 (R3), cysts surrounded by follicle cells. (A,B) htsF and 1B1 antibodies label fusomes in the germline (arrow) and follicle cell membranes (arrowhead). (C) htsM antibody labels only follicle cell membranes. (D) htsRC antibody labels puncta and then RCs starting in Region 2a. (**E-G**) htsF, 1B1 and htsM antibodies label follicle cell membranes exclusively; there is no localization in the germline. (**H**) htsRC antibody labels only RCs. (**I-K**) Transgene expression in germaria co-stained with 1B1 (red). (I) ShAdd::Ven localizes to the fusome until Region 3 and then is present at a very low level in the cytoplasm. (J) Cer::Ovhts localizes to the fusome in the germaria based on staining with anti-GFP antibodies. (K) Ovhts::GFP starts as puncta in Region 2 and then localizes to RCs throughout oogenesis. (**L**,**L'**) Higher magnification of a germaria expressing Ovhts::GFP. Puncta appear in Region 2a (arrowhead) near the fusome and start to become RCs (arrow) in Region 2b. Scale bars: A-D,I-L,10 μm; E-H, 20 μm.

Consistent with RNA in-situ data (Whittaker et al., 1999), Ovhts is germline-specific and Add1/2 are follicle-cell-specific. As *shadd* mRNA is also exclusively found in the germline, and the htsF antibody only labels the fusome in the germline, ShAdd is likely a fusome component (see below). Although both *shadd* and *ovhts* mRNAs are expressed in the germline throughout oogenesis (Whittaker et al., 1999), their protein products detected with 1B1 and htsF antibodies were only present in the germarium. This suggests that the proteins are either not translated or are not stable once egg chambers are formed.

Because we were unable to make a useful peptide antibody specific for ShAdd, we made a ShAdd transgene expressing ShAdd fused to Venus, a modified EYFP (Nagai et al., 2002). When ShAdd::Ven was expressed with the *ovarian tumor* (*otu*) promoter in the germline of wild-type flies, it localized specifically to spectrosomes and fusomes (Fig. 2I). As the fusome began to degrade in Region 2, the localization of ShAdd::Ven also became more dispersed (Fig. 2I). Thus, ShAdd::Ven provided additional evidence that ShAdd is a fusome protein.

HtsRC antibody (Robinson et al., 1994), which recognizes the C-terminus of Ovhts, had a completely different localization pattern. Starting in Region 2a of germaria, htsRC labeled discrete puncta, which resolved into RCs in Region 2 (Fig. 2D). htsRC antibody labeled RCs throughout the rest of oogenesis (Fig. 2H).

In order to verify the different labeling patterns of antibodies against the N- and C-termini of Ovhts, we made tagged Ovhts transgenes that were expressed specifically in the germline. In separate constructs containing the native *ovhts* UTRs, the N-terminus of Ovhts was tagged with Cerulean, a modified ECFP, and the C-terminus was tagged with GFP. The Cer::Ovhts transgene did not produce a fluorescent product; however, upon labeling with anti-GFP antibodies, Cer::Ovhts was detected on the fusomes in germaria (Fig. 2J). Like the N-terminus of Ovhts as seen by antibody labeling, Cer::Ovhts localized to both spectrosomes and branched fusomes. Co-staining with 1B1 showed that as the fusome began to break down in Region 2, Cer::Ovhts became dispersed and lost colocalization with 1B1.

GFP fluorescence from Ovhts::GFP localized specifically to RCs (Fig. 2K). As with htsRC antibody, protein was first detected in Region 2a as puncta that appeared to be near, although not within the fusome (Fig. 2K,L′, arrowhead). By Region 2b Ovhts::GFP was in rings (Fig. 2K,L′, arrow). Ovhts::GFP was seen on RCs in all subsequent stages until stage 13 (data not shown). Thus, localization of tagged Ovhts transgenes recapitulated antibody labeling, with the N-terminus present on fusomes and the C-terminus localizing to RCs.

Western analysis of Ovhts shows cleavage products

Although the predicted size of the Ovhts protein is 128 kDa, ovary extract never contained a protein of this size. Instead, htsF (Fig. 3A) and 1B1 (data not shown) antibodies detected a doublet of ~90 kDa, which are the Add1/2 proteins. When protein extracts were analyzed from virgin female ovaries, which contain fewer late-stage egg chambers and therefore are enriched for germarial tissue, a band of ~80 kDa was detected (Fig. 3A). As reported previously (Robinson et al., 1994), the htsRC antibody detected a ~60 kDa doublet (Fig. 3B). These results suggested that Ovhts is cleaved into two smaller proteins. Moreover, when ovary extracts of flies expressing Ovhts::GFP or Cer::Ovhts were blotted with anti-GFP antibody, only bands that would represent the cleavage products (with the added GFP tag) were seen (Fig. 3C). These results, along with protein localization data, show that Ovhts is cleaved. Based on the fact that antibodies to the N-terminus of Ovhts label fusomes and antibodies to the C-terminus label RCs, we designated the cleavage products Ovhts-Fus and Ovhts-RC, respectively.

To further investigate Ovhts cleavage, we expressed Ovhts in S2 cells, which do not express endogenous Ovhts. Western analysis with either 1B1 to visualize the N-terminus or htsRC to visualize the C-terminus, revealed 80 kDa and 60 kDa proteins like those detected in ovary extracts (Fig. 3D,E). Additionally, an ~140 kDa band that represents full-length Ovhts was apparent in S2 cell extract (Fig. 3D,E). As the full-length protein can only be detected when Ovhts is overexpressed in S2 cells and not in ovary extracts, the cleavage process in ovaries must be very efficient.

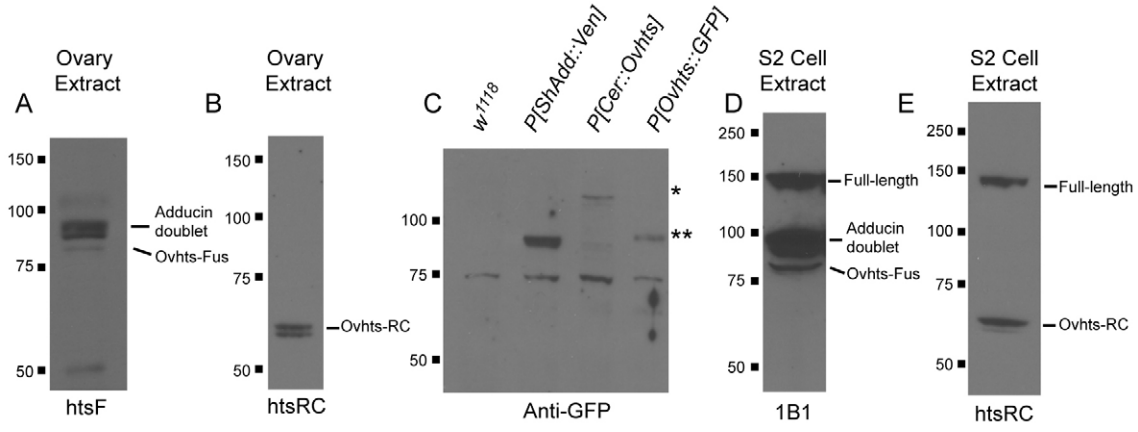

Fig. 3. Western immunoblot analysis of Hts proteins in ovary and S2 cell extracts. (**A**,**B**) Ovary extracts from virgin w^{1118} flies blotted with htsF and htsRC. (A) Both the Add1/2 doublet band at 90 kDa and the 80 kDa Ovhts-Fus band can be seen. (B) Only the Ovhts-RC doublet is detected. (**C**) Ovary extract from flies expressing tagged *hts* transgenes blotted with anti-GFP antibody. A 75 kDa nonspecific band is present in all lanes. The ShAdd::Ven band is of the predicted size. The bands in Cer::Ovhts-Fus (~110 kDa, *) and Ovhts-RC::GFP (~90 kDa, **) are of the sizes predicted for the respective cleavage products fused to the fluorescent proteins. (**D**,**E**) Protein extracts from S2 cells expressing Ovhts blotted with 1B1 and htsRC. (D) S2 cells express endogenous Add1/2 proteins that can be seen at 90 kDa. Bands representing both full-length Ovhts protein and Ovhts-Fus are indicated. (E) Bands representing both full-length Ovhts protein and Ovhts-RC are indicated. All Hts proteins consistently ran 10-20 kDa larger than predicted.

Characterization of new *hts* alleles

In order to elucidate the functions of the individual *hts* proteins, new alleles of *hts* were characterized. All previously described alleles of *hts* were *P*-element insertions or imprecise excisions that reduce expression of all *hts* transcripts. We examined two new EMS-induced *hts* alleles. hts^{W532X} (gift from Trudi Schüpbach) contains a single nonsense mutation, W532X, in the tail domain (Fig. 1B). $hts^{\Delta G}$ was in the Zuker collection (Koundakjian et al., 2004), and DNA sequencing showed a deletion of a single G in the last part of the Tail domain (G2346 of the *ovhts* transcript). This results in a frame shift followed by six novel amino acids and a stop codon. Conceptual translation of $hts^{\Delta G}$ results in a truncated protein that does not contain any of the normal C-terminal domains (Fig. 1B). These mutations are downstream of the entire ShAdd coding sequence.

The phenotypes of these truncation alleles were indistinguishable from the *P*-element alleles. Both were female sterile and showed a loss of oocyte specification, too few nurse cells, and no actin on RCs (data not shown). However, labeling of $hts^{\Delta G}$ and hts^{W532X} with Hts antibodies and western analysis showed a distinct difference between the alleles. Even though both truncation alleles should encode the epitope for the htsF antibody, protein was detected only in $hts^{\Delta G}$ (see Fig. S1E,I in the supplementary material). Western analysis showed that whereas $hts^{\Delta G}$ expressed a single truncation product, hts^{W532X} produced no detectable protein (see Fig. S1M in the supplementary material) and is therefore a null allele. Additionally in $hts^{\Delta G}$, antibodies 1B1 and htsF labeled a cytoplasmic protein that persisted in egg chambers after they emerged from the germarium, which is never seen in wild type (see Fig. S2 in the supplementary material; data not shown). Therefore, the mutant truncated protein was aberrantly stable in germline cells that normally do not have Ovhts-Fus. Mutant follicle cells labeled with 1B1 antibody showed a significant, but not complete loss of Add1/2 localization to lateral membranes (see Fig. S2D in the supplementary material).

Tagged transgenes rescue ring canals but not the fusome

To determine the functional requirements of the different Hts proteins in the germline, we crossed tagged *hts* transgenes expressed from the *otu* promoter into both $hts^{\Delta G}$ and hts^{W532X} mutant backgrounds for rescue experiments. Both *P{Ovhts::GFP}* and *P{Cer::Ovhts}* rescued recruitment of Ovhts-RC and actin on RCs (Fig. 4B,C, data not shown). However, other *hts* phenotypes were not rescued. Labeling with htsF, 1B1 or α-spectrin antibodies showed no fusome-like structure (Fig. 4B,C, data not shown). Anti-GFP labeling in mutants expressing *P{Cer::Ovhts}* only showed cytoplasmic labeling (Fig. 4A). Additionally, the egg chambers still had too few cells and degenerated. We tested whether the addition of ShAdd would improve rescuing activity. When *P{ShAdd-Ven}* was expressed alone in $hts^{\Delta G}$, Venus fluorescence was diffuse in the cytoplasm, and *hts* phenotypes were not rescued (Fig. 4D). Expression of *P{ShAdd::Ven}* with either *P{Cer::Ovhts}* or *P{Ovhts::GFP}* in a $hts^{\Delta G}$ background showed the same phenotype as the single rescue (Fig. 4E,F): only RCs were rescued, but not the fusome or any of the phenotypes resulting from the loss of the fusome.

Recent work has shown that the fusome precursor, the spectrosome, first begins to form during stage 11 of embryogenesis (Wawersik and Van Doren, 2005). As the fusome develops from the spectrosome, it was possible that the *otu* promoter was not providing Ovhts at an early enough stage. However, earlier expression of Ovhts by driving *P{UASH-ovhts::GFP}* with either *P{nos-GAL4}* or *P{tub-GAL4}* produced the same result as the *otu* promoter (data not shown).

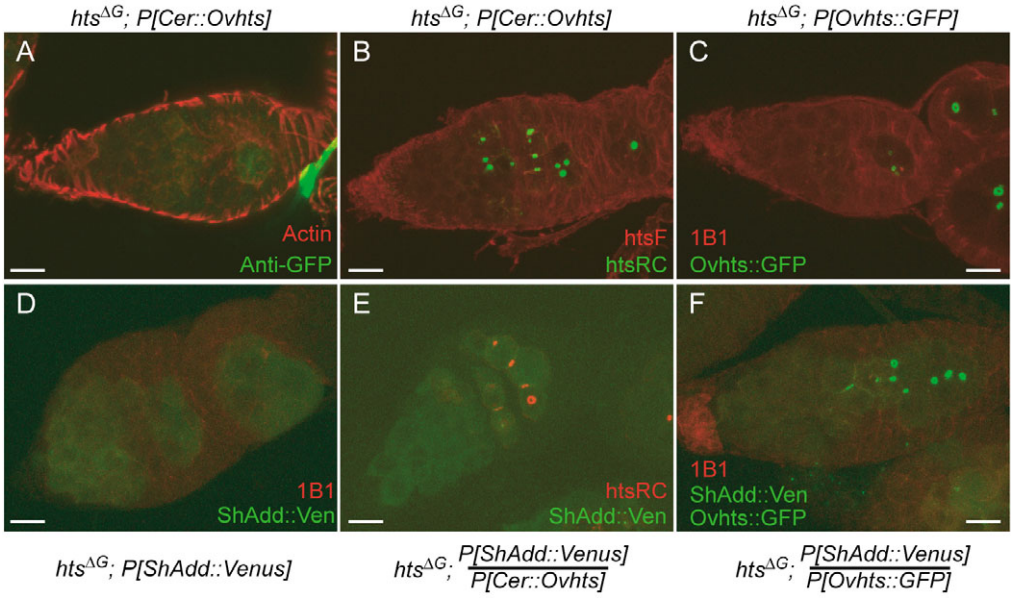

Fig. 4. Expression of transgenes can rescue RCs but not the fusome. (**A-F**) Germaria of $hts^{\Delta G}$ mutants expressing the designated *hts* transgenes. (A,B) Expression of Cer::Ovhts results in no fusome rescue by either anti-GFP antibody (A, green) or htsF (B, red) staining. However, there is rescue of Ovhts-RC localization to the RC shown by staining with htsRC antibodies (B, green). (C) Expression of Ovhts::GFP results in no fusome rescue (1B1 staining, red) but does rescue Ovhts-RC::GFP to RCs (green). (D) Expression of ShAdd::Ven results in no fusome rescue (1B1 staining, red). ShAdd::Ven (green) is cytoplasmic. (E,F) Expression of ShAdd::Ven (green) with either Cer::Ovhts (E) or Ovhts::GFP (F, green) does not rescue the fusome (E and F, green) or fusome staining with 1B1 (F, red), but does rescue RCs as seen with either transgenic GFP (F, green) or staining with htsRC antibody (E, red). Scale bars: 10 μm.

To determine whether there is a somatic contribution to the *hts* phenotype that results in our inability to rescue the fusome, we performed clonal analysis with $hts^{\Delta G}$. Germline clones showed the same phenotype as homozygous mutants, whereas egg chambers that had only follicle cell clones were normal (data not shown). Therefore, the loss of the fusome and RCs is caused solely by the loss of full-length Ovhts in the germline.

Ring canal formation is delayed in mutants rescued by *hts* transgenes

During the rescue experiments with *P{Ovhts::GFP}*, we noticed that localization of Ovhts-RC::GFP to RCs was delayed. In wild-type flies expressing this transgene, GFP was always present in Region 2 of germaria (Fig. 5A). In contrast, in either $hts^{\Delta G}$ (Fig. 5C) or hts^{W532X} (Fig. 5B) flies expressing *P{Ovhts::GFP}*, GFP was often absent from germaria and only appeared later. Quantitation of this phenotype revealed that in hts^{W532X} there was a delay in 30% of germaria, and in $hts^{\Delta G}$ there was a delay in 91% of germaria (Fig. 5D). Thus, in *hts* mutants the recruitment of Ovhts-RC and actin, and therefore the establishment of RCs, did not occur at the correct developmental stage, suggesting that the fusome is necessary for the timing of RC development.

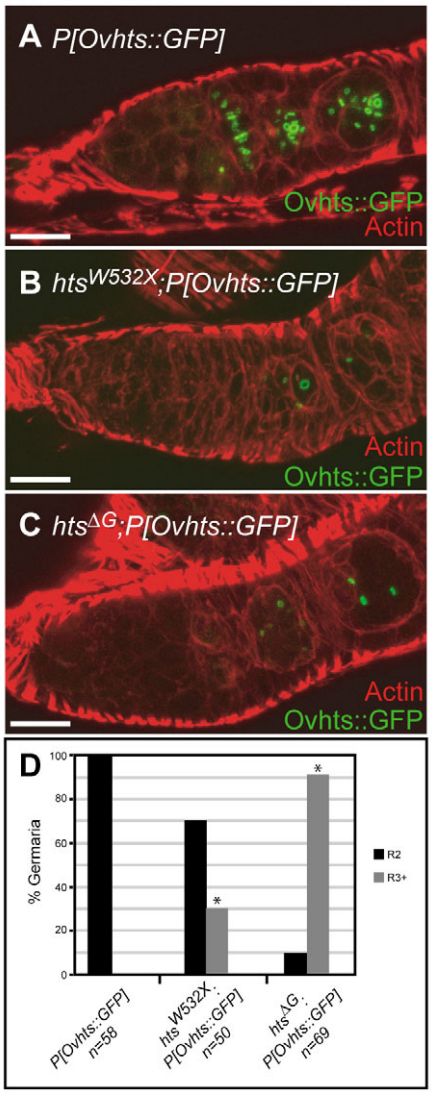

Fig. 5. Localization of Ovhts::GFP to the RC is delayed in *hts* mutants. (**A-C**) Expression of Ovhts::GFP in wild-type, hts^{W532X} and $hts^{\Delta G}$ ovaries. (A) In the control background, Ovhts::GFP localizes to the RCs in Region 2a. (B,C) In hts^{W532X} and $hts^{\Delta G}$, Ovhts::GFP localization is delayed until Region 3. (**D**) Quantitation of the localization timing of Ovhts::GFP at either Region 2a (black bars) or at a later stage (gray bars). There is a 30% and 91% delay in hts^{W532X} and $hts^{\Delta G}$, respectively. *Significant χ^2, $P<0.001$. Scale bars: 10 μm.

Behavior of uncleavable Ovhts

We took advantage of the ability of S2 cells to cleave Ovhts to identify amino acids necessary for its cleavage. We made a series of small in-frame deletion mutations in the region of the predicted cleavage site, four of which are shown in Fig. 6A, and expressed them in S2 cells. The Δ1 deletion, which removes 20 amino acids in the Tail domain, was cleaved at a wild-type level (Fig. 6B). Deletion Δ2 removed the last 20 amino acids of the Tail domain, and Δ3 removed the last nine amino acids of the Tail and the first 11 amino acids of the RC domain. The Δ2 protein was cleaved, although less efficiently than wild-type protein, whereas the Δ3 protein was not cleaved (Fig. 6B). This result demonstrates that the first 11 amino acids (ALVSQLAQKYA) of the RC domain are required for cleavage.

To study the effect of uncleavable Ovhts in the ovary, we made a *P{Ovhts-Δ3::GFP}* transgene that was expressed from the *otu* promoter. Except for the 20 amino acid deletion, this transgene was identical to the *P{Ovhts::GFP}* transgene. Western analysis of Ovhts-Δ3::GFP from ovary extracts demonstrated that, as in S2 cells, this protein is not cleaved (Fig. 6C). When expressed in wild-type flies, Ovhts-Δ3::GFP was present not only on RCs, but also on the fusome (Fig. 6E,E′). Additionally, the 1B1 antibody, which normally only labels the fusome, now also labeled RCs (Fig. 6E,E″). Therefore, the uncleaved protein localized to both structures where the cleavage products are normally found.

Although wild-type flies expressing Ovhts-Δ3::GFP were fertile and produced apparently normal egg chambers, uncleaved Ovhts did cause a subtle, but completely penetrant dominant defect in the disappearance of the fusome. In wild-type germaria, the fusome begins to disappear where the RCs are starting to form (Fig. 6D,D″,F). This results in unobstructed RCs with fragmented fusome material between them, but not through them. In flies expressing *P{Ovhts-Δ3::GFP}*, fusome material was present within the RCs as late as stage 2 and 3 egg chambers (Fig. 6G). In some cases, GFP-positive rings were occluded with GFP-negative fusome material that could be visualized with 1B1 antibody (Fig. 6G′,G″ arrowhead). Therefore, at least some of the aberrant fusome contained only wild-type Ovhts-Fus protein and not the N-terminal portion of *P{Ovhts-Δ3::GFP}*. Additionally, RC rims were thicker than normal, less organized and misshapen (Fig. 6, compare F with G). These results suggest that the cleavage and proper maintenance of the Ovhts domains may play a role in the transition from a fusome to RCs.

We also tested whether the *P{Ovhts-Δ3::GFP}* transgene could rescue *hts* mutants, $hts^{\Delta G}$ and hts^{W532X} (data not shown). As with expression of *P{Ovhts::GFP}*, *P{Ovhts-Δ3::GFP}* rescued RCs but not the fusome. The rescued RCs were also labeled with 1B1 antibody indicating that the N-terminus of Ovhts was present.

Ovhts is expressed throughout all of oogenesis

Our ability to rescue RCs with Ovhts transgenes provided an opportunity to investigate the function of Ovhts-RC. To determine when full-length Ovhts needs to be expressed for RC localization, stage-specific induction of Ovhts expression was done. Wild-type flies carrying a *P{UASH-Ovhts::GFP}* transgene were crossed to

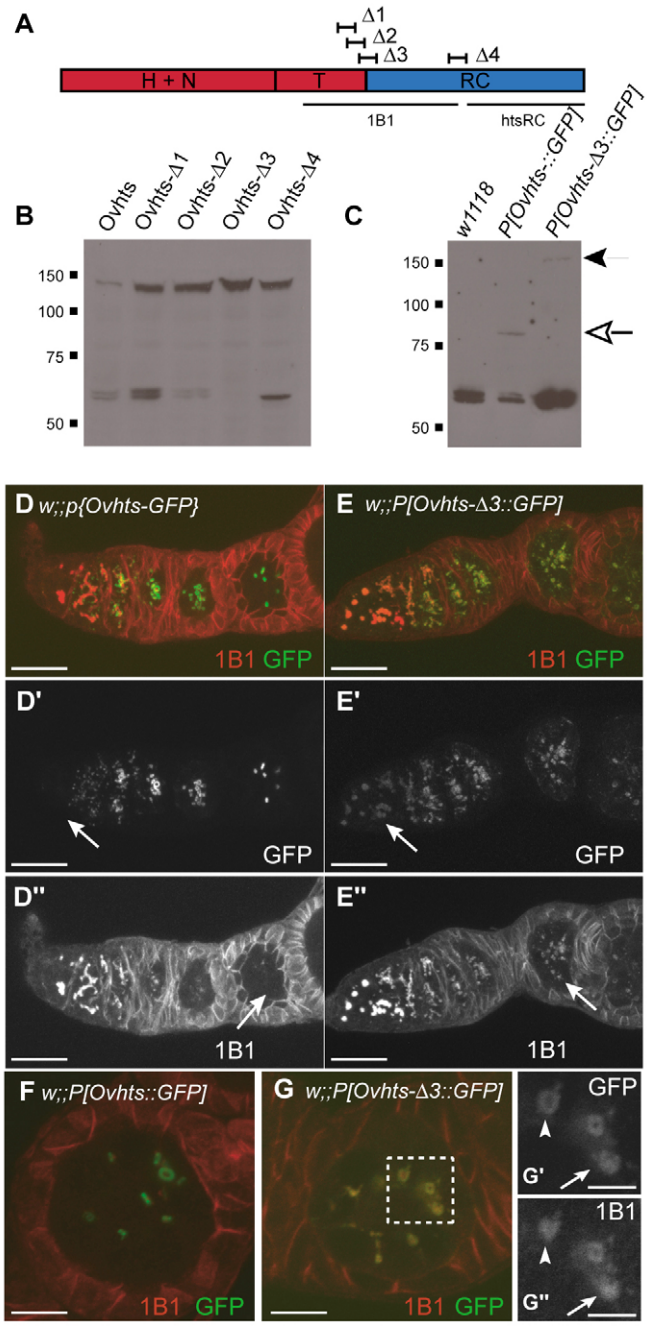

Fig. 6. Expression of an uncleavable form of Ovhts disrupts the transition from fusome to RC. (**A**) Schematic of the location of the four 20-amino acid deletions. (**B**) Western blot of deletion constructs Δ1 through Δ4 expressed in S2 cells, probed with htsRC antibody. Ovhts-Δ3 protein is not cleaved. (**C**) Ovary extracts from flies expressing either Ovhts::GFP or Ovhts-Δ3::GFP blotted with htsRC antibody. Whereas only a cleavage product is present in Ovhts::GFP extract (open arrow), Ovhts-Δ3::GFP is only present in a full-length band (arrowhead). (**D-D"**) Germaria from flies expressing Ovhts::GFP show a GFP signal on RCs but not on fusome (arrow D′), and 1B1 localization only to fusome and not to RCs (arrow D″). (**E-E"**) Flies expressing Ovhts-Δ3::GFP show localization of GFP (E′) and 1B1 (E″) to both the fusome and RCs (arrows). (**F**,**G**) Stage 2 egg chambers expressing either Ovhts::GFP or Ovhts-Δ3::GFP stained with 1B1 antibody (red). (F) RCs show localization of Ovhts::GFP in tight clear rings that do not have 1B1 staining. (G) RCs show a colocalization of 1B1 staining and Ovhts-Δ3::GFP fluorescence on RCs (yellow). Additionally, RCs are malformed. (**G′**,**G"**) Magnification of boxed region in G shows that rings are not round, appear thicker and less organized, and can be fully occluded with fusome material that does (arrow) or does not (arrowhead) include Ovhts-Δ3::GFP. Scale bars: C,D, 20 μm; F,G, 10 μm; G′,G″, 5 μm.

two different Gal4 lines: *MTD-Gal4* (see Materials and methods) that induces strong expression throughout oogenesis or *P{bam-Gal4}* that induces expression only in Region 1 of the germarium. In ovaries from *P{UASH-Ovhts::GFP}, MTD-Gal4* flies, Ovhts::GFP was on RCs in all stages of oogenesis (Fig. 7A). However, in ovaries from *P{UASH-Ovhts::GFP}; P{bam-Gal4}*, Ovhts::GFP was only in the germaria, and on rare occasions on RCs in stage 2 egg chambers (Fig. 7B). Thus, continuous expression of Ovhts-RC protein is needed to maintain localization to RCs.

Ovhts-RC is necessary for actin localization to ring canals

The continual expression and localization of Ovhts-RC throughout oogenesis indicates that it may be important for actin maintenance at the RC. To test this possibility, we expressed *P{UASH-Ovhts::GFP}* *in hts$^{\Delta G}$* flies using the *P{nos-GAL4}* driver whose expression is high in the germarium, low during stages 2-6, and then high again starting approximately at stage 7. This allowed rescue of RCs when they form in the germaria of mutants, followed by about a day where little or no new Ovhts protein is produced. As expected Ovhts::GFP was present on RCs in the germaria, absent in mid-stage egg chambers, and present again in later egg chambers within the same ovariole (Fig. 7C). Both the amount of F-actin and its organization at RCs mirrored the presence of Ovhts::GFP (Fig. 7C′). When Ovhts::GFP was present, RCs appeared wild type. In egg chambers lacking Ovhts::GFP, there were no clear F-actin-containing RC rims. There were, however, actin-rich areas that may be disintegrating RC rims (Fig. 7C′,D). Thus, continued expression of Ovhts is needed for the recruitment and/or maintenance of F-actin on RCs.

DISCUSSION

The *Drosophila hts* gene is known to play key roles in early oogenesis (Lin et al., 1994; Yue and Spradling, 1992); yet its exact functions have not been characterized completely. We provide evidence that Ovhts is made as a full-length precursor protein that is cleaved, allowing the N-terminal Adducin-like domain (Ovhts-Fus) to localize to the fusome and the C-terminal unique domain (Ovhts-RC) to localize to RCs. Ovhts-Fus is stable only in proliferating cells whereas Ovhts-RC is present only after mitosis is complete, which further emphasizes the importance of post-translational regulation for this protein. We created an uncleavable form of the protein and show that it can dominantly delay early egg chamber development. Finally, expression of transgenes at specific developmental time points shows that Ovhts-RC is necessary for F-actin localization to RCs during all stages of oogenesis.

Ovhts is a polyprotein whose products are differentially maintained

Our data demonstrate that Ovhts is made as a full-length precursor protein, which is cleaved to form two mature proteins with separate functions. The detection of cleavage products by western blotting and the differential localization of the cleaved proteins in egg chambers provide strong support for this conclusion. We have ruled

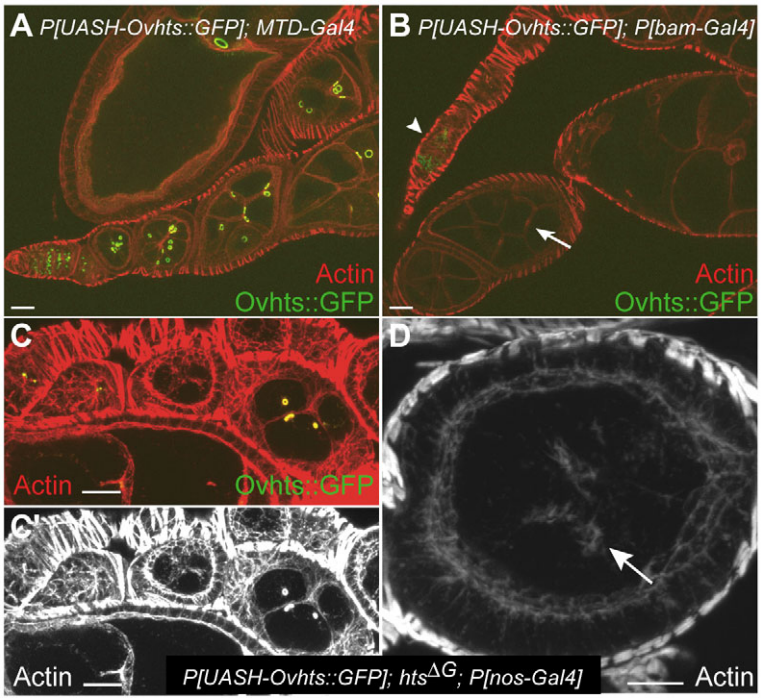

Fig. 7. Continuous Ovhts expression is necessary for Ovhts-RC and actin localization to RCs. (**A**) Expression of *P{UASH-Ovhts::GFP}* with *MTD-Gal4* results in expression of Ovhts::GFP during all stages of oogenesis and continuous localization to RCs. (**B**) Expression of *P{UASH-Ovhts-GFP}* with the *P{bam-GAL4}* driver in wild-type ovaries shows expression and localization of Ovhts::GFP to RCs in the germaria and faintly in stage 2 (arrowhead). There is no Ovhts::GFP protein on RCs at later stages that are actin-positive (arrow). (**C-D**) Expression of *P{UASH-Ovhts::GFP}* in $hts^{\Delta G}$ with *P{nos-Gal4}. The P{nos-Gal4}* driver produces Ovhts::GFP expression (green) in early and late stages, but no expression in intermediate stages. Staining with Rhodamine-conjugated phalloidin shows that F-actin localization to RCs mirrors Ovhts::GFP expression. (D) Higher magnification of an intermediate stage egg chamber that is not expressing Ovhts::GFP. F-actin is in clumps that may be degenerating RCs. Scale bars: A,B, 20 μm; C,D, 10 μm.

out the alternative mechanism of producing the downstream protein, Ovhts-RC, by the use of an internal ribosomal entry site (IRES). There is no evidence of an IRES in the sequence of the *ovhts* mRNA. Furthermore, both of the new *hts* alleles described in this paper have point mutations 5′ to any potential IRES for Ovhts-RC translation, yet neither mutant produces Ovhts-RC.

Endoproteolytic cleavage is a common method of protein regulation. A number of proteins are made as inactive pro-proteins that are activated by cleavage. In these cases part of the protein is removed and degraded to allow the now mature protein to function. In contrast, both mature Ovhts proteins are functional after cleavage, making Ovhts more analogous to a polyprotein, which are translated as a single polypeptide and subsequently cleaved into different proteins. Two types of polyproteins have been extensively described: those of some RNA viruses and prohormones such as vasopressin (for a review, see Luke and Ryan, 2001). Viral polyproteins are translation products of whole viral genomes, including both replication enzymes and structural proteins (Stanway et al., 2000). Viral polyproteins undergo a number of different cleavages, some of which use polyprotein-encoded proteases, and some of which occur through autoproteolysis (Palmenberg, 1990). The Vasopressin polyprotein includes vasopressin, neurophysin (NP) and a third protein of unknown function. The products of the Vasopressin polyprotein are released by at least two different uncharacterized cellular endoproteases (Acher and Chauvet, 1988).

The Ovhts polyprotein is likely to be cleaved by an endoprotease rather than undergoing autoproteolysis. The active site for autoproteolysis contains one of three amino acids: Ser, Thr and Cys (Perler et al., 1997). The Ovhts cleavage region contains one Ser residue, which when mutated to either a Cys or Ala did not affect Ovhts cleavage (L.N.P. and L.C., unpublished). This suggests Ovhts is not cleaved autoproteolytically. Therefore, the more likely mechanism of Ovhts cleavage is through a cellular endoprotease. Because cleavage can occur in S2 cells as well as the ovary, the protease is likely to be widely expressed; however, the cleavage region does not contain any known protease cleavage sites.

The linkage of the RC domain to Adducin may facilitate *Drosophila* ring canal development

The arrangement of the Ovhts polyprotein is conserved in *Drosophila*. Twelve *Drosophila* species have been sequenced to date (Grumbling and Strelets, 2006). We annotated the *hts* homolog from each species, and found that the overall gene organization was highly conserved (L.N.P. and L.C., unpublished). In all cases, an exon encoding the novel RC domain was similarly positioned near the 3′ end of the gene. Whereas the Adducin domains are greater than 90% identical, the RC domain is only 43% conserved, indicating that it is diverging much more quickly. The RC domain is not present as an independent gene or as part of the *Adducin* gene of other sequenced insect genomes. Thus, it appears that the Ovhts polyprotein was acquired after the divergence of *Drosophila*.

To explain the existence of an Ovhts polyprotein, we propose a model in which the Ovhts polyprotein functions to provide Ovhts-RC at a critical time and place and in sufficient abundance through its linkage to Ovhts-Fus. At all stages of oogenesis the Ovhts full-length precursor is translated and cleaved. In Region 1 of germaria, the Ovhts-Fus product is maintained and localizes to the fusome. However, the Ovhts-RC product cannot be detected in Region 1, indicating that it is rapidly degraded. Starting in Region 2, the Ovhts-RC domain begins to be maintained and localizes to RCs. This coincides with the completion of mitotic cell cycles, and perhaps also the expression of an Ovhts-RC binding partner required for its stability. Ovhts-RC first appears as puncta around the fusome, suggesting that the cleavage of the polyprotein may take place on or near the fusome. Another possibility is that the Ovhts-RC stabilizing factor is itself associated with the fusome. In either case the Ovhts-RC protein is in close proximity to the arrested cleavage furrows where it is needed to initiate RC development.

An open question is why the transgenes we tested do not restore the fusome in *hts* mutants. We ruled out the effects of secondary mutations by using several combinations of *hts* alleles from different backgrounds (i.e. *P*-element insertions, point mutations and deficiencies), and we deem the existence of another protein isoform

unlikely as we performed 3′ RACE and found only the known transcripts. The most likely possibility is that expression of *ovhts* is required at a specific time early in development for the formation and stability of spectrosomes in germline progenitor or stem cells, and we did not recapitulate this expression pattern in the rescue experiments.

Ovhts cleavage facilitates a smooth transition of fusome to ring canals

The presence of the uncleavable Ovhts-Δ3::GFP protein affects the transition from a fusome to an RC, even though it localizes to both structures. Therefore, cleavage is not required for localization but is necessary for the full function of the Ovhts polyprotein products. The expression of uncleavable Ovhts-Δ3::GFP causes the aberrant persistence of fusome material in RCs. Strikingly, the fusome material includes endogenous wild-type Ovhts-Fus protein that has failed to degrade. As it is known that mammalian Adducins function as heterotetramers, a direct interaction of wild-type Ovhts-Fus or ShAdd with Ovhts-Δ3::GFP may occur and result in pulling fusome material into the lumen of the RCs. This hypothesis is supported by the fact that RCs in *hts* mutants that lack wild-type Ovhts-Fus are rescued by Ovhts-Δ3::GFP and do not have fusome plugs or misshapen rims. The dominant-negative effect of Ovhts-Δ3::GFP on wild-type RCs disappears by stage 3, suggesting that uncleaved Ovhts is tolerated in egg chambers that have degraded the fusome. Therefore, it is the transition from fusomes to RCs that is particularly sensitive to the presence of uncleaved Ovhts.

Ovhts-RC is required throughout oogenesis

RC formation has been described as a multi-step process beginning with the arrested cleavage furrow followed by the recruitment of Filamin, Ovhts-RC and F-actin, and completed with the addition of other actin binding proteins (Hudson and Cooley, 2002). We found that the recruitment of Ovhts-GFP to RCs is delayed in *hts* mutants lacking a fusome, supporting a role for the fusome (and possibly prelocalization of Ovhts-RC) in the initiation of RC development. Furthermore, without continual expression of Ovhts-RC (Fig. 7), F-actin was lost from the inner rim of RCs. As the F-actin cytoskeleton of RCs is highly dynamic (Kelso et al., 2002), the absence of Ovhts-RC in *hts* mutant RCs may tip the balance of actin dynamics toward depolymerization, resulting in the depletion of RC F-actin. Thus, Ovhts-RC is needed continuously either to stabilize polymerized actin or to promote actin polymerization.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/4/703/DC1

References

Acher, R. and Chauvet, J. (1988). Structure, processing and evolution of the neurohypophysial hormone-neurophysin precursors. *Biochimie* **70**, 1197-1207.
Ashburner, M. (1989). *Drosophila: A Laboratory Handbook*. New York: Cold Spring Harbor Laboratory Press.
Chen, D. and McKearin, D. M. (2003). A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. *Development* **130**, 1159-1170.
de Cuevas, M. and Spradling, A. C. (1998). Morphogenesis of the Drosophila fusome and its implications for oocyte specification. *Development* **125**, 2781-2789.
de Cuevas, M., Lee, J. K. and Spradling, A. C. (1996). alpha-spectrin is required for germline cell division and differentiation in the Drosophila ovary. *Development* **122**, 3959-3968.
de Cuevas, M., Lilly, M. A. and Spradling, A. C. (1997). Germline cyst formation in Drosophila. *Annu. Rev. Genet.* **31**, 405-428.
Ding, D., Parkhurst, S. M. and Lipshitz, H. D. (1993). Different genetic requirements for anterior RNA localization revealed by the distribution of Adducin-like transcripts during Drosophila oogenesis. *Proc. Natl. Acad. Sci. USA* **90**, 2512-2516.
Dubielecka, P. M., Stebelska, K., Jazwiec, B. and Sikorski, A. F. (2003). Cystocyte and lymphocyte derived fusomes/spectrosomes: analogies and differences: a mini-review. *Cell. Mol. Biol. Lett.* **8**, 221-229.
Grumbling, G. and Strelets, V. (2006). FlyBase: anatomical data, images and queries. *Nucleic Acids Res.* **34**, D484-D488.
Hudson, A. M. and Cooley, L. (2002). Understanding the function of actin-binding proteins through genetic analysis of Drosophila oogenesis. *Annu. Rev. Genet.* **36**, 455-488.
Huynh, J. R. and St Johnston, D. (2004). The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte. *Curr. Biol.* **14**, R438-R449.
Kelso, R. J., Hudson, A. M. and Cooley, L. (2002). Drosophila Kelch regulates actin organization via Src64-dependent tyrosine phosphorylation. *J. Cell Biol.* **156**, 703-713.
King, R. C. (1970). *Ovarian Development in Drosophila melanogaster*. New York, London: Academic Press.
Koundakjian, E. J., Cowan, D. M., Hardy, R. W. and Becker, A. H. (2004). The Zuker collection: a resource for the analysis of autosomal gene function in Drosophila melanogaster. *Genetics* **167**, 203-206.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Lin, H., Yue, L. and Spradling, A. C. (1994). The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120**, 947-956.
Lindsely, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. Burlington, MA: Elsevier Science and Technology Books.
Luke, G. A. and Ryan, M. D. (2001). Translating the message. *Biologist London* **48**, 79-82.
Matova, N. and Cooley, L. (2001). Comparative aspects of animal oogenesis. *Dev. Biol.* **231**, 291-320.
Matsuoka, Y., Li, X. and Bennett, V. (1998). Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons. *J. Cell Biol.* **142**, 485-497.
Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K. and Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**, 87-90.
Palmenberg, A. C. (1990). Proteolytic processing of picornaviral polyprotein. *Annu. Rev. Microbiol.* **44**, 603-623.
Pepling, M. E., de Cuevas, M. and Spradling, A. C. (1999). Germline cysts: a conserved phase of germ cell development? *Trends Cell Biol.* **9**, 257-262.
Perler, F. B., Xu, M. Q. and Paulus, H. (1997). Protein splicing and autoproteolysis mechanisms. *Curr. Opin. Chem. Biol.* **1**, 292-299.
Rizzo, M. A., Springer, G. H., Granada, B. and Piston, D. W. (2004). An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* **22**, 445-449.
Robinson, D. N. and Cooley, L. (1997). Examination of the function of two kelch proteins generated by stop codon suppression. *Development* **124**, 1405-1417.
Robinson, D. N., Cant, K. and Cooley, L. (1994). Morphogenesis of Drosophila ovarian ring canals. *Development* **120**, 2015-2025.
Robinson, D. N., Smith-Leiker, T. A., Sokol, N. S., Hudson, A. M. and Cooley, L. (1997). Formation of the Drosophila ovarian ring canal inner rim depends on cheerio. *Genetics* **145**, 1063-1072.
Roper, K. and Brown, N. H. (2004). A spectraplakin is enriched on the fusome and organizes microtubules during oocyte specification in Drosophila. *Curr. Biol.* **14**, 99-110.
Rorth, P. (1998). Gal4 in the Drosophila female germline. *Mech. Dev.* **78**, 113-118.
Snapp, E. L., Iida, T., Frescas, D., Lippincott-Schwartz, J. and Lilly, M. A. (2004). The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts. *Mol. Biol. Cell* **15**, 4512-4521.
Sokol, N. S. and Cooley, L. (2003). Drosophila filamin is required for follicle cell motility during oogenesis. *Dev. Biol.* **260**, 260-272.
Stanway, G., Joki-Korpela, P. and Hyypia, T. (2000). Human parechoviruses – biology and clinical significance. *Rev. Med. Virol.* **10**, 57-69.
Tracey, W. D., Jr, Ning, X., Klingler, M., Kramer, S. G. and Gergen, J. P. (2000). Quantitative analysis of gene function in the Drosophila embryo. *Genetics* **154**, 273-284.
Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr. Biol.* **8**, 243-246.

Wawersik, M. and Van Doren, M. (2005). nanos is required for formation of the spectrosome, a germ cell-specific organelle. *Dev. Dyn.* **234**, 22-27.
Whittaker, K. L., Ding, D., Fisher, W. W. and Lipshitz, H. D. (1999). Different 3′ untranslated regions target alternatively processed hu-li tai shao (hts) transcripts to distinct cytoplasmic locations during Drosophila oogenesis. *J. Cell Sci.* **112**, 3385-3398.
Yue, L. and Spradling, A. C. (1992). hu-li tai shao, a gene required for ring canal formation during Drosophila oogenesis, encodes a homolog of adducin. *Genes Dev.* **6**, 2443-2454.
Zaccai, M. and Lipshitz, H. D. (1996). Differential distributions of two adducin-like protein isoforms in the Drosophila ovary and early embryo. *Zygote* **4**, 159-166.