Formation of the Drosophila Ovarian Ring Canal Inner Rim Depends on *cheerio*

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ABSTRACT

In Drosophila oogenesis, the development of a mature oocyte depends on having properly developed ring canals that allow cytoplasm transport from the nurse cells to the oocyte. Ring canal assembly is a step-wise process that transforms an arrested cleavage furrow into a stable intercellular bridge by the addition of several proteins. Here we describe a new gene we named *cheerio* that provides a critical function for ring canal assembly. Mutants in *cheerio* fail to localize ring canal inner rim proteins including filamentous actin, the ring canal-associated products from the *hu-li tai shao* (*hts*) gene, and kelch. Since hts and kelch are present but unlocalized in *cheerio* mutant cells, *cheerio* is likely to function upstream from each of them. Examination of mutants in *cheerio* places it in the pathway of ring canal assembly between cleavage furrow arrest and localization of hts and actin filaments. Furthermore, this mutant reveals that the inner rim cytoskeleton is required for expansion of the ring canal opening and for plasma membrane stabilization.

GERM cells of many species, from insects to mammals, develop as syncytia where sister cells are interconnected by stable intercellular bridges. In Drosophila oogenesis, these stable intercellular bridges, called ring canals, provide the passageway for cytoplasm transport between specialized nurse cells and the oocyte. Transport of cytoplasmic contents requires several cytoskeletal components, including the cytoskeleton of the ring canals (reviewed in COOLEY and THEURKAUF 1994). We are interested in how ring canals are formed and in their role in the development of a mature egg.

In the anterior-most region of the Drosophila ovary called the germarium, a stem cell divides producing a daughter stem cell and a daughter cystoblast (for a review of oogenesis, see SPRADLING 1993). The cystoblast undergoes four mitotic divisions characterized by incomplete cytokinesis generating a syncytium of sixteen germline cells: 15 nurse cells and one oocyte. The nurse cells contribute mRNAs, proteins and organelles to the oocyte beginning as soon as the fourth mitosis is complete and finishing several days later with a final rapid phase of transport in which the remaining nurse cell cytoplasm is "dumped" into the oocyte. Ring canals develop as the germline syncytium develops. The cleavage furrows arrest at a diameter of 0.5–1.0 μm. The plasma membrane of the arrested cleavage furrows thickens forming the outer rim (MAHOWALD 1971). After the fourth mitosis, a proteinaceous inner rim forms subcortically to the outer rim (KOCH and KING 1969; MAHOWALD 1971). Throughout oogenesis, the ring canals increase in diameter reaching 10 μm by the rapid phase of transport.

Several proteins have been identified so far that are involved in ring canal assembly. Each is localized to ring canals at a specific time in development, suggesting that each has a unique function in ring canal morphogenesis. The first protein, anillin (FIELD and ALBERTS 1995), can be detected on cleavage furrows. Anillin has been shown to associate with the cleavage furrows of many cell types in Drosophila including the cellularization front in blastoderm embryos, dividing cells in older embryos and in dividing Schneider cells, a Drosophila embryonic cell line. Anillin cycles between the nucleus in interphase and the cleavage furrow in anaphase of mitosis. This behavior, coupled with its actin filament binding activity, indicates that anillin may be involved in the signal that initiates organization of the acto-myosin contractile ring. During the final mitotic division, a second protein, which immunoreacts with antiphosphotyrosine antibodies, can be detected on the outer rim of the ring canals (ROBINSON *et al.* 1994). The immunoreactivity of ring canals with antiphosphotyrosine antibodies persists throughout oogenesis. The identity of this protein(s) remains to be elucidated.

Upon completion of the four mitoses, two new proteins are localized simultaneously to the ring canals: one product of the *hu-li tai shao* (*hts*) gene and filamentous actin (WARN *et al.* 1985; ROBINSON *et al.* 1994). Other products from the *hts* gene are localized elsewhere in the ovary (LIN *et al.* 1994; ROBINSON *et al.* 1994). In this paper, hts-RC refers to the *hts* product that is found on ring canals. The localization of actin

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depends on hts-RC since mutants that lack this protein fail to localize actin to the ring canal (YUE and SPRADLING 1992; ROBINSON *et al.* 1994). Electron microscopy has shown that during this phase of ring canal development, an inner rim accumulates subcortically to the ring canal outer rim (KOCH and KING 1969; MAHOWALD 1971); the inner rim contains abundant actin filaments (TILNEY *et al.* 1996). The assignment of at least one phosphotyrosine-containing protein to the outer rim and the hts-RC protein and filamentous actin to the inner rim is supported by the *hts*^{1} mutant that fails to form an inner rim but still has antiphosphotyrosine staining of the ring canal (YUE and SPRADLING 1992; ROBINSON *et al.* 1994).

In the final phase of ring canal assembly, at least one product of the *kelch* gene is localized to the inner rim of ring canals (XUE and COOLEY 1993). Kelch arrives on ring canals several hours after the other known ring canal proteins. Mutants that lack the kelch protein do acquire the phosphotyrosine protein, hts-RC and actin on their ring canals, suggesting *kelch* is not required for initial assembly (ROBINSON *et al.* 1994). However, *kelch* is required during ring canal growth; the actin filaments and hts-RC in the inner rim become disorganized during growth of *kelch* mutant ring canals, extending into the lumen of the ring canal (ROBINSON *et al.* 1994; TILNEY *et al.* 1996). This disorganization partially obstructs the lumen of the canal, resulting in the inability to transport cytoplasm properly and in a female sterile phenotype.

We present in this paper the characterization of a new ring canal mutant called *cheerio*, which functions very early in the assembly of a ring canal. Immunocytochemistry analysis places it in the ring canal pathway between cleavage furrow arrest and formation of the inner rim. Examination of *cheerio* provides further evidence that the function of the ring canal cytoskeleton is to allow growth of the ring canal while maintaining an opening sufficient for the bulk flow of cytoplasm transport.

MATERIALS AND METHODS

Fly strains: Canton S, w^{1118} or *cn;ry* (LINDSLEY and ZIMM 1992) flies were used as wild type in these experiments. The *cher*^{1} allele was generated in a germline transformation experiment performed in the lab of S. ARTAVANIS-TSAKONAS, and the inserted *P* element was mapped to polytene interval 66A (R. RAMOS and S. ARTANVANIS-TSAKONAS, personal communication). B. MARIONI observed that flies homozygous for the *P* insert were female sterile with a dumpless phenotype (personal communication). However, we determined that the *P* element did not map to the female sterile mutation. The *cher*^{2} allele was identified in a new collection of *P*-element-induced female sterile mutations generated in the lab of R. SCOTT HAWLEY. Deficiency strains used in these experiments were obtained from the Bloomington Drosophila Stock Center at Indiana University. Fly stocks were maintained under standard conditions.

Western analysis: Drosophila ovaries were dissected in Drosophila Ringer solution (COOLEY *et al.* 1992) and ground in 1× LAEMMLI's buffer under reducing conditions (LAEMMLI 1970). Protein concentrations of the ovary extracts were measured using the BioRad Protein Assay. Proteins separated by SDS-polyacrylamide gel electrophoresis (LAEMMLI 1970) were transferred to Hybond-ECL nitrocellulose (TOWBIN *et al.* 1979; Amersham). Following transfer, membranes were blocked in Blotto-Tween (5% powdered milk, 0.2% Tween, and PBS) for 2 hr, then incubated in hybridoma cell supernatant (1:10 in Blotto-Tween) for 1 hr to overnight. Membranes were washed four times for 10 min in 0.2% Tween in PBS at room temperature. Membranes were incubated for 30 min at room temperature in goat anti-mouse IgG secondary antibody conjugated to horseradish-peroxidase (Pierce Chemical Co.) in Blotto-Tween (1:10,000). After four washes, signals were detected using ECL Western blotting detection reagents (Amersham) according to manufacturer's specifications.

Immunolocalization and confocal imaging: Whole ovaries were dissected in Drosophila IMADS buffer (SINGLETON and WOODRUFF 1994), fixed in 1% formaldehyde saturated with heptane (COOLEY *et al.* 1992), rinsed three times in PBS and washed three times for 10 min in PBT [0.3% Triton-X-100 (Sigma), 0.5% BSA (Sigma) in PBS] at room temperature. For actin visualization, ovaries were incubated in 1–2 units of rhodamine-conjugated phalloidin (Molecular Probes) for 2 hr at room temperature. For antibody staining, ovaries were incubated in hts amino-terminal antibody, which immunoreacts with the hts fusome protein (hts-F) (LIN *et al.* 1994; ROBINSON *et al.* 1994) (1:1000), hts-RC antibody hybridoma supernatant (ROBINSON *et al.* 1994) (1:1), kelch hybridoma supernatant (XUE and COOLEY 1993) (1:1), or anti-phosphotyrosine antibody (ICN Biochemicals, PY20) (1:1000) diluted in PBT. The ovaries were incubated with rocking for 2 hr at room temperature then overnight at 4°. After washing four times for 15 min in PBT, bound antibodies were detected using a goat anti-mouse IgG secondary antibody conjugated to fluorescein isothiocyanate (Jackson Laboratories). Immunolocalizations were visualized by collecting 1–2 μm optical sections on a laser-scanning confocal microscope (BioRad MRC 600) then optical sections were compiled and displayed using the CoMOS software package (BioRad). A ×25 (0.8 NA), ×40 (1.0 NA) or a ×63 (1.4 NA) objective was used in each case. Images were processed using Adobe Photoshop.

Examination of membrane integrity: Staining of subcortical actin was carried out as described above. For nuclear staining, we dissected and fixed ovaries from 10–20 females per genotype as described for immunocytochemistry. During the third from final wash in PBT, DAPI (Molecular Probes) was added to the PBT at 1 μg/ml. The specimens were washed two more times and were examined and photographed using a Zeiss Axiophot with a ×40 objective (1.3 NA). Stage 11 and older egg chambers were scored for the presence of and the number of nurse cell nuclei in the oocyte.

RESULTS

***cheerio* mutant egg chambers are dumpless:** Females homozygous for the *cher*^{1} allele were sterile, while females homozygous for *cher*^{2} were weakly fertile. Both produced egg chambers with impaired cytoplasm transport from the nurse cells into the oocyte. *cheerio* mutant egg chambers appeared defective early in their development. The oocyte in early *cheerio* mutant egg chambers was not as large as it was in wild type, indicating that the initial slow phase of cytoplasm transport was defective. The subsequent rapid phase of cytoplasm transport

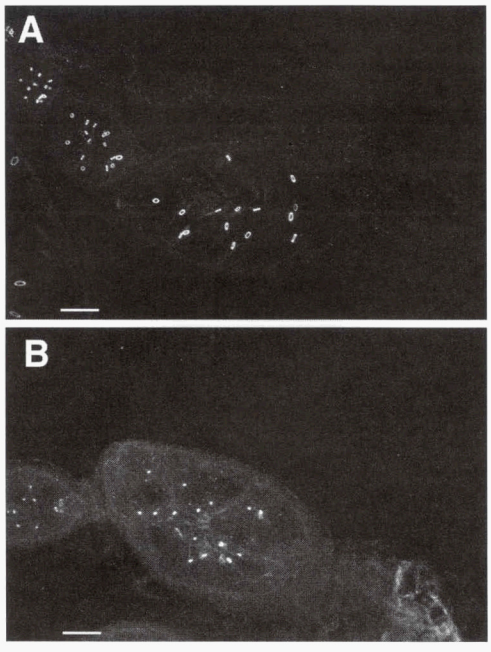

FIGURE 1.—Ring canals are defective in *cher*^{1} mutants. (A) Wild-type egg chambers immunostained with antiphosphotyrosine antibodies have robust ring canal staining. (B) *cher*^{1} mutant egg chambers have antiphosphotyrosine stained ring canals but the ring canals are smaller than wild type. All scale bars are 15 μm.

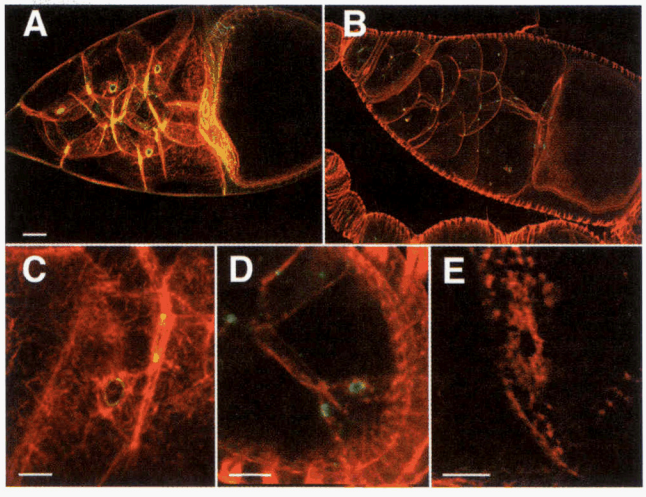

FIGURE 2.—*cher*^{1} mutants fail to form ring canal inner rims. (A–D) Specimens were stained with rhodamine-conjugated phalloidin (red) to identify actin filaments and with antiphosphotyrosine antibodies (green). Yellow indicates colocalization of both proteins. (A) In this wild-type stage 10 egg chamber, the two proteins colocalize in ring canals (yellow). (B) A *cher*^{1} stage 10 egg chamber has subcortical actin in the nurse cells and the oocyte but only phosphotyrosine epitopes in the ring canal. (C) A high magnification view of a wild-type ring canal shows that the two proteins colocalize in the inner rim of the ring canal. (D) A high magnification view of *cher*^{1} ring canals shows that only the phosphotyrosine protein is found associating with the ring canals. (E) A high magnification view of a *cher*^{1} ring canal labeled only with rhodamine-conjugated phalloidin shows that there is subcortical actin near the ring canal but there is no defined actin inner rim. Scale bars are 10 μm.

was also affected in these mutants, causing the oocyte to remain small and the nurse cells to persist at the anterior of the oocyte (not shown), a "dumpless" phenotype. In fact, the *cheerio* egg chamber morphology was virtually indistinguishable from the *kelch* egg chamber morphology (XUE and COOLEY 1993). Fertility in *cheerio* (and *kelch*) mutant males was not affected. Extensive analysis indicated no differences between egg chambers from *cher*^{1} homozygotes and those from flies heterozygous for *cher*^{1} and a deficiency that uncovers the *cheerio* locus [*Df(3R)C4*]. *cher*^{2} homozygous egg chambers had a less severe phenotype than *cher*^{1} homozygotes. However, egg chambers from flies heterozygous for *cher*^{2} and *Df(3R)C4* or *cher*^{1} had an intermediate phenotype. We conclude that *cher*^{1} is a genetic null allele and *cher*^{2} is a hypomorphic allele.

***cheerio* mutant egg chambers have defective ring canals:** Since the *kelch* phenotype results from defective ring canals (ROBINSON *et al.* 1994; TILNEY *et al.* 1996), we examined the ring canals in *cheerio* mutant egg chambers using immunofluorescence. Immunostaining of ovaries with antiphosphotyrosine antibodies showed that ring canals in *cher*^{1} mutants accumulated phosphotyrosine protein (Figure 1B), but these ring canals did not grow to the appropriate size. Ring canals in *cher*^{1} mutant egg chambers were ~4 μm in diameter at stage 9, while wild-type ring canals reached ~8 μm in diameter at the same stage (Figure 1, A and B).

To examine the inner rims of *cher*^{1} mutant ring canals, we performed double-labeling experiments with antiphosphotyrosine antibodies and rhodamine-conjugated phalloidin. All of the ring canals appeared yellow in wild-type egg chambers, indicating that phosphotyrosine (green) and actin (red) were both present (Figure 2, A and C). In *cher*^{1} mutant egg chambers, however, none of the ring canals appeared yellow (Figure 2, B and D). Small ring canals were seen at the nurse cell-oocyte boundary, but these appeared green, indicating that only phosphotyrosine was present. Small fragments of stained material were seen associated with the nurse cell membranes or in the cytoplasm (Figure 2B). Some fragments appeared yellow, demonstrating that both phosphotyrosine and actin were present, while other fragments appeared green (indicating phosphotyrosine alone) or red (indicating actin alone). Close examination of *cher*^{1} mutant ring canals showed that subcortical actin surrounded the ring canal, but no actin was seen on the ring itself (Figure 2E). Subcortical actin appeared unaffected at all stages in *cher*^{1} mutant egg chambers (Figure 2B). Cytoplasmic actin bundles were present at stage 11 in *cher*^{1} mutant egg chambers as well as in wild type (not shown). These results suggested that the lack of actin on ring canals in *cher*^{1} mutants was not due to a general disruption of the actin cytoskeleton, but instead to a ring canal-specific defect.

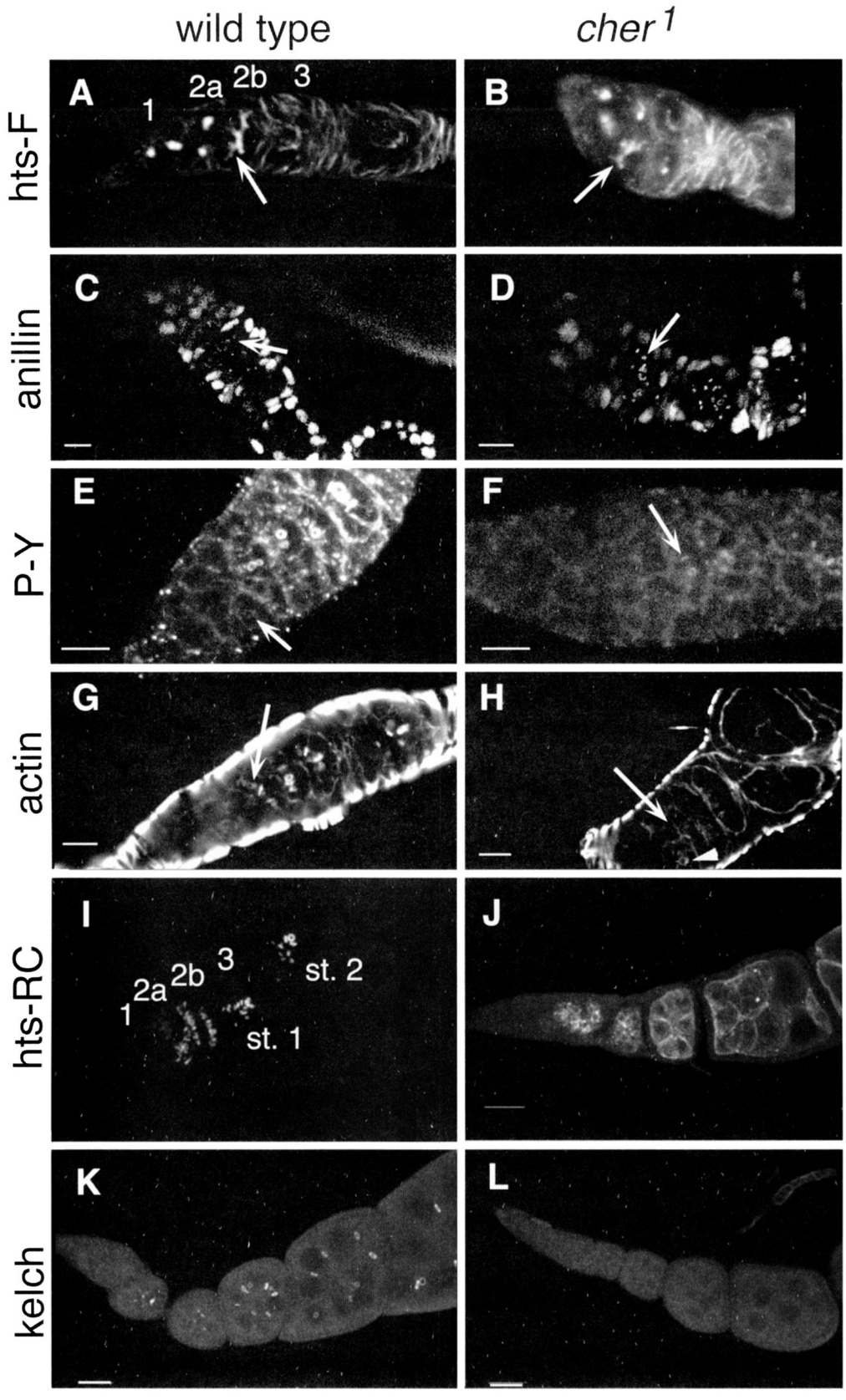

FIGURE 3.—*cher*^{1} mutants cannot localize inner rim components. Antibodies that immunoreact with the hts fusome protein (hts-F) were used to assess the formation of fusomes. Both wild-type (A) and *cher*^{1} mutant (B) germaria had normal fusomes early, and these developed into normal branched polyfusomes (A and B, arrows). Antianillin antibodies were used to assess the formation of arrested cleavage furrows. Both wild type (C) and *cher*^{1} (D) had anillin staining of arrested cleavage furrows (arrows). Anillin staining of ring canals was easily detected by region 2a. However, sometimes it was possible to detect anillin on cleavage

Ring canals in *cheerio*^{1} mutants do not accumulate inner rims: The localization of phosphotyrosine protein to the ring canals in *cher*^{1} mutants suggested that the earliest events of ring canal formation proceeded normally. We examined this in more detail by immunostaining with antibodies specific for the assembly phase of ring canal biogenesis. The fusome is a membranous organelle that is required for the correct number of germline divisions in the developing germline syncytium and is present during initial ring canal assembly. Branches of the fusome link the germ cells through their nascent ring canals during the original four mitotic divisions (Lin *et al.* 1994). One of the products of the hts gene encodes a protein that localizes to the fusome (Lin *et al.* 1994; Robinson *et al.* 1994). Immunostaining with anti-hts fusome (hts-F) antibodies showed normal fusomes in *cher*^{1} mutants; the fusome could be seen very early in the germarium and perdured until region 2A (Figure 3, A and B). Cleavage furrow arrest also appeared to be normal in *cher*^{1} mutant egg chambers. Immunostaining with antianillin antibodies demonstrated that anillin was present on arrested cleavage furrows in region 2A of the germaria in both wild-type and *cher*^{1} mutants (Figure 3, C and D). In fact, anillin staining was more intense on *cher*^{1} furrows than on wild-type furrows. The phosphotyrosine protein appeared on ring canals early in the germarium in both wild-type and *cher*^{1} mutant ring canals, although the staining on *cher*^{1} mutant rings was less intense (Figure 3, E and F). Staining with rhodamine-conjugated phalloidin revealed actin on the arrested cleavage furrows very early in the germarium in both wild-type and *cher*^{1} mutants (Figure 3H, arrowhead). This probably represents contractile ring actin.

We have demonstrated that actin was not present on the inner rims of *cher*^{1} ring canals in later staged egg chambers (Figure 2). Similarly in young egg chambers stained with rhodamine-conjugated phalloidin, robust rings of inner rim actin, began to accumulate on ring canals in region 2A of the germarium in wild-type cells (Figure 3G, arrow) but was never seen on *cher*^{1} mutant ring canals (Figure 3H, arrow). The same results were seen for the inner rim proteins, hts-RC and kelch. Antibodies specific for hts-RC stained wild-type egg chambers beginning in region 2A of the germarium (Figure 3I). Hts-RC proteins were not localized to *cher*^{1} mutant ring canals, although hts-RC antibodies did stain nurse cell membranes of these mutant egg chambers (Figure 3J). Immunostaining with antikelch antibodies showed that kelch protein was not present on *cher*^{1} mutant ring canals, although it can be seen on wild-type ring canals in region 3 of the germarium (Figure 3, K and L). Taken together, these results suggested that the inner rim did not form in *cher*^{1} mutant egg chambers.

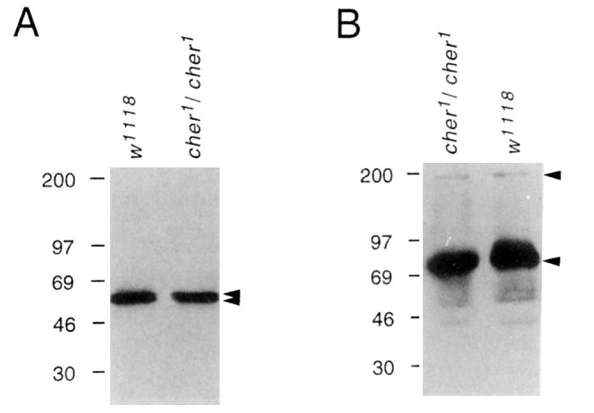

Figure 4.—Steady state levels of hts-RC and kelch are unchanged in *cher*^{1} mutants. (A). Western analysis of the level of hts-RC in w^{1118} and *cher*^{1} mutant ovary protein extracts revealed that the protein levels are similar. The relative mobility of the 60×10^3 D hts-RC proteins (arrowheads) is also unchanged. (B) Western analysis of kelch proteins revealed similar levels of the 80×10^3 and 170×10^3 D kelch species (arrowheads) in both w^{1118} and *cher*^{1} ovary protein extracts. Proteins smaller than 80×10^3 are degradation products. Protein concentrations were measured using the BioRad protein assay reagent and equal amounts of proteins were loaded in each case.

Unlocalized hts-RC and kelch proteins accumulate in *cheerio* mutant egg chambers: The hts-RC proteins at the nurse cell membranes in *cher*^{1} mutants suggested that the hts-RC was present but could not localize to ring canals. Western immunoblot analysis of ovary protein extracts from wild-type and *cher*^{1} mutants also revealed the accumulation of hts-RC in *cher*^{1} mutants (Figure 4A). A doublet of proteins with a relative molecular mass of 60×10^3 D was seen in both wild-type and *cher*^{1} mutant extracts. Similar analysis using antikelch antibodies demonstrated that two kelch proteins with relative molecular masses of 80×10^3 and 170×10^3 D were present in both wild-type and *cher*^{1} mutant protein extracts (Figure 4B). This showed that the *cheerio* mutation did not affect the production of known inner rim proteins but instead interfered with localization to the ring canals.

furrows earlier in region 1. Antiphosphotyrosine staining of wild type (E) and *cher*^{1} (F) germaria showed ring canal staining. Phosphotyrosine epitopes could be detected in wild type (E, arrow) early in region 1 in a place where the third mitosis should be complete. Phosphotyrosine epitopes were not easily detected until slightly later in region 2b in *cher*^{1} (F, arrow). In wild type, actin filaments began to accumulate by region 2a (G, arrow), whereas no actin was seen accumulating in *cher*^{1} mutant germaria (H, arrow). Actin could be detected on the arrested cleavage furrows in *cher*^{1} germaria (H, arrowhead). Hts-RC localized to ring canals by region 2a in a wild type (I) germarium, but in a *cher*^{1} (J) germarium, the protein did not localize to ring canals. Instead, it localized to the subcortical cytoskeleton of the germline cells. Kelch was localized to ring canals in wild-type (K) egg chambers beginning at late region 3 or stage 1. No localization of kelch protein to ring canals was observed in *cher*^{1} mutant egg chambers (L). The germarial region identification and egg chamber staging in A and I concur with the standard nomenclature. Scale bars are 10 μm.

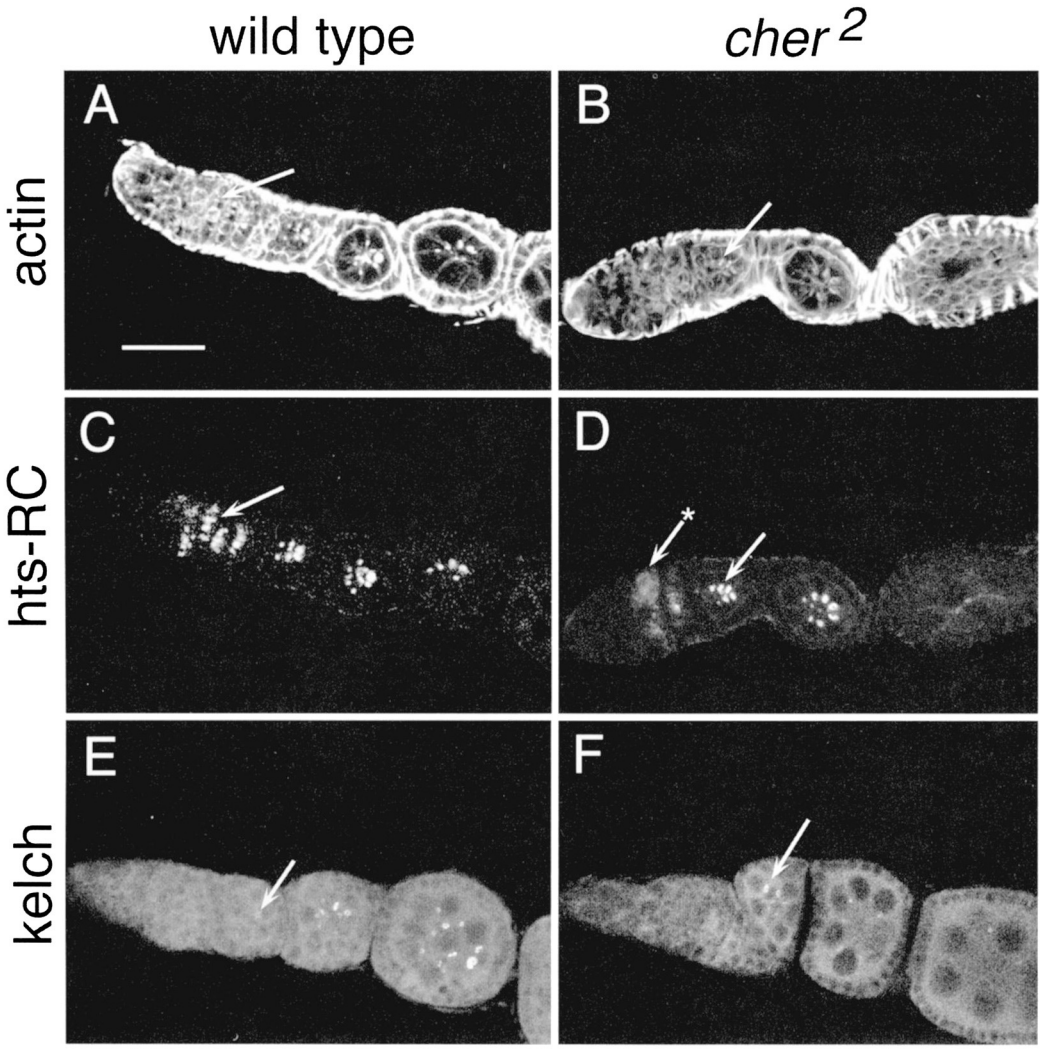

FIGURE 5.—*cher*^{2} mutants form ring canals that are unstable. Double-labeling of wild type egg chambers showed that inner rim actin (A) and hts-RC (C) colocalized beginning in region 2a and had accumulated by region 2b (A, C, arrows). In *cher*^{2} mutant germaria, inner rim actin (B, arrow) also accumulated on the inner rims at the same time as hts-RC (D, arrow). However, the accumulation in ring canals began slightly later than wild type as evidenced by the localization of hts-RC to the subcortical cytoskeleton in region 2a (D, arrow with asterisk). By stage 4 in *cher*^{2}, most of the ring canal localization of actin (B) and hts-RC (D) is gone. Kelch is localized beginning in region 3 in wild type (E, arrow) and accumulates over the next few stages. In *cher*^{2}, kelch begins to be localized slightly later (F, arrow) but soon disappears from ring canals. Scale bar is 15 μm.

Ring canal inner rims in a weaker *cheerio* allele are formed but not maintained: Immunostaining of *cher*^{2} germaria with antiphosphotyrosine antibodies demonstrated that phosphotyrosine epitopes were present on ring canals throughout oogenesis as in *cher*^{1} (not shown). Double-labeling experiments with rhodamine-conjugated phalloidin and anti-hts-RC antibody demonstrated that the inner rim proteins actin and hts-RC initially accumulated on early *cher*^{2} ring canals (Figure 5, B and D). The accumulation of actin and hts-RC appeared to be slightly delayed compared with wild type. In wild type, hts-RC and actin first appeared on ring canals in region 2A (Figure 5, A and C, arrows); hts-RC did not stain membranes (Figure 5C). In *cher*^{2} mutants, however, faint hts-RC ring canal staining and significant membrane staining were seen in region 2A (Figure 5D, arrow with asterisk). This membrane staining could correspond to the membrane accumulation of hts-RC in *cher*^{1} mutants (see Figure 3J). Although actin and hts-RC staining appeared robust in regions 2B and 3 of the germaria (Figure 5, B and D, arrows), these proteins were not maintained on *cher*^{2} ring canals. By stage 4, these proteins were absent from the majority of the ring canals in *cher*^{2} egg chambers. The few ring canals that were stained in later stages by hts-RC and actin were stained faintly (not shown). Antikelch antibodies gave a similar staining pattern. Kelch staining was first seen on wild-type ring canals in stage 1 (Figure 5E, arrow) and continued throughout oogenesis. Kelch was readily detectable on *cher*^{2} ring canals by stage 2 (Figure 5F, arrow). As with hts-RC and actin, kelch disappeared from *cher*^{2} ring canals by stage 4, and only faint staining on a small number of rings was seen in later egg chambers (Figure 5F).

Germ cell plasma membranes lose integrity in *cheerio*^{2} mutants: We found that the plasma membranes of the nurse cells and oocytes in *cheerio* egg chambers were destabilized (Figure 6). This often resulted in the transport of nurse cell nuclei into the oocytes of *cheerio* mutant post-stage 10 egg chambers (Figure 6, Table 1). Thirty-one percent of *cher*^{2}/*cher*^{2} homozygous females had nurse cell nuclei in the oocyte. On average, approximately four nuclei per egg chamber were in the oocyte when the egg chamber was affected. Females with the genotypes, *cher*^{2}/*Df(3R)C4* and *cher*^{2}/*cher*^{1}, also had nurse cell nuclei in the oocyte (20 and 12%, respec-

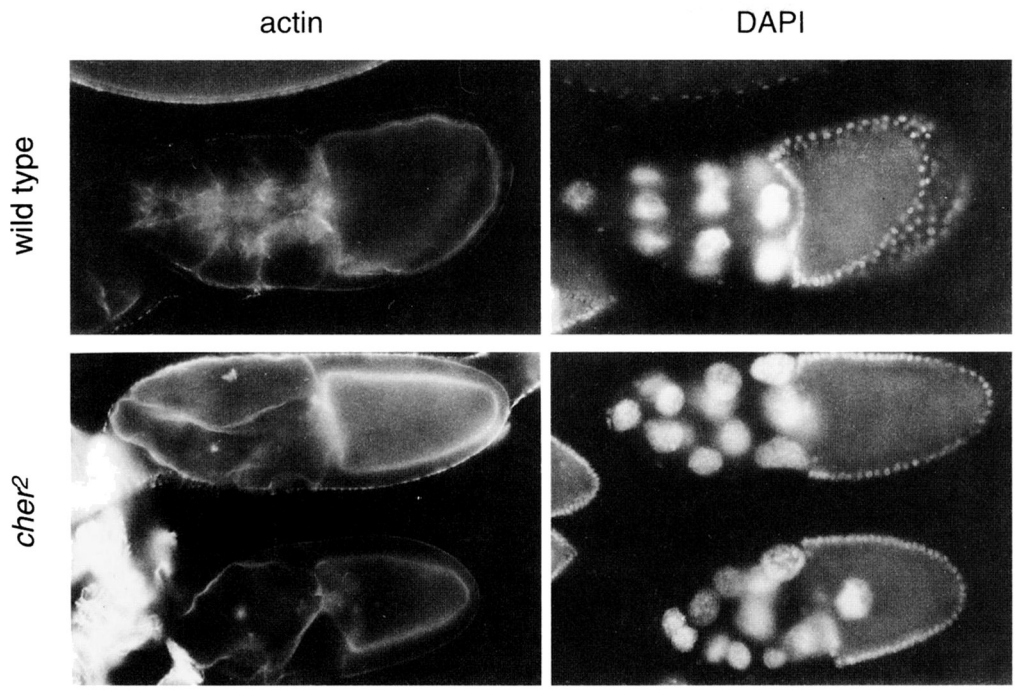

FIGURE 6.—Germ cell plasma membrane destabilization in *cheerio*. Wild-type egg chambers stained with rhodamine-phalloidin to identify actin have intact germ-cell membranes. DAPI staining reveals that each nurse cell contains an appropriately positioned nucleus. In $cher^2$ egg chambers stained with rhodamine-phalloidin, plasma membranes have degenerated allowing the cells to fuse. DAPI staining reveals multinucleate nurse cells and some egg chambers have nurse cell nuclei inappropriately transported into the oocytes.

tively), but only two nuclei were transported into the oocyte on average. This defect was not seen with the $cher^1$ allele. Females homozygous for $cher^1$ had no nuclei in 57 oocytes examined, and only one affected egg chamber out of 59 was seen for the genotype $cher^1/Df(3R)C4$. In general, approximately twice as many egg chambers had destabilized plasma membranes than showed nurse cell nuclei in the oocyte; however, examination of nurse cell nuclei in the oocyte is easily scored and proved to be a strong indicator of general germ cell membrane integrity.

***cheerio* is found on the right arm of the third chromosome:** Recombination analysis using a multiply marked third chromosome showed $cher^1$ to be located on the right arm of the third chromosome. We refined the genetic map position of *cheerio* by performing additional recombination experiments between $cher^1$ and the markers *st* and *cu*. These data demonstrated that *cheerio* is found at 60 cM (not shown). The genetic map position was corroborated with deficiency mapping, giving the physical map position. *Df(3R)C4* uncovers *cheerio* but other deficiencies in the region do not, indicating that *cheerio* is located between 89E and 90A (Figure 7).

TABLE 1

Nurse cell nuclei are tranported into the oocytes of *cher* mutants

Genotype	Affected egg chambers/total examined	Average no. of nuclei in the oocyte/ affected egg chamber
Canton S	0/88 (0)	0
$cher^2/cher^2$	23/74 (31)	3.7
$cher^2/Df$	12/58 (20)	1.6
$cher^2/cher^1$	6/49 (12)	1.3
$cher^1/cher^1$	0/57 (0)	0
$cher^1/Df$	1/59 (1.8)	3

Values in parentheses are percentages.

DISCUSSION

Ring canal development: A ring canal develops in a series of defined steps that transform arrested cleavage furrows into stable intercellular bridges (Figure 8) (reviewed in ROBINSON and COOLEY 1996). The cleavage furrows contain contractile ring actin filaments, anillin (FIELD and ALBERTS 1995) and most likely myosin II. Just before the final germline division, phosphotyrosine-containing proteins become localized to the cleavage furrow. This is the first event that distinguishes the ring canal from the contractile ring. During the arrest of the cleavage furrow, the plasma membrane is thickened forming an electron dense outer rim (MAHOWALD 1971). This region of the ring canal stains with periodic acid/Schiff reagent indicating enrichment of glycosylated proteins in the outer rim (KOCH and KING 1969).

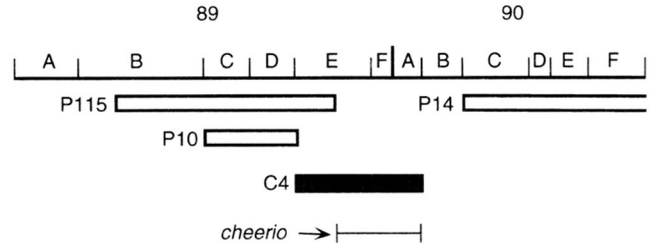

FIGURE 7.—Physical map of the region to which *cheerio* maps. *Df(3R)P115*, *Df(3R)P14*, and *Df(3R)P10* (open boxes) complement *cher* mutants. *Df(3R)C4* (black box) fails to complement *cher* mutants. This places the *cher* locus in the 89E to 90A interval.

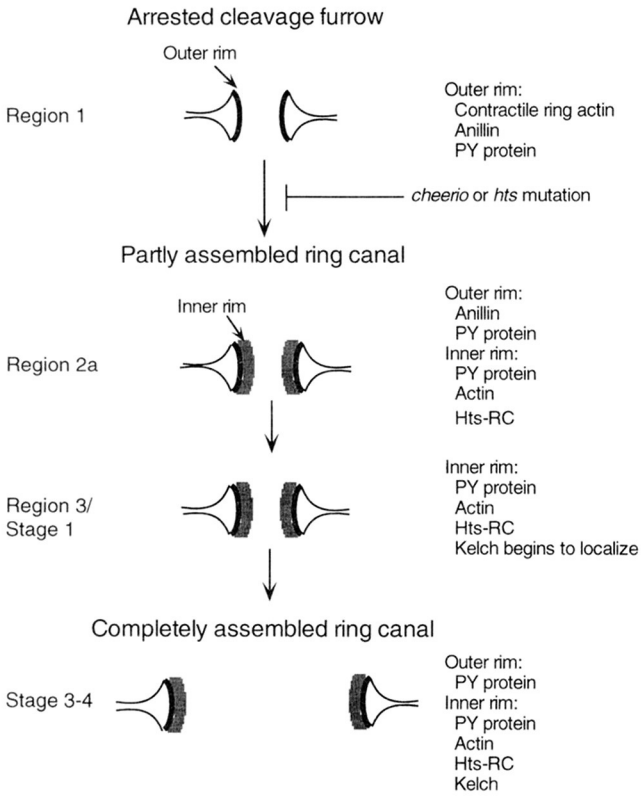

FIGURE 8.—The ring canal assembly pathway. A ring canal is assembled from an arrested cleavage furrow. Initially, contractile ring actin (and we presume myosin II) and anillin are localized to the cleavage furrows. Antiphosphotyrosine epitopes (PY protein) soon begin to accumulate before the completion of the fourth mitotic division. All of these proteins are localized to the outer rim of the ring canal. After the fourth mitosis, the inner rim components including additional PY protein, actin, and hts-RC proteins are localized. Several hours later in development in region 3 or stage 1, kelch begins to localize. By stage 3 or 4, kelch has reached every ring canal in the egg chamber. By this time, the ring canal diameter is 3–4 μm, and the maximal number of actin filaments has associated with the ring canal (TILNEY *et al.* 1996). The ring canal is now ready to increase its diameter throughout the rest of oogenesis.

After the completion of the fourth mitosis, the inner rim forms with the recruitment of additional actin filaments, hts-RC and kelch (ROBINSON *et al.* 1994; TILNEY *et al.* 1996). As kelch is localized, anillin can no longer be detected on ring canals indicating that they are discreet organelles separate from contractile rings.

Mutations in the *hts* and *cheerio* genes disrupt the addition of the inner rim of the ring canal, although incomplete cytokinesis and the localization of phosphotyrosine epitopes to the ring canal outer rim occurs. The function of hts-RC may be to interact directly or indirectly with the actin filaments to localize them to the ring canal. Hts-RC may also interact with a membrane protein to tether the actin filaments to the membrane. *cheerio* is likely to be required upstream of *hts* since hts-RC is present in *cheerio* mutant egg chambers but unlocalized. Based on the phenotype of $cher^1$, one possible function of *cheerio* might be to encode a ring canal structural protein. If so, it could bind ring canals at the stage where only an outer rim is present, perhaps by recognizing the phosphotyrosine-containing protein. *cheerio* bound to the outer rim could then interact directly or indirectly with actin and/or hts-RC to facilitate inner rim formation. Alternatively, *cheerio* could be required with hts-RC to organize actin in the inner rim. Another possibility is that the *cheerio* gene product regulates ring canal assembly through a signal transduction pathway. Molecular analysis of *cheerio* is now underway and will provide insight into the possible function of its product.

Ring canal inner rim function: *cheerio* and *kelch* mutants disrupt ring canal structure in dramatically different ways but with overall similar consequences to oogenesis in Drosophila. In *kelch*, ring canals initially are formed normally, but the inner rims do not remain properly organized as the ring canals increase in diameter from 3–4 to 10 μm (ROBINSON *et al.* 1994; TILNEY *et al.* 1996). Instead, the actin filaments and hts-RC extend into the lumen of the ring canal, presumably slowing the flow of cytoplasm. Insufficient cytoplasm transport into the oocyte causes *kelch* mutant egg chambers to be "dumpless" and the females to be sterile. In $cher^1$ mutant egg chambers, the ring canal inner rim does not form at all. The maximum size of the ring canals that are formed in these mutants is 3–4 μm, although most do not reach this size or they degenerate. The overall egg chamber phenotype of severe *cheerio* mutant egg chambers is indistinguishable from *kelch* mutant egg chambers with slowed cytoplasm transport that results in dumpless egg chambers. In both mutants, certain proteins are transported to the oocyte at correct times in oogenesis, indicating that global transport is not completely blocked (ROBINSON and COOLEY, unpublished results).

Unlike *cheerio* and *kelch*, *hts* function is not specific to ring canal assembly. Mutations of *hts* that block ring canal inner rim formation also disrupt the formation of the fusome. A reduction in expression of the hts fusome protein (hts-F) probably accounts for the defects in egg chamber cell number and the failure to differentiate an oocyte (YUE and SPRADLING 1992; LIN *et al.* 1994). The simultaneous reduction in the hts ring canal-specific protein (hts-RC) compounds the *hts* phenotype by blocking the addition of actin filaments to ring canal inner rims (YUE and SPRADLING 1992; ROBINSON *et al.* 1994); inner rim actin can be restored to *hts* mutant egg chambers by providing hts-RC transgenically (T. SMITH-LEIKER and L. COOLEY, unpublished data). The pleiotropic effects of hts mutations make it difficult to identify the role of the ring canal inner rim cytoskeleton in the development of an otherwise normal egg chamber.

Examination of *cheerio* reveals that maintenance of ring canal inner rims is important for nurse cell plasma

membrane integrity. In *cher*^{2}, the ring canals form and grow until midoogenesis, at which point the inner rims degenerate. This apparently results in a partial breakdown of the nurse cell plasma membranes, including those adjacent to the oocyte, and the movement of a few nurse cell nuclei into the oocyte late in oogenesis. The defect in plasma membrane stability is inversely related to the strength of the *cheerio* mutation. Placing the *cher*^{2} allele over *Df(3R)C4*, which should have less *cheerio* activity than *cher*^{2}/*cher*^{2}, results in fewer egg chambers with nurse cell nuclei transported into the oocyte. *cher*^{1}/*cher*^{1} and *cher*^{1}/*Df(3R)C4* mutants, in which ring canal inner rims never form, had almost no affected egg chambers. These data imply that the transient presence of ring canal inner rims and the accompanying partial growth of ring canals is ultimately more deleterious to overall nurse cell integrity than the complete absence of an inner rim cytoskeleton. In strong *cheerio* mutants, the failure to construct an inner rim and the persistence of relatively tiny ring canals might leave the nurse cell membranes less vulnerable to the forces placed on the plasma membranes during rapid cytoplasm transport at the end of oogenesis.

Other data also point to a connection between ring canal structure and plasma membrane integrity. Similar to *cher*^{2}, a dominant-negative kelch mutant protein causes ring canals to fall apart during their growth phase, and this is accompanied by nurse cell plasma membrane breakdown and the transport of nurse cell nuclei into the oocyte (D. N. ROBINSON and L. COOLEY, unpublished observations). Nurse cell plasma membrane breakdown is also seen in female sterile alleles of Drosophila protein kinase A (PKA) (LANE and KALDERON 1993), dominant-negative and constitutively active Cdc42 (MURPHY and MONTELL 1996), and dominant-negative RhoL (MURPHY and MONTELL 1996). In these mutants, ring canals are released from the membranes and multinucleate cells are formed. However, in the cases of PKA, Cdc42, and RhoL mutations, nurse cell nuclei are reported not to be transported into the oocytes, suggesting that the nurse cell-oocyte boundary is not affected as severely as in *cheerio*. The deleterious effects of PKA, Cdc42, and RhoL mutant proteins are likely to be on the overall actin cytoskeleton, whereas *cher*^{2} and dominant-negative kelch are most likely affecting ring canal integrity specifically. However, it is also possible that *cheerio* function is required in the subcortical actin cytoskeleton. Perhaps the subcortical actin in *cher*^{1} nurse cells cannot undergo contraction during dumping, while enough residual *cheerio* protein is present in *cher*^{2} for partial contraction resulting in the movement of nuclei into the oocyte.

In summary, the comparison of the phenotypes from *kelch* and *cheerio* egg chambers indicate that the function of the inner rim of the ring canal is to provide a substantial opening in the nurse cell membranes for cytoplasm transport and to maintain the opening so that the membranes remain intact. Failure to form a ring canal inner rim (severe *cheerio* alleles) or to organize it during growth (*kelch*) results in small openings that restrict cytoplasm transport, causing the egg chambers to be dumpless. Formation and growth of a ring canal opening followed by loss of the ring canal (weak *cheerio* allele, dominant-negative kelch) results in loss of integrity of the germ cell plasma membrane and subsequent leakage of the nurse cell nuclei into the oocyte. The function of the ovarian ring canal inner rims is to provide stable but expandable junctions capable of accommodating proper cytoplasm transport. Cloning *cheerio* and determining the subcellular localization of cheerio protein will help show whether this gene is specific for ring canal development or has additional roles in the actin cytoskeleton.

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