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## Stay connected: a germ cell strategy

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### Abstract

Germ cells develop as a cyst of interconnected sibling cells in a broad range of organisms in both sexes. A well-established function of intercellular connectivity is to transport cytoplasmic materials from ‘nurse’ cells to oocytes, a critical process for developing functional oocytes in ovaries of many species. However, there are situations where connectivity exists without a nursing mechanism, and the biological meaning of such connectivity remains obscure. In this review, we summarize the current knowledge on the formation of intercellular connectivity, and discuss its meaning by visiting multiple examples of germ cell connectivity observed in evolutionarily distant species.

### Keywords

germ cell connectivity; *Drosophila*; fusome; DNA damage; genome integrity

### Why do germ cells develop as interconnected cysts?

As early as the 19<sup>th</sup> century, cysts of interconnected germ cells were described by biologists of the time including Sertoli (1877) and von Ebner (1878) [1]. Since then, it became evident that stable intercellular bridges connecting the cytoplasm of germ cells is a common feature of developing germ cells in both female and male metazoans [2–4]. In addition, some somatic cells have also been shown to be connected through intercellular bridges [5].

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The mechanisms that underlie the formation of interconnected germ cell cysts have been intensively investigated [4]. However, *why* interconnected cysts are formed in the germline of many organisms has remained unclear, except for in meroistic ovaries where only a subset of germ cells are specified as oocytes while the others differentiate into nurse cells (Figure 1). In this case, intercellular bridges allow nurse cells to transport their cytoplasmic contents into oocytes, facilitating the development of oocytes [6]. Oocytes acquire much of their cytoplasmic materials such as organelles and fate determining mRNAs from nurse cells, whereas their own nuclei remain mostly quiescent.

The *Drosophila* ovary, where 15 nurse cells support the development of an oocyte, has been the premier model of meroistic ovaries to study how germ cell connectivity is established and how it contributes to oocyte development. Accordingly, our knowledge has been framed around the idea of a ‘nursing mechanism’ carried out by the nurse cells to nurture oocytes. However, broad evolutionary conservation of germ cell connectivity such as in panoistic ovaries (where all germ cells become oocytes) and in the male germline (Figure 2) urges us to think that the nursing mechanism is unlikely the only reason for how and why germ cell connectivity might have arisen during the evolution of multicellular organisms.

In this review, we attempt to discuss the functionality of germ cell connectivity across multiple systems beyond the meroistic ovaries. First, we will briefly review the molecular mechanisms by which intercellular connectivity is established in the ovary of *Drosophila*. Then we will describe germ cell connectivity in evolutionarily distant species. Finally, we discuss recent findings on germ cell connectivity in the *Drosophila* male germline and propose that germ cell connectivity might help protect the genomic integrity of germ cells.

## Mechanisms to establish germ cell connectivity: ring canal and fusome

The structure of germ cell intercellular bridges has been best studied in the *Drosophila* female germline. Asymmetric division of germline stem cells (GSCs) produces one self-renewing GSC and one cystoblast, the latter of which proceeds to differentiate. The cystoblast undergoes 4 mitotic divisions with incomplete cytokinesis, resulting in formation of a cyst with 16 interconnected germ cells (Figure 1). During these mitotic divisions, the contractile ring is stabilized without completely pinching off sister cells. These stabilized contractile rings result in intercellular bridges referred to as ring canals. Ring canals are outlined by the actin cytoskeleton, which grow considerably in size from 0.5  $\mu\text{m}$  to  $\sim 10 \mu\text{m}$  in diameter [7]. The first step in ring canal formation is the appearance of phospho-tyrosine residues along the contractile ring during mitosis [8, 9]. The ring canal is initially composed of contractile ring components such as Anillin, kinesin motor MKLP (mitotic kinesin-like protein, Pavarotti in *Drosophila*), and Myosin II. As ring canals grow, these initial components disappear, whereas the core components of mature ring canals, such as Hts-RC and Kelch, are loaded onto the ring canals [10–12]. The expansion of ring canals is critical for oocyte development by allowing for the intercellular transport of cytoplasmic materials from nurse cells to the oocyte.

In the *Drosophila* male germline, four mitotic divisions with incomplete cytokinesis yield a cyst of 16 spermatogonia, all of which subsequently undergo the meiotic program as

spermatocytes (Figure 2). Ring canals do not grow in size, likely reflecting the lack of need for the large scale cytoplasmic transport that is observed during oocyte development. Ring canals in the male germline do not have Hts-RC or Kelch, critical ring canal components in the female germline. Instead, male ring canals remain associated with contractile ring components such as Anillin, Septins (Peanut, Sep1, Sep2), and Pavarotti [13, 14].

In mammals, germ cells also develop intercellular bridges during gametogenesis in both males and females [1]. Intercellular bridges are observed in germ cells in fetal ovaries and testes, forming germ cell cysts [1, 15]. Cysts subsequently fragment, resulting in individual primary oocytes or prospermatogonia in neonatal gonads [16]. In adult testes, germ cells again undergo incomplete cytokinesis through spermatogonial and meiotic divisions [17]. Spermatogonial populations undergo repeated cycles of cyst fragmentation and branching (Figure 2) [17]. The molecular composition of the intercellular bridges in adult testes was revealed by proteomic analysis. Several proteins that are involved in somatic cytokinesis (such as MKLP1, RacGap, SEPT2, SEPT7, SEPT9, and Anillin) were shown to be localized to intercellular bridges in adult mouse testes [18]. Mice with germ cell-specific knockout of RacGAP or non-muscle Myosin IIB showed a male infertility phenotype [19, 20]. Germline-specific components of intercellular bridges, such as *Tex14* and RBM44, were also discovered [21, 22]. *Tex14* was identified as a key protein that blocks the cytokinesis machinery containing CEP55, TSG101 and ALIX, thus leading to stable intercellular bridges in germ cells in adult testes [23]. Male mice with a *Tex14* null mutation are infertile [21]. Although *Tex14* also localizes to the intercellular bridges in female germ cells, *Tex14* mutant female mice are fertile [24].

In *Drosophila*, ring canals are filled with a membranous organelle called the fusome (Figure 1). The fusome, found both in males and females, is a germline-specific membranous organelle that is considered to be a derivative of the endoplasmic reticulum [25]. The fusome adopts a branched morphology that runs through all germ cells within a cyst (Figure 1). The core fusome components are Hts-Fus/Adducin-like (a product of the *hu-li tai shao* gene, which produces a peptide that is cleaved into Hts-Fus and Hts-RC, a component of ring canals as described above),  $\alpha$ - and  $\beta$ -Spectrin, and Ankyrin [26–28]. The fusome likely functions to facilitate communication among germ cells within a cyst. It is known to be associated with cell cycle regulators (Cyclin A, B, E, Cdk1, and Cyclin degradation factors) [29–32], and loss of the fusome results in disruption of cell cycle synchronization within the cyst. These results show that the fusome plays a critical role in promoting the sharing of information among the germ cells within a cyst, leading to cell cycle synchronization. Moreover, fusome-associated Aurora B and Survivin may contribute to the formation of cyst by preventing complete abscission via inhibition of Cyclin B, which promotes abscission [30, 33].

Fusome-like structures have been observed in germ cells of multiple vertebrate systems, including the *Xenopus* ovary and the dogfish testis [34, 35], suggesting that ring canals and fusomes are ubiquitous mechanisms for achieving germ cell connectivity. However, EM studies on mouse ovaries suggest that there is no fusome-equivalent structure [36]. Mouse male germ cells also appear to lack fusome-like structure [1]. Despite the apparent lack of fusome, mouse ovarian germ cell cysts are synchronized in meiotic progression, suggesting

that there are yet-to-be identified mechanism(s) that allow synchronization among germ cells with the cysts [16].

In summary, connectivity/cyst formation is a ubiquitous phenomenon observed in germ cells of both sexes in a broad range of organisms.

### Function of germ cell connectivity: meroistic ovaries

The function of germ cell connectivity is best understood in meroistic ovaries, in which a subset of germ cells is chosen to become the oocyte while the remaining germ cells differentiate as nurse cells. In the *Drosophila* ovary, after 4 mitotic divisions, only one germ cell develops as the oocyte, whereas the remaining 15 germ cells become nurse cells. During and soon after the mitotic divisions, a polarity within connected germ cells is set up such that the oocyte can collect materials (e.g. mRNAs and organelles such as mitochondria) from nurse cells [37–39]. During this process, polarization of microtubules within the cyst plays a fundamental role: all the centrosomes move into the oocyte, thus the minus ends of microtubules are concentrated in the oocyte [40]. This allows dynein-mediated directional transport of cargos (such as fate-determining mRNAs), leading to oocyte determination. Failure to set up this polarity or in early transport of oocyte fate determinant(s) results in defective oocyte specification, leading to 16 nurse cells and no oocyte [41]. Even if oocyte fate is correctly specified, later defects in transporting nurse cell contents into oocyte results in defective oocyte growth, leading to underdeveloped oocytes and thus sterility [41].

Recent studies demonstrated that mouse ovaries are also meroistic, where some germ cells function as nurse cells to donate their cytoplasm to their sister cells that will develop into oocytes [42]. The function of intercellular bridges in allowing cytoplasmic transport during mammalian oocyte differentiation was initially suggested by studies using EM. Organelles such as mitochondria, ER and free ribosomes were found within the bridge in rabbit and mouse fetal ovaries [36, 43, 44]. Recent study by lineage-labeling further revealed that organelles (centrosomes, Golgi complexes and mitochondria) redistribute extensively within germline cysts during mouse oocyte differentiation, where the future oocytes collect organelles whereas the remaining cells donate them [42]. Subsequently, in the neonatal mouse ovary, cysts fragment into individual germ cells and those that have collected organelles from sibling cells to form a Balbiani body (aggregation of centrosomes, Golgi and mitochondria) become the primary oocytes. The germ cells that lack a Balbiani body, presumably nurse cells, undergo apoptosis. When cytoplasmic transport is blocked by inhibitors of microtubule polymerization or dynein, primary oocytes contain less cytoplasm and are defective in their ability to develop into later stage oocytes, suggesting that cytoplasmic augmentation via intracyst transport is critical for oocyte development [42].

These studies establish the importance of germ cell connectivity in oocyte development in meroistic ovaries.

## Evolution of germ cell connectivity: nursing mechanism does not explain everything

Whereas meroistic ovaries present a clear case for the function of intercellular bridges, the nursing mechanisms is unlikely the only reason for forming intercellular bridges. For example, male germ cells in a broad range of species (insects, fish [45], mammals [46, 47]) develop as a cyst of interconnected cells, yet these male germ cells obviously do not utilize a nursing mechanism (Figure 2). It has been suggested that intercellular connectivity might help complement genomic contents of haploid cells: after meiosis, half of the spermatids lack the X chromosome and the other half lack the Y chromosome. As the X chromosome is essential for cells' viability and the Y chromosome is essential for male fertility, haploid gametes may need access to the other chromosomes' products, which may be supported by germ cell connectivity [48–51]. Also, it has been postulated that germ cell connectivity might prevent meiotic drive, although no evidence has been found thus far [52]. However, the need for connectivity in post-meiotic haploid cells does not explain why connectivity in pre-meiotic diploid spermatogonia exists.

In another example, primitive insects such as grasshoppers, cockroaches, and stoneflies have panoistic ovaries, where all germ cells develop as oocytes without ever developing nurse cells. This implies that oocyte development does not always require intercellular bridges to aid cytoplasmic transport from nurse cells. Yet curiously, in several species with panoistic ovaries, mitotic germ cells are interconnected with cytoplasmic bridges, followed by separation of individual germ cells, each of which become an oocyte [53–56].

These observations clearly point to the possibility that cytoplasmic connectivity might have more functions beyond 'oocyte nursing'. Although no comprehensive effort has been made to determine when germ cell connectivity arose during evolution, there are several examples where researchers describe germ cell connectivity in diverse species, including early-diverging animals such as Cnidarians, Ctenophores, and Poriferans (i.e. non-Bilaterian phyla). In hydra (belonging to Cnidaria, a phylum that diverged roughly 720 million years ago [57]), germ cells are connected both in spermatogenesis and oogenesis, and the utilization of a nurse cell mechanism during oocyte development already makes an appearance [58, 59].

In Poriferans (e.g. sponges, calcarea), germ cells are often generated from transdifferentiation of somatic cells. When they undergo gametogenesis, it appears that only post-meiotic spermatids are connected by cytoplasmic bridges without evidence for earlier spermatogonial connectivity [60, 61], an observation consistent with the hypothesis that male germ cells need to complement their haploid genome by connectivity [48].

Interestingly, evidence of intercellular connectivity goes back possibly before the emergence of metazoans. Choanoflagellates are believed to be the closest relative of animals. During their life cycle, they alternate between the unicellular phase as single-flagellar swimming cells and the colony-forming phase. During the colony-forming phase of the choanoflagellate *S. rosetta*, a single cell undergoes multiple rounds of cell divisions with incomplete cytokinesis, leading to the formation of a colony of up to ~50 cells [62, 63]. It

was shown that intercellular bridges connect the cells within the colony, although it remains entirely unknown what purposes (if any) these bridges may serve. However, this suggests that stable intercellular bridges connecting mitotic cells emerged fairly early during evolution, likely predating the emergence of metazoans and before the evolution of an oocyte nursing mechanism. Future studies in choanoflagellates might provide insights into the potential roles of germ cell connectivity outside of the nursing mechanism.

### **Increasing sensitivity to DNA damage by germ cell connectivity**

Recent work in the *Drosophila* testis has shed light on an additional purpose for germ cell connectivity outside of the nursing mechanism. It was shown that in response to DNA damage by irradiation, all spermatogonia within a cyst die in synchrony even when only a subset of them exhibit detectable DNA damage (Figure 3A) [64]. Such synchronized cell death was disrupted in mutants of the fusome, suggesting that intracyst communication mediated by the fusome plays a critical role in synchronized spermatogonial death (Figure 3) [64]. It awaits future investigation to understand how the death-promoting signal may be shared among germ cells within the cyst. The signal may be transmitted through fusome-associated cell death regulators, as is the case for fusome-associated cell cycle regulators in synchronized cell cycle, as described above. Alternatively, death-promoting agents (signals or organelles) might be transported along the microtubules organized by the fusome.

Irrespective of the mechanisms, fusome-mediated sharing of death-promoting signal(s) leads to the death of spermatogonia that are not sufficiently damaged to die on their own. Sharing of such signals would effectively increase the sensitivity of the germline to the DNA damage by lowering the threshold of damage per cyst needed to commit to cell death. It might be that by increasing connectivity, germ cells might be able to ‘cast a bigger net’ to detect DNA damages (and possibly other cellular stresses) for an increased surveillance mechanism for the genomic integrity.

Sensitivity of the germline to DNA damaging agents compared to somatic cells is a long-held observation [65], and has been speculated to be a means by which organisms protect gametes’ genomic integrity. During the evolution of metazoans as germ cells segregated from somatic cells, it might have been necessary to introduce a ‘double standard’ in the surveillance mechanisms of genomic integrity. Whereas germ cells could enjoy the highest level of DNA damage surveillance (where any subpar genome can be culled), such stringent quality control could compromise the development of somatic tissues: cells in the developing embryo undergo a series of concerted fate determination processes (e.g. induction of cell fate in one cell type by another), requiring the presence of all participating cells to be in the right place and time thus limiting the ability to afford discarding too many cells. Therefore, somatic cells may have to tip the balance toward keeping cells alive rather than maintaining the highest level of genomic quality.

Thus, germ cell connectivity that allows intracyst communication to share death-promoting signals might serve as a mechanism that confer a high sensitivity to DNA damage, possibly discriminating germ cells from somatic cells.

## Conclusion and future direction

As described here, germ cell connectivity is a broadly conserved phenomenon with its evolutionary root possibly found in pre-metazoan species (e.g. choanoflagellates), although for what purpose intercellular connectivity serves in such pre-metazoan species remains unclear. It is possible that, once intercellular connectivity evolved in pre-metazoan species, it started serving many other purposes such as oocyte nursing and increased sensitivity to DNA damage. It awaits future investigation to understand for what purpose intercellular connectivity may serve in other examples of intercellular connectivity (see Outstanding Questions). It is of particular interest to understand the evolutionary origin of the purpose of intercellular connectivity, which will provide fundamental insights into universality and particularities of intercellular connectivity.

### Outstanding question

- What is the evolutionary origin for intercellular connectivity? It will be of particular interest to understand the function of intercellular connectivity in pre-metazoan species such as choanoflagellates.
- Are there any unknown functions for germ cell connectivity, and if so, what are they?
- What are the signals that mediate germ cell death, and how fusome mediates the propagation of such death signals?

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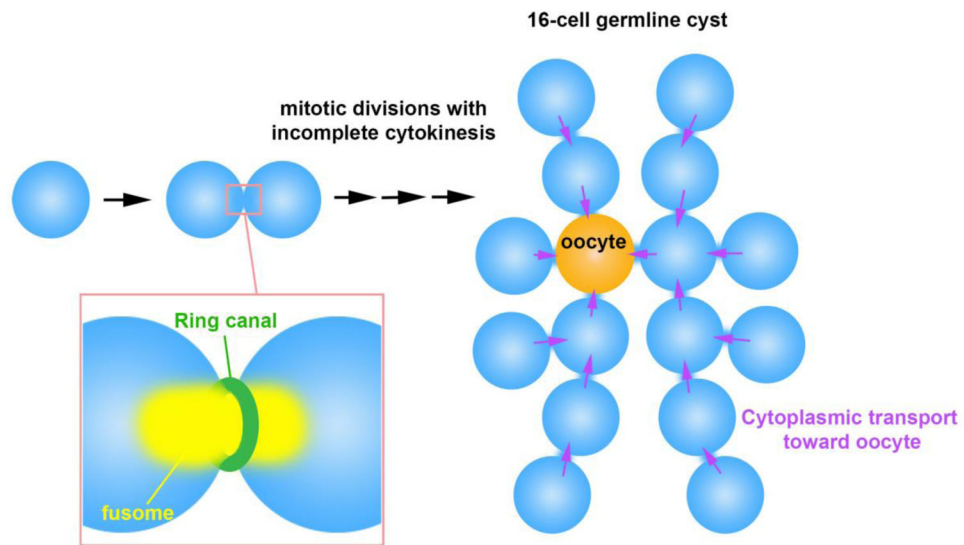
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### Trends Box

Germ cell connectivity is observed in broad range of organisms, including early diverging metazoans.

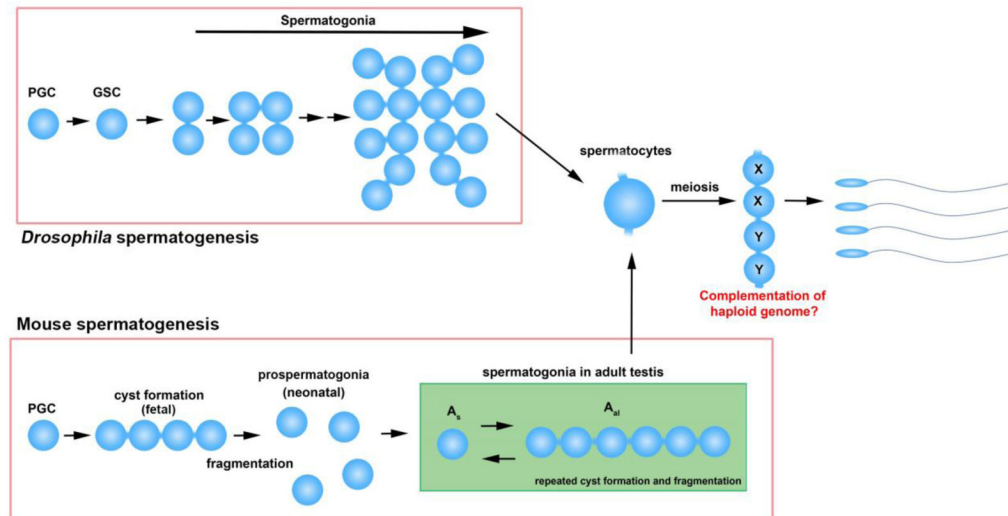
In meroistic ovaries, germ cell connectivity functions to transport cytoplasmic material from nurse cells to oocyte.

In *Drosophila* male germline, germ cell connectivity may serve as a mechanism to increase the sensitivity to DNA damage. When only a subset of germ cells within a cyst are damaged, all germ cells within the cyst die, increasing the net sensitivity to DNA damage.



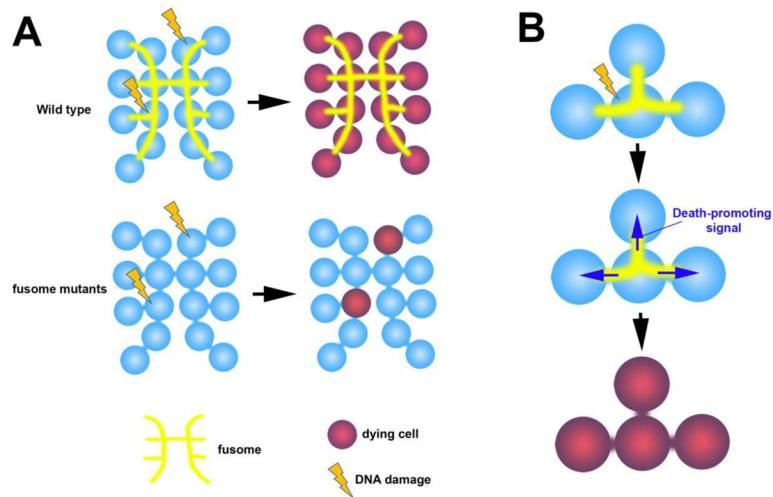
**Figure 1. Nursing mechanism through germ cell connectivity in meroistic ovaries**

In meroistic ovaries, where only a subset of germ cells within the cyst are chosen to become an oocyte, sister germ cells (nurse cells) donate their cytoplasm to the oocyte through ring canals. Shown is the example of the *Drosophila* female germline cyst, where 16 interconnected cells develop into one oocyte and 15 nurse cells. The fusome is a germline specific membranous organelle that runs through ring canal and disassembles before cytoplasmic transport begins.



### Figure 2. Germ cell cyst formation during spermatogenesis

Despite the lack of a nursing mechanism, male germ cells also develop germline cysts. Germ cell connectivity in post-meiotic spermatids has been proposed to help complement haploid genomes (X chromosome products shared by Y-containing spermatids, and vice versa). Note that spermatocytes are still interconnected with sibling spermatocytes while undergoing meiosis and spermiogenesis (although depicted as a single cell). Mitotically-dividing spermatogonia are also interconnected with sibling cells both in *Drosophila* and mice.



**Figure 3. Fusome-mediated sharing of death-promoting signal may enhance sensitivity of spermatogonia to DNA damage**

A. In the *Drosophila* testis, all spermatogonia within a cyst die in synchrony even when only a subset of spermatogonia have DNA damage. This is mediated by the fusome, as spermatogonia die individually in fusome mutants.

B. The fusome might facilitate the sharing of death-promoting signal among spermatogonia, leading to all-or-none cell death.