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Article

Mutations that affect channel opening of innexin hemichannels in the *C. elegans* gonad

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Abstract: In *C. elegans*, gap junctions couple cells of the somatic gonad with the germline to support germ cell proliferation and gametogenesis. We previously characterized a strong loss-of-function mutation (T239I) affecting the second extracellular loop (EL2) of the somatic INX-8 hemichannel subunit. These mutant hemichannels form non-functional gap junctions with germline-expressed innexins. Here we describe the characterization of mutations that restore germ cell proliferation in the T239I EL2 mutant background. We recovered seven intragenic mutations located in diverse domains of INX-8 but not the EL domains. These second-site mutations compensate for the original channel defect to varying degrees, from nearly complete wild-type rescue, to partial rescue of germline proliferation. One suppressor mutation (E350K) supports the innexin cryo-EM structural model that the channel pore opening is surrounded by a cytoplasmic dome. Two suppressor mutations (S9L and I36N) may form leaky hemichannels that support germline proliferation but cause the demise of somatic sheath cells. Phenotypic analyses of three other suppressors reveal an equivalency in the rescue of germline proliferation and comparable delays in gametogenesis but a graded rescue of fertility. These latter mutations may be useful to probe interactions with the biochemical pathways that produce the molecules transiting through soma-germline gap junctions.

Keywords: gap junctions; innexins; soma-germline interactions.

1. Introduction

Gap junctions are nearly ubiquitous in multicellular animals. The molecular constituents of gap junctions differ in chordates (connexins) and non-chordates (innexins), but their properties and biological functions are remarkably similar (recently reviewed in [1]). Although it is still unclear why a different class of gap junction molecule emerged within vertebrates, the ubiquity of gap junctions themselves suggests that being coupled is essential for most multicellular forms of life, and the functions of gap junctions are many. The sizes of connexin and innexin gene families within species further attest to a diversity of function. Coupling may coordinate activity of cells within a tissue, transfer metabolites and signals between cells, or conduct electrical currents. Gap junction coupling and the composition of junctional channels between cells can also dramatically change over developmental time, though the rationale driving these changes is mostly unknown. We are studying the role that gap junctions play in the somatic control of germline development in the *C. elegans* gonad. Two symmetric gonad arms extend anterior or posterior of a central uterus and vulva (Figure 1). Initially germ cells proliferate in a single small pool which becomes partitioned by migration of somatic cells from each of the developing gonad arms late in the second larval stage; these somatic cells eventually divide and develop to form the gonadal sheath, spermatheca, and uterus [2]. A somatic distal tip cell (DTC) occupies the leading edge of each expanding gonad arm, and the DTC establishes a stem cell niche supporting germ cell proliferation by producing Delta-class ligands LAG-2 and APX-1, which activate GLP-1/Notch receptors on germline stem cells [3–6]. At adulthood

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the soma of a gonad arm includes the DTC, 5 pairs of sheath cells and the spermatheca. The Sh5 pair of sheath cells is the most proximal (closest to the uterus) and is connected to a constriction of the distal portion of the spermatheca. A series of sheath cell contractions, coordinated with the dilation of the distal constriction of the spermatheca, results in the ovulation of a maturing oocyte into the spermatheca, where fertilization and the completion of the meiotic divisions occur. Embryos exit the spermatheca through the spermathecal-uterine valve and enter the uterus, where embryogenesis generally proceeds for a short time before egg laying through the vulval opening (Figure 1).

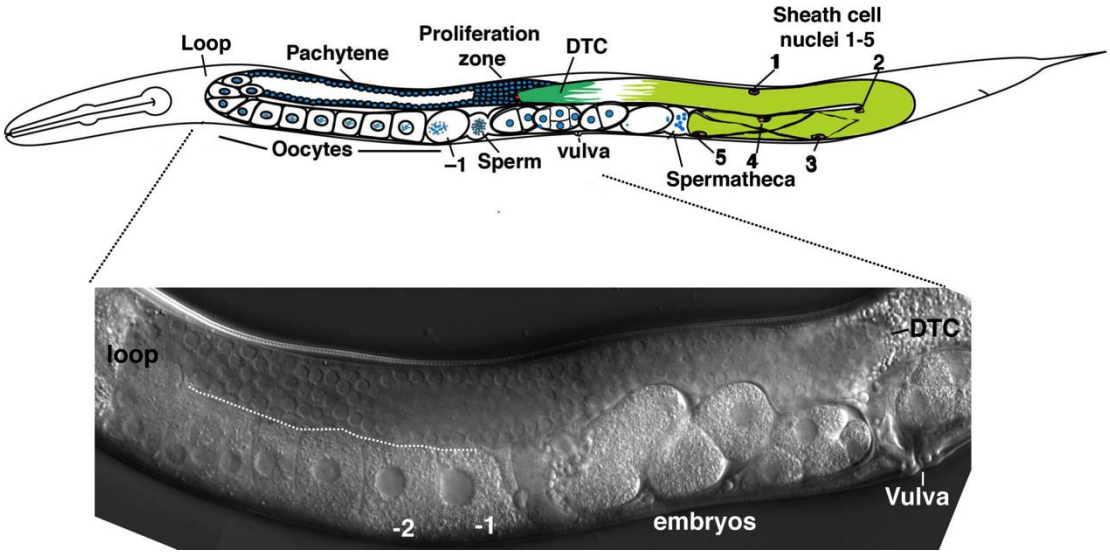

Figure 1. Diagram of the symmetric gonad arms in *C. elegans* (upper) and photo of corresponding gonad arm from the wild-type strain N2. DTC, distal tip cell; –1, most proximal oocyte.

From the primordial germ cells to the fully developed gonad, the soma and germline are coupled by two classes of gap junction channels [7]. The somatic DTC and sheath express the *inx-8* and *inx-9* pair of recently duplicated innexins, which constitute an operon (a single innexin is found at this locus in *C. briggsae)*. Either gene can rescue *inx-8(0) inx-9(0)* null mutants, and we regard the hemichannels that INX-8 and INX-9 subunits form as homomeric (although there may be a difference in phosphokinase recognition sites). Germ cells do not proliferate in *inx-8(0) inx-9(0*) mutants (~4 per gonad arm). Germ cells express *inx-14*, *inx-21*, and *inx-22*, and their corresponding protein products assemble into two classes of heteromeric hemichannels, composed of INX-14 in conjunction with either INX-21 or INX-22. As in *inx-8(0) inx-9(0)*, germ cells in *inx-14(0)* or *inx-21(0)* null mutants fail to proliferate, and animals are sterile. In *inx-22(0)* null mutant hermaphrodites, the germline appears unaffected; however, feminized *inx-22(0)* mutants fail to properly inhibit meiotic maturation in the absence of the major sperm protein (MSP) meiotic maturation signal, and unfertilized oocytes are ovulated into the uterus [8]. Clearly the two classes of germline hemichannels enable different processes, and presumably the nature (or quantities) of the molecules traversing the corresponding gap junction channels are different.

The failure of germ cells to proliferate in the absence of soma–germline gap junctions obscures any role that junctions may play during later development of the germline. In an attempt to

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address this issue, the *lag-2* promoter was used to express INX-8 in the DTC, but not sheath cells, in *inx-8(0) inx-9(0)* mutants using extrachromosomal arrays [7]. In these *inx-8(0) inx-9(0); Ex[inx-8(DTC+, Sheath–)]* animals, germ cell proliferation was restored to ~1/2 wild-type levels, but few progeny (avg. ~2) and dead embryos (~20) are produced compared to the wild-type (brood size ~300). This level of germline proliferation mirrors that observed when sheath cell precursors are ablated from developing gonad arms [9].

To explore specific roles that the soma plays in nurturing the germline, we have focused on manipulating *inx-8* somatic hemichannels in an *inx-9(0)* null background. Under these conditions, all somatic hemichannel functions are dependent on INX-8. Non-null reduction-of-function *inx-8* mutations may allow for germ cell proliferation and reveal later phenotypes associated with gap junction functions. Such mutant gap junctions might preferentially restrict the passage of certain molecules or exhibit unusual gating properties.

Here we describe a series of non-null *inx-8* mutations derived from a genetic suppressor screen based on restoration of germ cell proliferation in a severe *inx-8* loss-of-function background. The starting mutant INX-8 protein has a T239I change in the second extracellular loop. We previously showed that INX-8(T239I) is capable of supporting formation of gap junction channels with the germline, but these channels are non-functional [7]. Suppressor mutations were isolated at very low frequency, but, surprisingly, were found to be located widely in the molecule. The cryo-EM structure of the *C. elegans* INX-6 hemichannel was recently determined to be an octamer [10], and it has sufficient amino acid identity with INX-8 to allow for estimation of the positions of suppressor mutations in the INX-8 tertiary structure. Characterization of the phenotypes associated with suppressor mutations are consistent with this cryo-EM model of INX-6. We recently used one of these suppressor mutations to show a requirement for malonyl-CoA transfer from soma to germline to support early and continued embryogenesis [11]. Other suppressor mutations may be similarly useful for investigating genetic interactions with candidate biochemical pathways found in the soma that produce biomolecules that transit through gap junctions to control germline development.

2. Materials and Methods

2.1. Strains and Genetics

Worms were grown on standard NGM agar plates. All brood counts reported were done at 20°C. Bristol N2 was used as the wild type, and *mIs11* IV was used to balance *inx-8 inx-9* mutants. Strain DG3954 *inx-8(tn1513) inx-9(ok1502); tnEx195[sur-5::gfp; inx-8(+) inx-9(+)]; tnIs107[inx-8p::mCherry; str-1::gfp]* served as the foundation for the suppressor screen. To screen for suppressors, DG3954 was mutagenized with EMS following standard protocols (50 mM, 4 hrs). Mutagenized hermaphrodites were plated singly, grown at 20°C, and their F2 progeny were screened on a fluorescence dissecting stereomicroscope 5–6 days later for the presence of *sur-5::gfp(–)* animals with extended and reflexed *inx-8p::mCherry* gonad arms. Plates identified with such animals were then propaged to identify single hermaphrodites giving rise to candidate suppressors of *inx-8(tn1513) inx-9(0)*. In total, 34 separate mutageneses were performed and 37,300 mutagenized hermaphrodites were plated. In seven mutageneses, a subset of at least 300 plates were scored for sterility, and the overall percentage of sterile or near-sterile mutagenized animals averaged ~35% (range 15–50%). If we assign a very conservative brood size of just 5 F1 progeny produced on average, this screen would represent ~240,00 mutagenized haploid genomes. These suppressors

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therefore represent rare mutations. Allele designations for suppressor mutations and corresponding amino acid changes follow (*tn1789* was used as the representative for E350K in brood counts as this mutation was independently isolated twice).

1. *inx-8(tn1513 tn1553) inx-9(ok1502)*– INX-8(T239I, M117T)
2. *inx-8(tn1513 tn1555) inx-9(ok1502)*– INX-8(T239I, D24N)
3. *inx-8(tn1513 tn1771) inx-9(ok1502)*– INX-8(T239I, S9L)
4. *inx-8(tn1513 tn1789) inx-9(ok1502)*– INX-8(T239I, E350K)
5. *inx-8(tn1513 tn1790) inx-9(ok1502)*– INX-8(T239I, A288V)
6. *inx-8(tn1513 tn1791) inx-9(ok1502)*– INX-8(T239I, E350K)
7. *inx-8(tn1513 tn1792) inx-9(ok1502)*– INX-8(T239I, I36N)

Delayed ovulation for INX-8(T239I, A288V) was determined as previously described [11]. Photos of phenotypes primarily employed a Zeiss motorized Axioplan 2 microscope with a 63x PlanApo (numerical aperture 1.4) objective lens and an AxioCam MRm camera with AxioVision acquisition software. Germ cell counts were made by photographing dissected, fixed, and DAPI-stained gonad arms. Sperm nuclei were not included in the final counts (highest counts were ~60 sperm nuclei).

2.2 Site-directed mutagenesis and microinjection

inx-8 mutations to be assayed for rescue of *inx-8(tn1474null) inx-9(ok1502null)*—abbreviated *inx-8(0) inx-9(0)*—on extrachromosomal arrays were synthesized using *Pfu* Ultra (Agilent). In most cases, a *Pst*I/*Nhe*I restriction fragment encompassing most of the *inx-8* coding region was used as template, and after sequencing to verify the desired changes, this fragment was subcloned back into an *inx-8::gfp* plasmid previously shown to rescue *inx-8(0) inx-9(0)* [7]. Plasmids were injected into N2 at 1–2 ng/µl along with a *str-1::gfp* marker (80–100 ng/µl). After stable lines were obtained, extrachromosomal arrays were crossed into *inx-8(0) inx-9(0)/mIs11* heterozygotes, and *inx-8(0) inx-9(0)* homozygotes expressing the array were examined for rescue. All constructs except INX-8(D24N)::GFP rescued *inx-8(0) inx-9(0)* to some degree, indicating they possessed a capability of forming junctions. To verify that INX-8(D24N)::GFP could not form gap junctions, we constructed the strain *inx-8(0) inx-9(0)/mIs11; tnEx*222*[lag-2p::inx-8::gfp; myo3p::mCherry]; tnEx*223*[str-1::gfp; inx-8(tn1555)::gfp].* In *inx-8(0) inx-9(0)* homozygotes, *lag-2p::inx-8::gfp* rescues germline proliferation but is only expressed in the DTC and not sheath cells. Gonad arms could then be dissected and antibody-stained for GFP and INX-22 [7], and any GFP signal in the proximal arm could be attributed to *inx-8(tn1555)::gfp.* Both GFP and INX-22 expression was evident in proximal arms, but no gap junction-like puncta were detected, suggesting that INX-8(D24N)::GFP cannot localize INX-22 into junctions.

3. Results

We previously isolated a strong loss-of-function *inx-8(tn1513lf)* allele in a null *inx-9(0)* background [7], producing on average ~21 germ cells per gonad arm (cf. >1000 per wild-type arm

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[9]). t*n1513* encodes a T239I change in the second extracellular loop (EL2). A tagged INX-8(T239I)::GFP construct was introduced as a multi-copy extrachromosomal array into *inx-8(0) inx-9(0); Ex[inx-8(DTC+, Sheath–)].* In this background, *lag-2p* drives INX-8::GFP expression in the DTC and rescues germ cell proliferation; any expression of INX-8::GFP in the sheath (especially apparent in the proximal arm) can be attributed to INX-8(T239I)::GFP. We showed that this proximal arm expression was sufficient to support localization of germline INX-22 to gap junction plaques, indicating that INX-8(T239I) is capable of assembling into somatic hemichannels and forming gap junctions with germline hemichannels; the resultant channels, however, fail to rescue proximal arm gap junction function [7]. Potentially INX-8(T239I) causes a partial blockage in the channel it forms with the germline (total blockage might be expected to prevent formation of channels with germline hemichannels). *inx-8(tn1513) inx-9(0)* was therefore an attractive candidate for a suppressor screen to find compensatory mutations that might "unblock" INX-8(T239I) channels.

To carry out the screen, a strain was constructed including the following features: (1) the *inx-8(tn1513lf) inx-9(0)* mutation to be suppressed; (2) a means of visualizing the gonad at the dissecting microscope level, for which we designed an mCherry construct driven by the *inx-8* promoter (*inx-8p::mCherry*) that was integrated into the genome as a multi-copy array; and (3) an extrachromosomal array carrying wild-type copies of the *inx-8 inx-9* genomic region to rescue *inx-8(tn1513lf) inx-9(0)*, along with a co-injection marker (*sur-5::gfp*, expressed in the nuclei of all somatic cells), to visualize the presence of the array (Figure 2). Extrachromosomal arrays are frequently lost meiotically (and mitotically), resulting in a mixture of rescued and mutant progeny.

Hermaphrodites were mutagenized with EMS, placed singly on growth plates, and their F2 progeny were screened for signs of suppression. This was done by scanning for evidence of germ cell proliferation—larger gonad arms—in animals which had lost *sur-5::gfp* (Figure 2). Seven independent suppressor mutations were isolated (from >200,000 mutagenized genomes, see Materials and Methods), representing six unique mutations. All were eventually confirmed as *inx-8* intragenic changes by DNA sequencing. Somewhat surprisingly, suppressor mutations mapped to diverse regions of INX-8 but not to either of the extracellular loops. (For simplicity, we will refer to the suppressor strains by their amino acid changes rather than their genetic allele designations, which are listed in Materials and Methods.) For each suppressor mutation (SUP), we carried out the following analyses: (1) the phenotype of the INX-8(T239I, SUP) suppressor strain was characterized; (2) a corresponding INX-8(T239I, SUP)::GFP construct was introduced as a multi-copy extrachromosomal array into an *inx-8(0) inx-9(0)* background to see if the original suppression phenotype could be recapitulated; and (3) INX-8(SUP)::GFP lacking T239I was introduced on extrachromosomal arrays into *inx-8(0) inx-9(0)* to assay for rescue. The GFP tag allowed for verification of expression in the event that mosaicism might be suspected.

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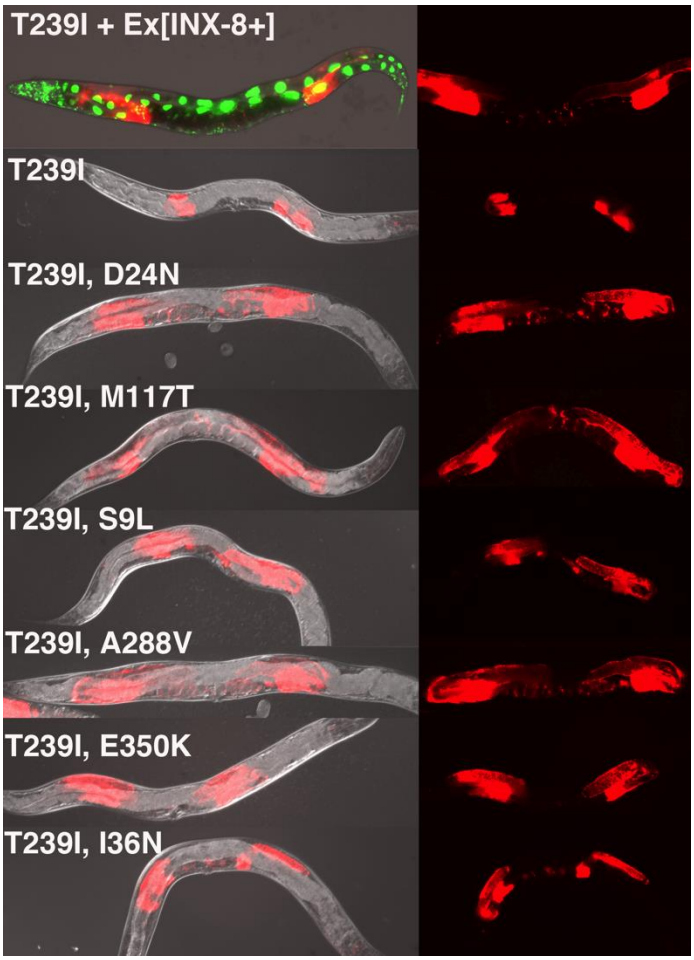

Figure 2. Suppressor mutants. The starting mutant, T239I, is rescued by an extrachromosomal array that carries wild-type *inx-8(+) inx-9(+)* and a *sur-5::gfp* co-injection marker. Meiotic loss of the array allows for visualization of the *Pinx-8::mCherry* somatic gonad marker. Broods with F2 progeny exhibiting increased gonad size in relation to T239I were further examined

The identity of amino acid changes associated with the suppressor mutations, and their predicted topological locations based on homology to INX-6, are indicated in Figure 3. E350K was isolated twice, suggesting the screen was beginning to approach saturation. Mutations were found in three of the four TM domains, the N-terminus and the C-terminus. Although we expected that the starting T239I change in EL2 might cause blockage of the channel in the region of hemichannel–hemichannel association, none of the suppressor mutations were located in the extracellular loops. Our interpretation is that suppressor mutations are relieving channel blockage by effecting a change in the EL tertiary structure within the INX-8 molecule, or by increasing access of molecules through the hemichannel to the site of the T239I blockage. Though speculative, the latter explanation seems more likely for most of the suppressor mutations. Suppressors are organized based on shared phenotypes.

Suppression approximating recovery to wild type

M117T (TM2) is the strongest suppressor, and the corresponding INX-8(T239I, M117T) hemichannel functions at nearly wild-type levels (Table 1). The cryo-EM model for INX-6 predicts

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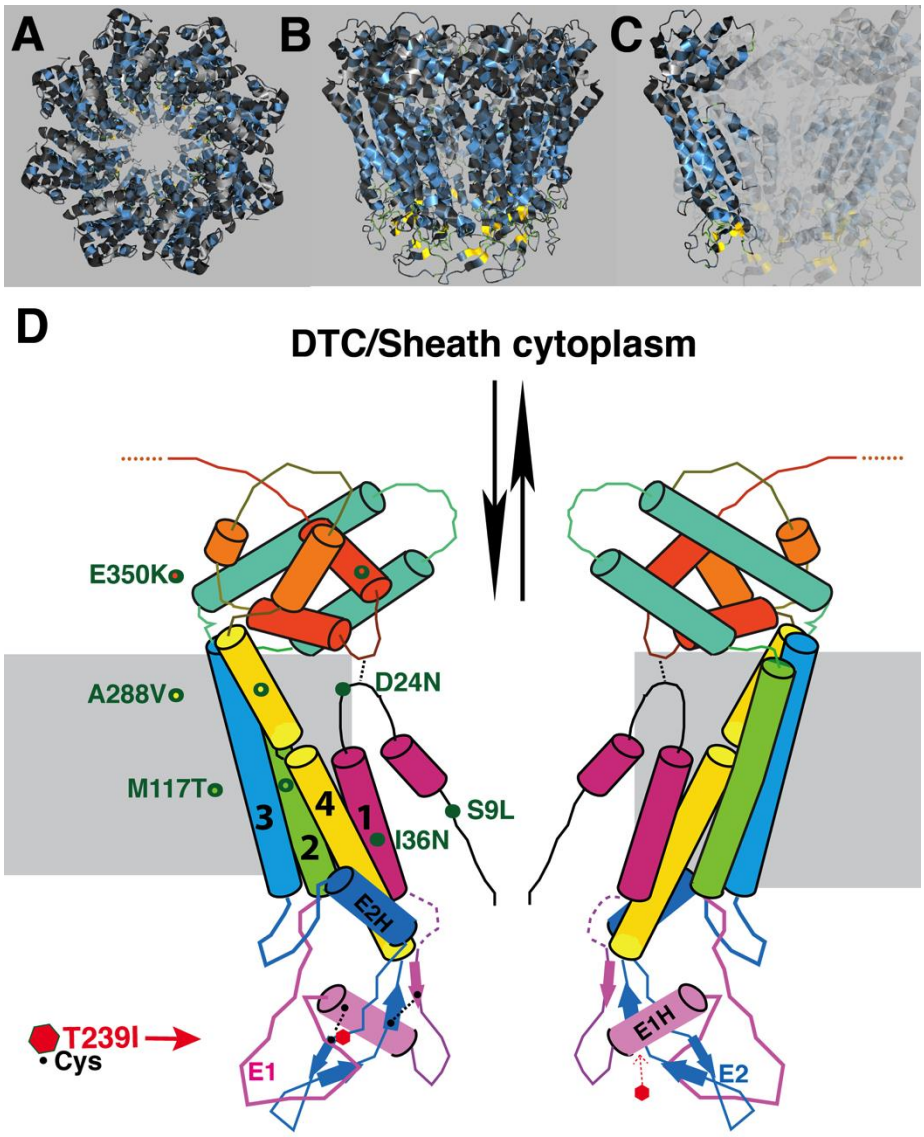

Figure 3. Position of T239I suppressor mutations. (A–C) Proposed structural model of INX-8 hemichannels based on homology to INX-6 [10, 12]. (D) Simplified representation of (C)) to highlight suppressor positions in relation to T239I. TM domains are labeled 1–4.

that the narrowest constrictions in the innexin channel are in the region lined by the N-terminus, and the region spanned by the extracellular loops (specifically a helix in EL1; [10]). Evidence from tryptophan scanning mutagenesis of the Drosophila innexin *shaking-B(lethal)* TM1 domain suggested that TM domains are less tightly packed in the innexin channel than in connexin channels [13]. If so, specific alterations in TM domains might open the channel through shifting the packing arrangement of the TM regions, or by influencing the position of the N-terminus lining the channel. Because T239I, M117T hemichannels approach wild type in functionality, it is possible that the T239I blockage itself has been relieved by a resultant change in tertiary structure as well. Multi-copy arrays of INX-8(T239I, M117T)::GFP rescued *inx-8(0) inx-9(0)* to high levels of fertility.

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Table 1. Germline and Brood Sizes of INX-8(T239I) Suppressor Mutants

INX-8 amino acid changes	# Viable progeny	Germ cells/arm
INX-8(T239, M117T)	256 ± 51 (n=10)	ND
INX-8(T239I, D24N)	108 ± 40 (n=90)[1]	322 ± 18 (n=3)
INX-8(T239I, A288V)	69 ± 20 (n=15)	469 ± 31 (n=3)
INX-8(T239I, E350K)	2 ± 2 (n=15)	440 ± 85 (n=3)
INX-8(T239I, S9L)	0 (n>20)	173 ± 17 (n=3)
INX-8(T239I, I36N)	0 (n>20)	245 ± 38 (n=3)
inx-8(0) inx-9(0); with extrachromosomal arrays containing		
[INX-8(T239I, M117T)::GFP]	ND	
[INX-8(T239I, D24N)::GFP]	48 ± 22 (n=20)	
[INX-8(T239I, A288V)::GFP]	4 ± 4 (n=30)	
[INX-8(T239I, E350K)::GFP]	0 (n>15)	
[INX-8(T239I, S9L)::GFP]	0 (n>15)	
[INX-8(T239I, I36N)::GFP]	62 ± 28 (n=13)	
[INX-8(M117T)::GFP]	213 ± 62 (n=10)	
[INX-8(D24N)::GFP] 2 lines	0 (n>20)	
[INX-8(A288V)::GFP] line 1	78 ± 35 (n=22)	
" line 2	120 ± 48 (n=21)	
[INX-8(E350K)::GFP] line 1	199 ± 47 (n=9)	
" line 2	101 ± 36 (n=10)	
[INX-8(I36N)::GFP] line 1	110 ± 36 (n=19)	
" line 2	67 ± 43 (n=10)	
[INX-8(S9L)::GFP] 2 lines	0 (n>20)	

[1]From [11]

Suppression with reduced germline proliferation, delayed gametogenesis, and reduced brood size

These mutants share approximately equivalent restoration of germline proliferation, exhibit a marked delay in gametogenesis, but display a graded rescue of brood-size numbers. D24N is located in the N-terminus near TM1. It lies close to a second aspartate, D21, that by homology to INX-6, is predicted to contribute to anchoring of the N-terminus to the cytoplasmic dome [10]. We recently reported a detailed characterization of INX-8(T239I, D24N). Germline and brood sizes are ~1/3 of the wild-type N2 (Figure 4, and [11]). Gametogenesis and ovulation are delayed ~18 hrs in relation to the last larval (L4-to-adult) molt. Though delayed, once fertilization ensues it continues over several days, similar to the wild type. The rescue of *inx-8(0) inx-9(0)* by INX-8(T239I, D24N) extrachromosomal arrays recapitulates these phenotypes, though brood sizes are smaller (Table 1). Because INX-8(T239I, D24N) behaves as a strong reduction-of-function allele of *inx-8* that produces

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moderate brood sizes, it was used to show genetic interactions with conditional mutants in the fatty acid synthesis pathway; this led to further genetic experiments demonstrating requisite transfer of malonyl-CoA from the somatic sheath to the germline [11].

A288V lies in TM4, and INX-8(T239I, A288V) gonad size and germ cell proliferation is slightly higher than that of INX-8(T239I, D24N) (Table 1; Figure 4), with lower levels (~2/3) of fertility (Table 1). INX-8(T239I, A288V) animals show a marked delay in gametogenesis in relation to the fourth larval molt similar in length to that seen for INX-8(T239I, D24N) [11], although a small percentage of animals failed to ovulate within 24 hours of the molt (Figure 5). Multi-copy arrays of INX-8(T239I, A288V)::GFP rescued *inx-8(0) inx-9(0)* to low levels of fertility (Table 1). We conclude that in general expression levels from the extrachromosomal arrays in these experiments appear lower than the levels derived from chromosomal expression.

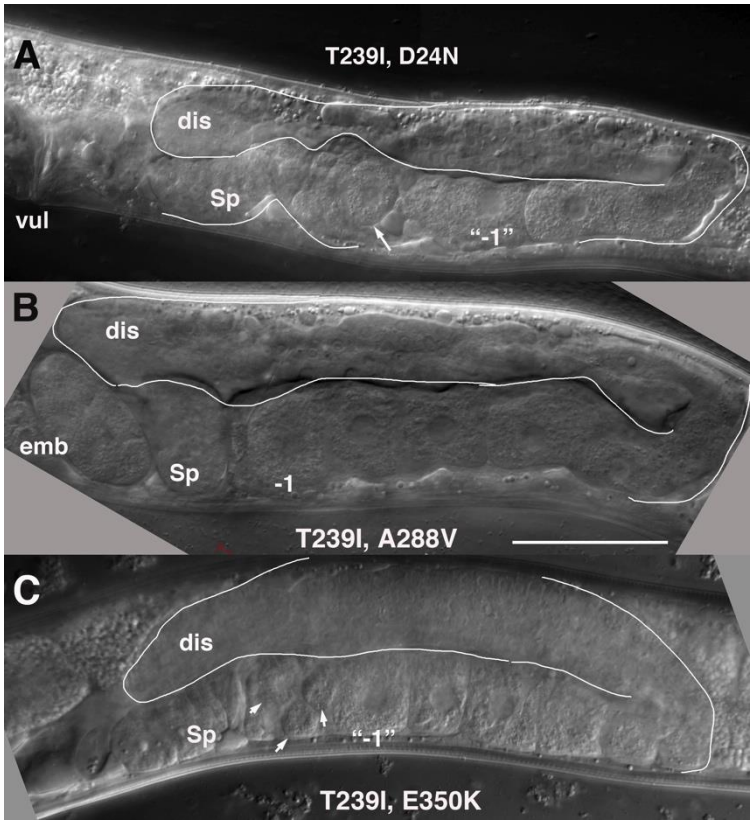

Figure 4. Representative gonad arms of suppressors with reduced fertility and delayed ovulation. Arrows in (A) and (C) point to unusually small oocytes in the most proximal position, a phenotype commonly seen in all three of these suppressors during early adulthood. Sp, spermatheca; dis, distal arm; vul, vulva; emb, embryos; –1, most proximal oocyte. Bar, 50 μm. A wild-type gonad is shown in Figure 1.

E350K is located in the C-terminus. INX-8(T239I, E350K) gonad size and germline proliferation is comparable to INX-8(T239I, A288V), but virtually no viable progeny are produced (Table 1). Lack of progeny prevented assessing the relationship of onset of ovulation to the L4 molt. The position of E350K is coincident with a site (Y356) in INX-6 proposed to contribute to inter-subunit interactions in the cytoplasmic domains of the hemichannel. These interactions support the formation of a dome-like structure that surrounds the hemichannel pore face. Disruption of this cytoplasmic dome

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by the E350K change may enlarge the pore face and allow increased access to the channel, which may allow for an increased probability of molecular transfer across the T239I-induced site of channel restriction. INX-8(T239I, E350K)::GFP extrachromosomal arrays rescue germline proliferation but not production of embryos in *inx-8(0) inx-9(0)* (Table 1).

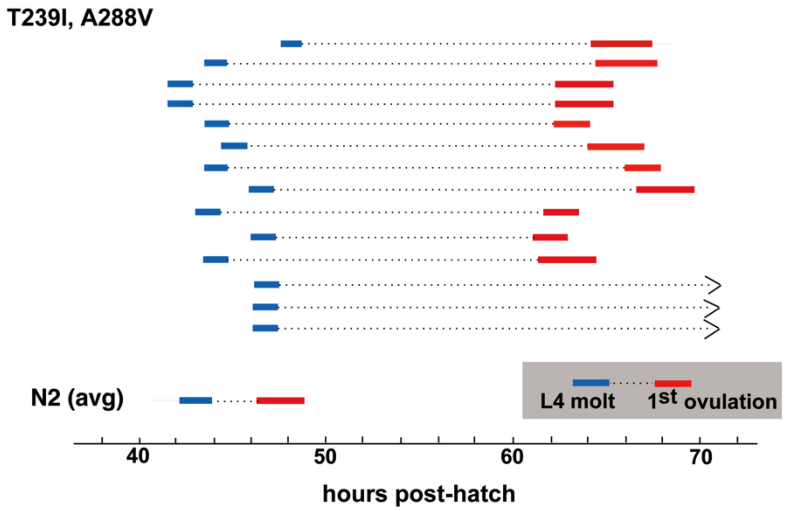

Figure 5. Ovulation is delayed in T239I, A288V mutants in relation to the L4 molt. (N2 avg. from [11]).

Suppressors with defects in Sh5 pair of somatic sheath cells

I36N (TM1) suppression of T239I is distinct from the other TM domain suppressors. Germ cell numbers are restored to ~1/5 wild-type levels in INX-8(T239I, I36N) (Table 1). Sperm and an occasional oocyte develop, but animals are sterile, due to a somatic defect. Late in the L4 larval stage, at a time when sperm are usually visible, it appears that the cytoplasm of the Sh5 pair of cells fills with fluid, and nuclei swell (Figure 6). Other fluid-filled foci can appear more distally in the gonad arm with time. Surprisingly, INX-8(T239I, I36N)::GFP expression from multi-copy arrays can rescue *inx-8(0) inx-9(0)* to low levels of fertility (Table 1). Of 36 gonad arms (18 animals) examined, only 2 arms resembled the phenotype of INX-8(T239I, I36N) animals. The nature of their isolation may preclude the recovery of extrachromosomal arrays that overexpress deleterious mutations; thus *Ex[INX-8(T239I, I36N)::GFP]* arrays that rescue *inx-8(0) inx-9(0)* may be selected for lower expression levels of the transgene.

S9L lies in the N-terminus, and INX-8(T239I, S9L) mutants share with INX-8(T239I, I36N) a very small germline, production of sperm but few oocytes, and swelling of somatic sheath cells. However, this swelling appears to be less restricted to the Sh5 pair of cells at onset (Figure 6). INX-8(T239I, S9L)::GFP arrays do not rescue *inx-8(0) inx-9(0)* to fertility, but unlike the original suppressor isolate, occasional fertilized embryos can be produced. Again, we hypothesize that the recovery of these arrays may be dependent on lower expression levels of the transgene compared to chromosomal expression of INX-8(T239I, S9L).

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Studies of tryptophan-substitution mutations in the TM1 domain of the Drosophila *shakingB(lethal)* innexin identified several sites which exhibited increased conductance in relation to wild-type ShakB(L) [13]. These mutants also displayed open-hemichannel activity when expressed in unpaired Xenopus oocytes, which compromised oocyte survival; however, when paired, the survival of oocytes expressing these constructs improved, likely due to reduction of free hemichannels as they assembled into gap junctions. Indeed, that opening of hemichannels may actually drive gap junction formation was originally proposed and demonstrated in a study of connexins expressed in Xenopus oocytes [14]. We speculate that I36N and S9L may enhance germline proliferation in a T239I background by increasing channel conductance in relation to wild-type INX-8; however, this may result in hemichannels that are leaky in the cell membrane when unpaired. Sh5 expresses INX-8 at very high levels, at least partly due to the fact that upon ovulation the oocyte endocytoses gap junctions formed with the soma, thus depleting Sh5 of a significant number of INX-8/9 subunits that must be replaced [7]. Sh5 may therefore be especially susceptible to a defect resulting in open hemichannels. Additionally, because oocytes have not developed and advanced into the proximal arm at the time of Sh5 swelling, it appears that when hemichannels initially arise in Sh5 there are no available pairing partners in the germline with which they might form gap junction channels (sperm do not form gap junctions with sheath cells). This may be exacerbated if there is a delay in gametogenesis as seen with other suppressors, though we have not yet attempted to document this for I36N or S9L. The more severe phenotype of INX-8(T239I, S9L) suggests that other somatic cells that express *inx-8* at levels lower than Sh5 are also susceptible to this gain-of-function in the corresponding hemichannels, and S9L may create a greater degree of "openness" than I36N.

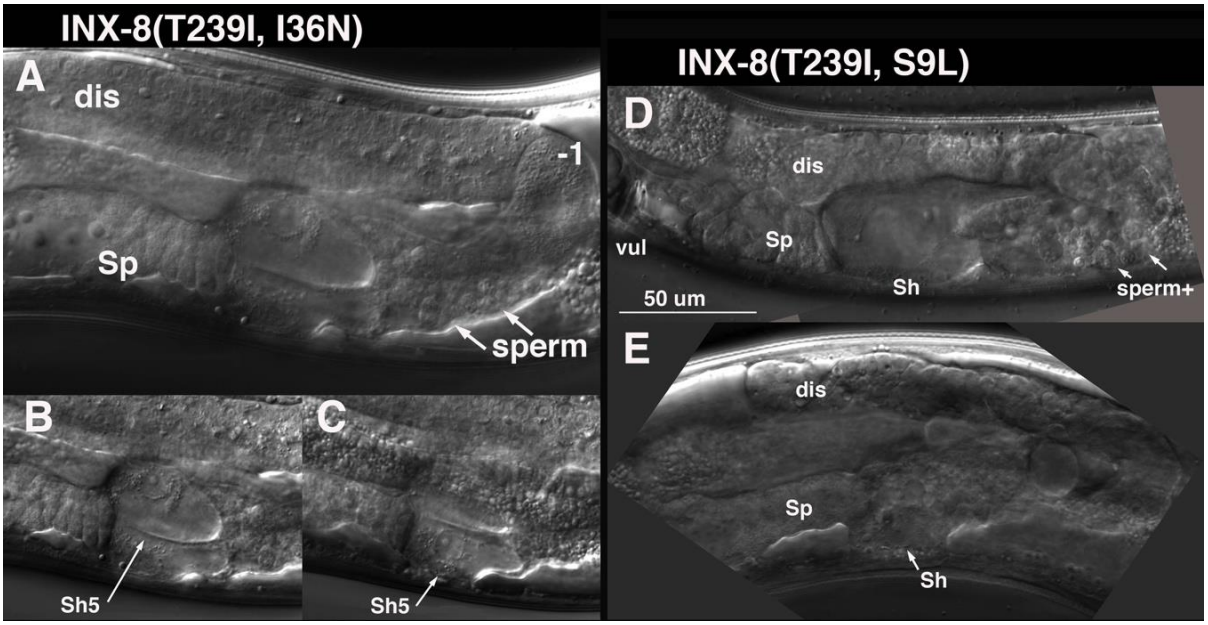

Figure 6. I36N and S9L affect cytoplasmic membrane integrity, especially of Sh5. (A–C) T239I, I36N mutants show swelling of both Sh5 cells, with nuclei visible within an expanded clear cytoplasm. (D, E) T239I, S9L hemichannels also affect Sh5 but loss of membrane integrity appears to include other cells or organelles. Sp, spermatheca; dis, distal arm; vul, vulva; Sh, sheath.

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Expression of suppressor mutations in the absence of T239I

Our interpretation of the nature of the suppressor mutations isolated in this screen is that they might result in increased conduction of the T239I hemichannel. As such, it was of interest to see if these mutations by themselves may confer unusual phenotypes. We expressed each of the *inx-8* single suppressor mutations on extrachromosomal arrays to rescue *inx-8(0) inx-9(0)* mutants (Table 1). Because expression from such arrays can be variable, we focused on potential qualitative rather than quantitative effects.

As might be predicted, INX-8(M117T)::GFP rescued *inx-8(0) inx-9(0)* mutants robustly. Tagged versions of A288V, E350K, and I36N also rescued, but to reduced fertility. Multiple independent lines for each of these mutations were generated, and no unusual phenotypes were identified that were seen consistently across each line representing a particular mutation.

INX-8(S9L)::GFP arrays were difficult to recover. One balanced line gave rise to homozygous *inx-8(0) inx-9(0)*; *Ex[INX-8(S9L)::GFP]* progeny in which germ cells proliferated but sheath cells displayed the characteristic swelling seen in INX-8(T239I, S9L) animals. A second independent INX-8(S9L)::GFP array in a balanced *inx-8(0) inx-9(0/++* background gave rise to animals with defective gonad arms and eventually could not be maintained except in a wild-type background, suggesting a dominant effect on wild-type INX-8. These results are consistent with the interpretation that S9L may lead to leaky hemichannel behavior that may be dominant to wild type in heteromeric hemichannels.

INX-8(D24N)::GFP showed no rescue of germ cell proliferation and was distinctive in being the only mutant tagged construct that appeared unable to contribute to channel formation. This was determined in two ways: (1) when expressed in an *inx-8(0) inx-9(0); Ex[inx-8(DTC+, Sheath–)]* background, INX-8(D24N)::GFP was unable to localize INX-22 to gap junction puncta in the proximal arm (see above for determination of T239I ability to form junctions, and Materials and Methods); and (2) whereas wild-type INX-8 and INX-8::GFP are endocytosed by maturing oocytes and can be identified in early embryos [7], INX-8(D24N)::GFP when expressed in a wild-type background was not detected in embryos. Therefore D24N neither appears to make hemichannels on its own nor associates at significant levels with wild-type INX-8 to contribute to hemichannel formation.

4. Discussion

Germ cells in the adult gonad progress in an assembly-line fashion, providing a snapshot of continual developmental progression at any single time point. In combination with a detailed understanding of its development from embryo through larval stages, the *C. elegans* gonad offers an excellent model for examining the gap junction relationships and requirements between cell types in a structure from its origin to its final functional form. Although the composition of hemichannels in soma and germline do not change, we expect that the specific requirements for molecules passing through gap junction channels changes with developmental progression from a mitotic state through the stages of meiosis and gametogenesis. Because the molecules that transit through gap junctions are small (<1000–2500 daltons), it is not easy to tag candidates and follow their intercellular passage *in vivo*. Genetic tools including mosaic analysis and mutations that perturb channel function have been useful in identifying candidates for traversing innexin gap junctions [11, 15]. One question

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entertained in characterizing these mutants was whether there was any evidence for preferential restriction of a particular class of molecules, e.g. negatively charged or positively charged molecules, or restriction by size. At least at this level of investigation none of the phenotypes associated with a particular suppressor mutant suggested such a strict gating, rather the observed phenotypes seemed to vary only by degree.

Only a limited set of suppressors of INX-8(T239I) was isolated, but the suppressors were widely distributed within INX-8. This may suggest that mutations throughout the innexin subunit may increase conduction, but the nature of our screen selected only those that still allowed germ cell proliferation. Three of these--D24N, A288V, and E350K--in the context of T239I behave as reduction-of-function *inx-8* alleles. They show similar degrees of support for germline proliferation but vary in their effectiveness of rescuing production of progeny, suggesting that the channel requirements for the latter are more demanding. They also support a gap junction requirement for timely gametogenesis, the nature of which is only a guess at present. It could represent the inability to accumulate a necessary factor, or remove an inhibitory factor. The extent of this delay—almost a full day—is sufficiently long that suppressor screens for the restoration of wild-type timing of egg-laying are feasible and may uncover bypass mutations in the responsible pathway.

Because INX-8(T239I, D24N) produces a moderate brood size it has been useful in establishing genetic interactions with fatty acid synthesis genes [11]. However, it may not be the best choice for investigating interactions with candidates required for germ cell proliferation. We propose that E350K likely disrupts the cytoplasmic dome surrounding the hemichannel pore, which might increase flux through the pore entry but would be unlikely to affect the channel constriction in T239I or the narrowest regions of the channel. As such E350K may be a more sensitive gauge for genetic interactions with other mutations affecting proliferation.

S9L and I36N suppression of T239I seem best explained as hemichannels that have open activity when unpaired. Clearly these hemichannels can form junctions, as they restore germ cell proliferation to some degree. The absence of a deleterious effect on the distal gonad arm suggests that most of the somatic hemichannels there are paired with germline hemichannels, or the levels of somatic hemichannels are sufficiently low that open hemichannel activity has a minimal effect on the plasma membrane. Expression levels of INX-8--graded from low in the distal arm to very high in the most proximal region [7]--are consistent with this being the determining factor in whether or not somatic sheath cell membrane integrity becomes compromised. A contributing factor for the Sh5 pair of cells, however, may be the absence of gap junction pairing partners for open hemichannels when they arise. It is possible such an explanation might apply to other widely expressed gap junction mutants for which defects are restricted to only a subset of expressing cells.

Other than being of structure-function interest, do these proposed open-hemichannel mutants have any utility for use in genetic interaction studies? Possibly. Because INX-8(T239I, I36N) arrays have been successfully isolated, by our interpretation due to lower expression levels, it would be possible to integrate these arrays into a chromosome and establish a reduction-of-function *inx-8* allele. Because these mutants are viable but display smaller germlines, they may also be candidates for genetic interaction inquiries with other mutants affecting germline proliferation. Additionally, it may be possible to determine if there are any ramifications to increased conduction across soma–germline gap junctions.

5. Conclusions

A genetic screen for rescue of germ cell proliferation in a strong loss-of-function *inx-8* mutant yielded a set of suppressor mutations that likely increases conduction through their corresponding hemichannels. These mutations highlight distinct requirements for soma–germline coupling in the distal and proximal gonad arms and have helped identify a gap junction requirement for timely ovulation and gametogenesis that nevertheless does not inhibit the production of healthy broods. One of these suppressors (D24N) has already proved useful for establishing genetic interactions with the fatty acid synthesis pathway and for providing evidence that malonyl-CoA transits through soma-germline gap junction channels [11]. These suppressor mutations may facilitate the elucidation of the molecules delivered through soma–germline gap junctions and their roles in promoting germline development.

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