A Genetic Test of the Mechanism of Wolbachia-Induced Cytoplasmic Incompatibility in Drosophila

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ABSTRACT

Cytoplasmic bacteria of the genus Wolbachia are best known as the cause of cytoplasmic incompatibility (CI): many uninfected eggs fertilized by Wolbachia-modified sperm from infected males die as embryos. In contrast, eggs of infected females rescue modified sperm and develop normally. Although Wolbachia cause CI in at least five insect orders, the mechanism of CI remains poorly understood. Here I test whether the target of Wolbachia-induced sperm modification is the male pronucleus (*e.g.*, DNA or pronuclear proteins) or some extranuclear factor from the sperm required for embryonic development (*e.g.*, the paternal centrosome). I distinguish between these hypotheses by crossing gynogenetic *Drosophila melanogaster* females to infected males. Gynogenetic females produce diploid eggs whose normal development requires no male pronucleus but still depends on extranuclear paternal factors. I show that when gynogenetic females are crossed to infected males, uniparental progeny with maternally derived chromosomes result. This finding shows that Wolbachia impair the male pronucleus but no extranuclear component of the sperm.

WOLBACHIA comprises a group of maternally transmitted cytoplasmic bacteria that have a variety of reproductive effects in arthropods (O'Neill *et al.* 1997; Werren 1997). These bacteria have been implicated as the cause of induced parthenogenesis in haplodiploids (Stouthamer *et al.* 1990), feminization of genetic males in isopods (Rousset *et al.* 1992), and, most notably, cytoplasmic incompatibility (CI; Yen and Barr 1971; Hoffmann and Turelli 1997). CI results when the sperm of Wolbachia-infected males fertilize eggs from uninfected females, causing embryonic death. Crosses between infected males and infected females produce no developmental anomalies. Antibiotic treatment of infected individuals simultaneously cures the Wolbachia infection and the associated reproductive effects (Wright and Barr 1981). The ability of Wolbachia strains to induce CI after experimental transfer between phylogenetically distant hosts (*i.e.*, between the mosquito *Aedes albopictus* and the fruit fly *Drosophila simulans*) suggests that the microbes disrupt an evolutionarily conserved target (Braig *et al.* 1994). But the mechanism by which Wolbachia cause CI remains unknown.

Although Wolbachia are abundant in the testes of infected males, they are not physically associated with mature sperm (Binnington and Hoffmann 1989; Bressac and Rousset 1993). Instead the bacteria are shed with the cytoplasm during individualization in spermatogenesis. Wolbachia do not therefore cause CI directly, but modify developing sperm, which then transmit the CI-inducing effects to eggs. Cytological studies of eggs from incompatible crosses reveal early mitotic defects and paternal chromosome loss following fertilization in the mosquitoes *Culex pipiens* (Jost 1970) and *Aedes polynesiensis* (Wright and Barr 1981), the wasp *Nasonia vitripennis* (Ryan and Saul 1968; Reed and Werren 1995), and the fruit fly *D. simulans* (Callaini *et al.* 1996, 1997; Lassy and Karr 1996). Haploid development results in production of male progeny in the haplodiploid Nasonia (Breeuwer and Werren 1990) but is lethal in mosquitoes and Drosophila. Only the presence of Wolbachia (or Wolbachia-derived products) in the egg cytoplasm can rescue such modified sperm.

To cause CI, Wolbachia must modify nuclear and/or extranuclear components of the sperm. For example, modification of paternal chromosomes—either DNA or paternal DNA-packaging proteins—during spermatogenesis might later disrupt the condensation cycle of the male pronucleus and/or karyogamy after fertilization. Alternatively, Wolbachia might modify extranuclear factors of sperm that are essential for embryonic development but unrelated to processing of the male pronucleus. There are several examples of such paternal factors. In most animals, for instance, the paternal centrosome is essential: centrosome elements of the sperm basal body must combine with those of the maternal centrosome to form the zygotic microtubule organizing center (MTOC). The MTOCs replicate and orchestrate assembly of the spindles needed for pronuclear apposition and segregation of chromosomes during mitosis (Schatten 1994). In addition to the centrosome,

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several essential paternal proteins have been identified in Drosophila (reviewed in Karr 1996; Fitch *et al.* 1998) and *Caenorhabditis elegans* (Browning and Strome 1996). Moreover, because the entire sperm enters the egg in many Drosophila species, it has been suggested that the sperm tail itself plays a critical role in the early embryo (Karr 1996). All such extranuclear paternal contributions represent potential targets for Wolbachia.

In fact, cytological work in *D. simulans* has uncovered both abnormal paternal chromosome behavior *and* irregular centrosome-mediated microtubule processes in embryos from incompatible crosses (Callaini *et al.* 1996; Lassy and Karr 1996). Two unpublished studies also found differences in sperm proteins between infected and uninfected Drosophila males (cited in Karr 1996; T. Sasaki cited in Wilkinson 1998). While suggestive, these findings do not conclusively identify the site of Wolbachia's action. Distinguishing between the nuclear *vs.* extranuclear possibilities thus represents an important step toward understanding the mechanism of CI.

Here I present genetic results that distinguish these possibilities, using a gynogenetic stock of *D. melanogaster* (Fuyama 1984, 1986a,b). Gynogenesis is like parthenogenesis in that diploid zygotes inheriting all chromosomes from their mother can develop without a *genetic* contribution from males. However, as shown in crosses below, even though gynogenetic diploid eggs do not require paternal chromosomes, they do require *extranuclear* factors from sperm—physical penetration of the egg alone is insufficient to initiate development. We can therefore ask whether diploid gynogenetic eggs can develop using the sperm of Wolbachia-infected males: If Wolbachia disrupt paternal chromosomes only, diploid gynogenetic eggs should develop; if, however, Wolbachia disrupt any extranuclear paternal factors required for development, diploid gynogenetic eggs should not develop (see Figure 1). Thus, by crossing uninfected gynogenetic females to infected males, I can determine whether Wolbachia disrupt the male pronucleus or essential extranuclear paternal factors.

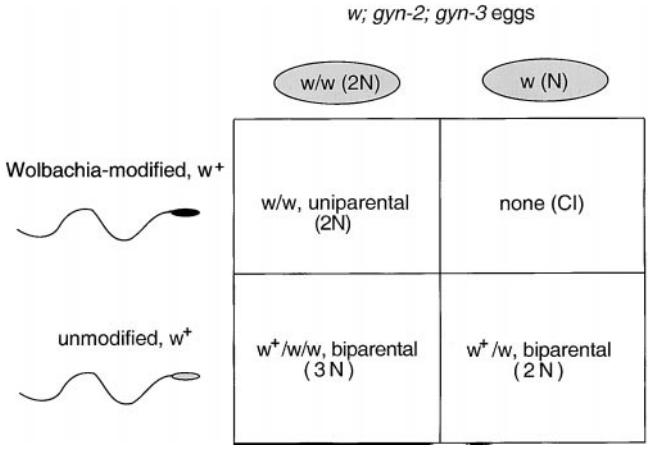

Figure 1.—The cross between gynogenetic *w; gyn-2; gyn-3* females and Wolbachia-infected males. *w; gyn-2; gyn-3* females produce both diploid eggs and haploid eggs; infected males produce both modified sperm and unmodified sperm. Cells indicate the possible genotypes of daughters. If CI is caused by modification of the male *pronucleus* only, white-eyed uniparental daughters will be seen (top left). If, on the other hand, CI is caused by modification of *extranuclear* paternal factors, these white-eyed uniparental daughters will not be seen—they instead will die as embryos.

MATERIALS AND METHODS

Stocks: Fly stocks were kindly provided by Drs. Y. Fuyama [*w; gyn-2; gyn-3* and *ms(3)K81*], K. Fitch [*ms(3)snky*], S. O'Neill [Wolbachia-infected *D. melanogaster* Canton-S], A. Hoffmann [*D. simulans* Riverside (DSR)], and M. Turelli [*D. simulans* Watsonville (DSW)].

Infection status and CI: Tetracycline curing of Wolbachia infections was carried out as described by Hoffmann *et al.* (1986). Flies were bred on standard medium with 0.3% tetracycline concentration for at least three generations. "DSRT" and "Canton-ST" refer to tetracycline-cured DSR and Canton-S flies.

The infection status of all stocks was confirmed by PCR using primers specific for *Wolbachia pipientis* and the reaction conditions described in O'Neill *et al.* (1992). An ~900-bp fragment from the bacterial 16S rRNA gene was amplified from Wolbachia-infected strains, while no product was obtained from uninfected strains.

Levels of CI were measured for both intraspecific and interspecific crosses at 25°. Single pairs of flies were set up in vials with standard medium for 24 hr. Females were then transferred to vials containing small spoons with grape juice-colored medium coated with a live yeast suspension (Hoffmann *et al.* 1986). Females were transferred every 24 hr for several days. The percentage egg hatch was scored 28 hr after females were removed from a vial. To control for background egg mortality (*i.e.*, mortality independent of Wolbachia), I calculated corrected CI values (CI_{corr}; Poinsot *et al.* 1998) as the rate of egg mortality from incompatible crosses (CI_{obs}) minus the rate of egg mortality observed in compatible crosses (CCM) between two uninfected individuals: $CI_{corr} = [(CI_{obs} - CCM)/(100 - CCM)]$.

Crosses: The gynogenetic *D. melanogaster* stock, *w; gyn-2; gyn-3*, is described in detail by Fuyama (1986b). *w; gyn-2; gyn-3* females can produce both biparental and uniparental progeny. Expression of a recessive visible mutation (in this case *white*) carried by the mother allows a simple check that the chromosomes of gynogenetically produced uniparental progeny are maternally derived, as all males used in this study had wild-type eye color, w^+.

I performed two versions of the experimental test, one using crosses between species and one using crosses within species. Because infected strains of *D. melanogaster* show only low levels of CI (*e.g.*, Hoffmann *et al.* 1994; A. Hoffmann, T. Karr, M. Turelli, F. Rousset, M. Solignac, personal communications), I initially crossed *w; gyn-2; gyn-3 melanogaster* females to males of another species, the infected Riverside strain of *D. simulans*. DSR shows high levels of CI within *D. simulans* (Hoffmann *et al.* 1986). Using species hybrids does not affect the level of CI: uninfected *D. melanogaster* females crossed to infected *D. simulans* males show levels of CI comparable to those seen within *D. simulans* (M. Green, personal communication; and below). Ten *w; gyn-2; gyn-3* virgin females were mass mated to 20 3- to 5-day-old virgin *D. simulans* males.

Males were removed from vials when there was evidence of fertilization (*i.e.*, dead eggs or larvae), usually within 2–3 days. *D. melanogaster* females crossed to *D. simulans* males produce hybrid daughters only; male hybrids die at the larval-pupal transition (Sturtevant 1920). Lethality of hybrid males in no way affects the present results, as tests of gynogenesis among *w; gyn-2; gyn-3* females involve scoring uniparental *w*/*w* daughters.

I repeated the experiment within species. *w; gyn-2; gyn-3* females were crossed to an infected strain of *D. melanogaster* created by introgressing Canton-S chromosomes into the infected cytoplasm of a *y w* stock (S. O'Neill, personal communication). Because CI is weak in *D. melanogaster*, I tried to increase its expressivity by using a slightly different crossing design from that used above. I set up individual *w; gyn-2; gyn-3* virgin females in vials with 3–5 1-day-old Canton-S virgin males and observed all crosses until copulation occurred, as CI levels decrease with male age (Hoffmann *et al.* 1986) and repeated copulation (Karr *et al.* 1998). Each female was then aspirated and placed in a fresh vial for oviposition. Females were transferred to new vials every 3–4 days. The eye color and sex of all progeny were scored.

When haploid *w; gyn-2; gyn-3* eggs are fertilized by unmodified w^+ sperm, red-eyed diploid daughters result (w^+/w; Figure 1). When these eggs are fertilized by Wolbachia-modified sperm, CI results (Figure 1). However, ~24% of *w; gyn-2; gyn-3* eggs are diploid (see Table 1 in Fuyama 1986b). When diploid *w; gyn-2; gyn-3* eggs are fertilized by umodified w^+ sperm, triploid red-eyed daughters result ($w^+/w/w$). When these eggs are fertilized by Wolbachia-modified sperm, and if only the paternal chromosomes are disrupted by Wolbachia, production of uniparental white-eyed daughters should result (Figure 1). We can roughly approximate the expected percentage of uniparental white-eyed daughters as

$$\%2N \text{ eggs} \cdot \%\text{ modified sperm}/\{(\%2N \text{ eggs} \cdot \%\text{modified sperm}) + [\%\text{unmodified sperm } (\%2N \text{ eggs} + \%N \text{ eggs})]\},$$

where %2*N* eggs = 24%, and the rate of sperm modification is estimated from corrected CI values (CI_{corr}), as explained above.

RESULTS AND DISCUSSION

Properties of the *gyn-2; gyn-3* stock: Before performing the key experiment, it is important to characterize the properties of *gyn-2; gyn-3* reproduction and to characterize the infection and CI status of the stocks employed.

First, both haploid *and* diploid eggs of uninfected *w; gyn-2; gyn-3* females use the nuclear and extranuclear contributions of unmodified w^+ sperm: *w; gyn-2; gyn-3* females crossed to uninfected Watsonville (DSW) males produce only red-eyed daughters (Table 1, line 1; see Table 2 for infection status). Because approximately one-third of *w; gyn-2; gyn-3* eggs are diploid (Fuyama 1986b), some red-eyed daughters are $w^+/w/w$ triploids.

Next, two facts must be established: (1) diploid *w; gyn-2; gyn-3* eggs do not require a paternal nuclear contribution; and (2) diploid *w; gyn-2; gyn-3* eggs *do* require an extranuclear paternal contribution. I confirmed that *w; gyn-2; gyn-3* females can reproduce gynogenetically by crossing them to homozygous *ms(3)K81* males (Fuyama 1984, 1986b). While functional in all other respects, *ms(3)K81* sperm cannot deliver the paternal pronucleus. Thus, if *w; gyn-2; gyn-3* females reproduce gynogenetically, the cross of *w; gyn-2; gyn-3* females to *ms(3)K81* males should produce abundant uniparental white-eyed daughters. This is precisely what occurred: virtually all progeny (99.8%) were white-eyed uniparental daughters (Table 1, line 2). In contrast, nongynogenetic Oregon-R females produced only dead eggs when crossed to *ms(3)K81* males (Table 1, line 3). Diploid *w; gyn-2; gyn-3* eggs do not therefore require a paternal nuclear contribution. The few sons produced (6.7%) in the first cross were sterile *XO* males (confirmed by testes dissections and failure to produce progeny) resulting from nondisjuction in one of the two egg pronuclei that fuse to restore diploidy in uniparental progeny [in a similar cross Fuyama (1986b) found ~2% *XO* males].

The ability of diploid *w; gyn-2; gyn-3* eggs to use *ms(3)K81* sperm shows that they do not need a nuclear contribution from males. But the fact that these eggs never develop without fertilization shows that they require something from the sperm. To test whether sperm penetration alone is sufficient to stimulate diploid *w; gyn-2; gyn-3* egg development, I crossed *w; gyn-2; gyn-3* females to *D. melanogaster* males homozygous for the paternal effect lethal mutation *ms(3)snky*. The plasma membrane of *snky* sperm fails to break down after pene-

TABLE 1

Gynogenetic reproduction

Cross (female × male)	Sperm contribution: Sperm penetration	Sperm contribution: Extranuclear factors	Sperm contribution: Paternal chromosomes	Daughters: w^+	Daughters: *w*	Sons: w^+	Sons: *w*	Total daughters	% White daughters
1 *w; gyn-2; gyn-3* × DSW	+	+	+	271	0	0	3	271	0.0
2 *w; gyn-2; gyn-3* × *ms(3)K81*	+	+	−	1	535	0	39	536	99.8
3 *Ore R* × *ms(3)K81*	+	+	−	0	0	0	0	0	0.0
4 *w; gyn-2; gyn-3* × *ms(3)snky*	+	−	−	1	0	0	0	1	0.0
5 *Ore R* × *ms(3)snky*	+	−	−	0	0	1	0	0	0.0

TABLE 2

Infection status as determined by PCR assay

Species	Stock	Infection status
D. melanogaster	*w; gyn-2; gyn-3*	Uninfected
D. melanogaster	*ms(3)K81*	Uninfected
D. melanogaster	*ms(3)snky*	Uninfected
D. melanogaster	Oregon-R	Uninfected
D. melanogaster	Canton-S	Infected
D. melanogaster	Canton-ST	Uninfected
D. simulans	DSW	Uninfected
D. simulans	DSR	Infected
D. simulans	DSRT	Uninfected

tration, trapping the nuclear *and* extranuclear paternal contributions within the membrane (Fitch and Wakimoto 1998; Fitch *et al.* 1998). If diploid *w; gyn-2; gyn-3* eggs only require sperm penetration, uniparental white-eyed daughters should appear. Instead, the cross of *w; gyn-2; gyn-3* females to *ms(3)snky* males produced only a single biparental daughter from thousands of eggs (Table 1, line 4). Thus, sperm penetration alone is not sufficient to initiate development—diploid *w; gyn-2; gyn-3* eggs require essential extranuclear factors from sperm. The single escaper reflects *ms(3)snky*'s known slight leakiness (Fitch and Wakimoto 1998; see also the single male produced in the control Oregon-R cross, Table 1, line 5).

Infection status and CI: Table 2 gives the infection status of all stocks. Table 2 also shows that Wolbachia infections were successfully cured by the tetracycline treatment.

Within-species levels of CI (Table 3) were similar to those in previous reports (*e.g.*, Hoffmann *et al.* 1986; Bourtzis *et al.* 1998). In *D. simulans*, DSR males cause strong CI when crossed to DSRT females (CI_{corr} = 79.9%). As expected for *D. melanogaster*, Canton-S males cause weak CI when crossed to cured Canton-ST females (CI_{corr} = 25.3%).

The level of CI induced by DSR males *between* species (Table 3, line 10) was similar to that seen within species: uninfected *D. melanogaster* Oregon-R females mated to DSR males showed CI_{corr} = 87.0%. Uninfected *D. melanogaster* Oregon-R females mated to cured DSRT males showed egg hatch rates similar to compatible crosses within species (Table 3, line 11). Species crosses between *D. melanogaster w; gyn-2; gyn-3* females and DSR males should therefore reflect normal DSR levels of CI.

Test of Wolbachia target: We now turn to the critical experiment. The crosses above show that *w; gyn-2; gyn-3* eggs use the paternal chromosomes and extranuclear factors of unmodified wild-type sperm (Table 1, line 1). They also show that diploid *w; gyn-2; gyn-3* eggs do not require paternal chromosomes (Table 1, line 2) but *do* require extranuclear factors from the sperm (Table 1, line 4). Given this, we can make two predictions: If Wolbachia impair the paternal chromosomes only, uniparental diploid white-eyed daughters should appear when *w; gyn-2; gyn-3* females are crossed to infected males. If, on the other hand, Wolbachia impair an essential extranuclear paternal factor, uniparental white-eyed daughters should *not* appear.

TABLE 3

Cytoplasmic incompatibility relationships among strains

Cross (female × male)	Eggs	Females	Eggs/female (SE)	% Unhatched eggs[a] (SE)
CI in *D. simulans*				
1 DSR × DSR	1182	14	84.4 (3.6)	24.4 (8.1)[a,b]
2 DSRT × DSRT	1093	15	77.7 (2.6)	9.3 (1.8)[a]
3 DSR × DSRT	1184	13	91.1 (6.4)	23.9 (6.7)[a,b]
4 DSRT × DSR	1362	14	97.3 (5.7)	81.8 (2.6)[c]
CI in *D. melanogaster*				
5 Canton-S × Canton-S	1142	15	76.1 (6.4)	11.2 (2.7)[a]
6 Canton-ST × Canton-ST	878	11	79.8 (9.6)	10.3 (3.4)[a]
7 Canton-S × Canton-ST	1158	13	89.1 (6.6)	13.0 (4.7)[a]
8 Canton-ST × Canton-S	1717	20	85.9 (4.5)	33.0 (4.4)[b]
CI in species cross				
9 Oregon-R × Oregon-R (control)	1139	12	94.9 (6.0)	9.2 (6.9)[a]
10 Oregon-R × DSR	1863	21	88.0 (6.8)	88.5 (4.5)[c]
11 Oregon-R × DSRT	951	13	72.1 (5.9)	11.5 (5.6)[a]

[a] Values with different superscripts differ significantly by Student's *t*-test ($\alpha = 0.05$). Data were arcsine transformed prior to analysis. Contrasts for compatible *vs.* incompatible crosses used directed *t*-tests (Rice and Gaines 1994), while all other contrasts use symmetrical tests. Probabilities were corrected for multiple tests using the sequential Bonferroni technique (Rice 1989).

TABLE 4

Crosses between gynogenetic females and infected males

Cross (female × male)	Daughters		Sons		Total daughters	% White daughters
	w^+	w	w^+	w		
Crosses to infected males						
1 *w; gyn-2; gyn-3* × DSR	511	554	0	20	1065	52.0
2 *w; gyn-2; gyn-3* × Canton-S	630	121	0	532	751	16.1
Crosses to uninfected males						
3 *w; gyn-2; gyn-3* × DSRT	947	0	0	0	947	0.0
4 *w; gyn-2; gyn-3* × Canton-ST	471	4	0	400	475	0.8

When *w; gyn-2; gyn-3* females are crossed to *infected* DSR males, many uniparental daughters are produced: 52% of daughters are white-eyed (Table 4, line 1). This result is not an artifact of the species cross, as similar results are obtained in the within-species test: when *w; gyn-2; gyn-3* females are crossed to *infected* Canton-S males, 16.1% white-eyed daughters appear (Table 4, line 2).

As expected, control crosses of *w; gyn-2; gyn-3* females to tetracycline-cured DSRT males failed to produce any white-eyed daughters (Table 4, line 3). Similarly, *w; gyn-2; gyn-3* females crossed to cured *D. melanogaster* Canton-ST males produce only rare escaper (<1%) white-eyed daughters (Table 4, line 4). Tetracycline treatment of infected males thus cures Wolbachia infection and simultaneously eliminates production of uniparental progeny.

The production of uniparental daughters in crosses to infected males definitively shows that *w; gyn-2; gyn-3* eggs successfully use the extranuclear components of Wolbachia-modified sperm but not the male pronucleus. Wolbachia do not, therefore, impair essential extranuclear components of the sperm.

It is worth noting that the levels of gynogenetic reproduction induced by DSR and Canton-S males are proportional to the levels of CI induced by males of these strains. This reflects the fact that both phenotypes—percentage uniparental progeny and percentage of unhatched eggs (CI)—are largely determined by the percentage of sperm modified. For example, given the 25.3% sperm modification rate of the Canton-S Wolbachia strain and the 24% diploid egg production of *w; gyn-2; gyn-3* females, we expect ~7.5% white-eyed daughters ($= (0.25 \times 0.24) / [(0.25 \times 0.24) + 0.75(0.24 + 0.76)]$). We observe 16% white-eyed daughters (Table 4, line 2). Similarly, given the 79.9–87.0% sperm modification rate of the DSR strain, we expect 48.8–61.6% white-eyed daughters. We observe 52% (Table 4, line 1). Finally, for crosses involving males from uninfected strains, no sperm modification occurs and virtually no uniparental daughters are produced.

Concluding remarks: The genetic tests performed here support the results of cytological studies in Drosophila and Nasonia suggesting that CI is caused by disruption of paternal chromosome processing in uninfected eggs (O'Neill and Karr 1990; Reed and Werren 1995; Callaini *et al.* 1996, 1997; Lassy and Karr 1996). Similarly, the fact that CI in the haplodiploid wasp Nasonia results in haploid males shows that the paternal genome is affected. However, none of these earlier findings ruled out the possibility that Wolbachia also impair extranuclear sperm components. In fact, previous cytological work showed that—in addition to chromosomal mitotic defects—abnormal spindle structures and supernumerary centrosomes appear in incompatible crosses (Callaini *et al.* 1996; Lassy and Karr 1996). The present results, however, rule out the possibility that Wolbachia critically impair the paternal centrosome, the sperm tail, or any other essential extranuclear paternal factors not related to paternal chromosome processing. Instead, Wolbachia critically impair the male pronucleus.

The present results do, however, suffer from at least one limitation: they tell us little about the cellular and molecular mechanisms of CI. The questions of how the male pronucleus is modified and how Wolbachia in the egg rescue this modification still remain. As noted by Karr and colleagues (O'Neill and Karr 1990; Karr 1996; Lassy and Karr 1996), the embryonic lethal phenotypes caused by *ms(3)K81* sperm and Wolbachia-modified sperm are strikingly similar. The present work shows that both lesions can induce gynogenetic reproduction in *gyn-2; gyn-3* females. Based on their genetic analysis of *ms(3)K81*, Yasuda *et al.* (1995) conclude that the K81 protein is likely required for one of three steps in postfertilization chromosome remodeling: decondensation of the sperm pronucleus, replication of the male pronucleus, or recondensation of the male pronucleus corresponding to its acquisition of maternally derived chromatin packaging proteins. Wolbachia might similarly interfere with any one of these critical steps.

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