

# Role of Delayed Nuclear Envelope Breakdown and Mitosis in *Wolbachia*-Induced Cytoplasmic Incompatibility

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The bacterium *Wolbachia* manipulates reproduction in millions of insects worldwide; the most common effect is cytoplasmic incompatibility (CI). We found that CI resulted from delayed nuclear envelope breakdown of the male pronucleus in *Nasonia vitripennis*. This caused asynchrony between the male and female pronuclei and, ultimately, loss of paternal chromosomes at the first mitosis. When *Wolbachia* were present in the egg, synchrony was restored, which explains suppression of CI in these crosses. These results suggest that *Wolbachia* target cell cycle regulatory proteins. A striking consequence of CI is that it alters the normal pattern of reciprocal centrosome inheritance in *Nasonia*.

*Wolbachia*, prevalent among arthropods worldwide, cause a variety of reproductive alterations in their hosts, including feminization, male killing, induction of parthenogenesis, and cytoplasmic incompatibility (CI) (1–4). As a consequence, *Wolbachia* are thought to be important in insect ecology and evolution (3, 5). CI, the most common effect of *Wolbachia* infection, arises when infected males mate with uninfected females and produce embryos that develop abnormally (CI embryos). CI is suppressed when both parents are infected. This cross is fertile, as are crosses between infected females and uninfected males.

In CI embryos, paternal chromosomes fail to properly condense and align on the metaphase plate during the first mitosis (6–9). Paternal chromosomes are ultimately lost. Only maternal chromosomes segregate normally, producing haploid embryos that develop into male progeny in *Nasonia* and are inviable in *Drosophila* (6, 10–13). In *Drosophila simulans* CI embryos, the centrosome, normally inherited from sperm at fertilization, often dissociates from nuclei (8, 13). These cellular studies suggest that *Wolbachia* disrupt chromatin remodeling (9, 11, 14–17) and centrosome function. However, models based on these observations do not readily explain how compatibility is restored when sperm from infected males fertilize infected eggs.

A timing model in which *Wolbachia* specifically disrupt the timing of events in the male pronucleus more readily addresses

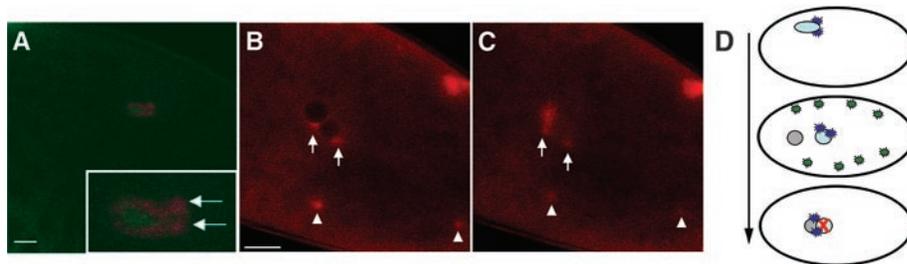
this aspect of CI (7–9, 14, 15). When *Wolbachia* are also present in the egg, CI is suppressed because the timing of events in the female pronucleus would be equivalently disrupted, restoring synchrony between the two pronuclei (9). Timing models have been difficult to test directly because these early events are rapid and occur immediately after fertilization in the interior of the embryo. We developed live cytological techniques to analyze fertilization and the first mitotic division in living *Nasonia* embryos (18), which allowed us to directly test these models.

To determine whether *Wolbachia* induce CI by disrupting centrosome function, we injected rhodamine-tubulin into CI embryos to follow centrosome and pronuclear behavior immediately after fertilization (18, 19). Fertilization and pronuclear migration and

apposition occurred normally in CI embryos (Fig. 1, A to C). Sperm from *Wolbachia*-infected males behaved normally in the egg (Fig. 1, A and B). Sperm from infected males were rod-shaped and associated with two centrosomes soon after they entered the egg (Fig. 1A), similar to sperm from uninfected males (18). In preparation for mitosis, these centrosomes separated normally (Fig. 1, A and B).

In *Nasonia*, centrosome inheritance occurs reciprocally; paternally derived centrosomes are inherited by fertilized female eggs, and maternally derived centrosomes are inherited by unfertilized male eggs (18). To determine whether CI-induced males inherit maternal or paternal centrosomes, we injected CI embryos with rhodamine-tubulin and performed time-lapse microscopy. In CI embryos, centrosome behavior was normal; maternally derived asters were present in the cytoplasm (Fig. 1B), and paternally derived centrosomes were associated with the male pronucleus (Fig. 1, A and B). Paternal centrosomes set up the first mitotic division (Fig. 1, B and C), whereas maternal cytoplasmic asters were excluded (Fig. 1, B and C). Thus, *Wolbachia* did not disrupt paternal centrosome function and inheritance, which is consistent with results in *D. simulans* (17). Furthermore, although segregation of paternally derived chromosomes was disrupted in CI (6–9), segregation of paternally derived centrosomes was not (Fig. 1D). Thus, CI produces a new pattern of centrosome inheritance; males inherit maternally derived chromosomes and paternally derived centrosomes, instead of the normal maternally derived centrosomes (Web fig. 1) (19).

To determine whether *Wolbachia* disrupt nuclear cycle timing, we performed



**Fig. 1.** *Wolbachia* did not disrupt paternal centrosome transmission. (A) CI embryo injected with rhodamine-tubulin (red; labeling centrosomes and nuclear periphery) and Oligreen (green; labeling DNA) showed that sperm from a *Wolbachia*-infected male was rod-shaped and associated with two paternally derived centrosomes (arrows, inset). Scale bar = 8  $\mu\text{m}$ . (B) In CI embryo, injected with rhodamine-tubulin, pronuclei were apposed and centrosome separation (arrows) occurred normally. Maternally derived centrosomes (arrowheads) were present in the cytoplasm. (C) In same embryo as in (B),  $T = +510$  s, paternally derived centrosomes (arrows) set up mitotic spindle; maternally derived asters degenerate. Scale bar = 16  $\mu\text{m}$  for (B and C). (D) Model of centrosome inheritance in CI males. Rod-shaped sperm (blue oval) associated with two paternally derived centrosomes (blue stars) enters egg. As female meiosis completes, maternal asters form (green stars) and female pronucleus (gray circle) migrates toward male pronucleus (blue circle). Paternal genome is lost (red X) but paternal centrosomes are maintained, producing haploid males.

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immunofluorescent analysis with an antibody specific for phosphorylated histone H3 (anti-PH3) (19). Anti-PH3 is an excellent marker for entry into mitosis, because phosphorylation of histone H3 requires active Cdk1 and is an initial event accompanying chromosome condensation (20, 21). Anti-PH3 specifically labels chromosomes from prophase through anaphase in *Drosophila* embryos (21).

In control embryos, PH3-positive chromosomes were observed from late prophase through anaphase (Fig. 2A), approximately corresponding to the period from nuclear en-

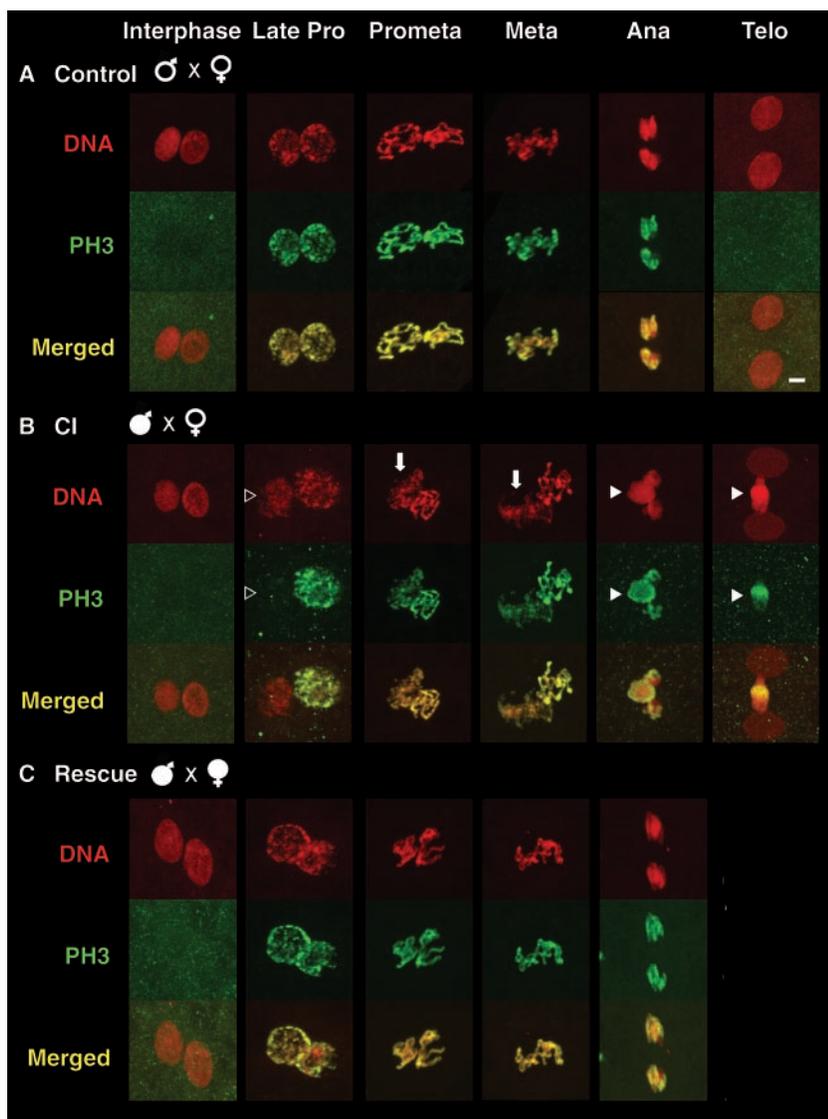
velope breakdown to nuclear envelope formation of the next cell cycle. The male and female pronuclei were synchronously labeled by PH3, which suggests that Cdk1 was synchronously activated in the two pronuclei. It was rare to see PH3 labeling only one of the two pronuclei (3 of 28 embryos at late prophase). Thus, chromosome condensation driven by Cdk1 activation proceeded synchronously in the two pronuclei. Male and female pronuclei normally entered mitosis and proceeded through anaphase at the same rate.

In CI embryos, we observed PH3-posi-

tive chromosomes from late prophase through telophase (Fig. 2B). In contrast to control embryos, CI embryos displayed significant asynchrony in PH3 staining of the two pronuclei. During late prophase, 11 of 22 embryos showed PH3 staining in only one pronucleus (Fig. 2B). Of these 11 embryos, 3 showed similar states of chromosome condensation between the two pronuclei, and 8 showed unequal chromosome condensation, with the less condensed pronucleus being negative for PH3 (Fig. 2B). Thus, Cdk1 activation and entry into mitosis were asynchronous in the two pronuclei in CI embryos. Because only male chromosomes are undercondensed and ultimately lost (6, 7, 11) (Fig. 2B), it is likely that the male pronucleus was delayed in activation of Cdk1 and entered mitosis later than the female pronucleus. As a consequence, paternal chromosomes were not properly condensed when the chromosomes aligned on the metaphase plate and improperly segregated at anaphase (Fig. 2B). At telophase, the paternal genome formed a bridge between the two mitotic products and remained PH3 positive (Fig. 2B), which further suggests that the male pronucleus was delayed in entering and exiting mitosis relative to the female pronucleus.

Thus, delayed entry into mitosis of the male pronucleus is likely to be the primary defect in CI embryos, with chromosome condensation defects a secondary consequence of this delay. To test this directly, we injected rhodamine-tubulin into control and CI embryos and monitored nuclear envelope breakdown (NEB) (19), an early event that signals entry into mitosis in insect embryos (22). In control embryos, NEB of male and female pronuclei occurred synchronously (within 30 s of each other) (Fig. 3). In 9 of 10 embryos, NEB of the two pronuclei was synchronous (Fig. 4). As this rapid event requires analyzing living embryos, see the movies available as supplementary information at *Science Online* (19).

In CI embryos, NEB of the two pronuclei never occurred synchronously (Fig. 3). For 8 of 10 embryos, relative timing of NEB differed by 31 to 90 s. In the remaining 2 embryos, NEB of one pronucleus occurred more than 90 s later than NEB of the second pronucleus (Fig. 4). Based on chromosomal defects and ultimate loss of the male pronucleus (6, 7, 11), we deduced that timing of the male pronucleus was delayed. Because male and female pronuclei are juxtaposed and share the first mitotic spindle, the female pronucleus drives spindle assembly for both. As a consequence of delayed NEB, the interval between NEB and spindle assembly is shorter for the male than for the female pronucleus. Consequently, there is insufficient time for paternal chromosomes to properly



**Fig. 2.** Relative timing of histone H3 phosphorylation (PH3) was disrupted in CI embryos. (A) Control ( $n = 109$ ), (B) CI ( $n = 89$ ), and (C) rescue ( $n = 144$ ) embryos (0 to 1 hour) were fixed and stained for DNA (red) and PH3 (green). Positive PH3 chromosomes indicate high Cdk1 activity. In control (A) and rescue (C) embryos, the timing of H3 phosphorylation at late prophase (late pro) and dephosphorylation at anaphase (ana) was synchronous between the two pronuclei. In CI embryos (B), often only one of the pronuclei was positive for PH3 (open arrowhead). At prometaphase (prometa) and metaphase (meta), the male pronucleus (closed arrows) was less condensed than the female pronucleus and at anaphase formed a bridge (closed arrowhead) between the segregating maternal chromosomes. At telophase (telo), the lagging male chromosomes remained PH3 positive (closed arrowhead). Scale bar = 4  $\mu\text{m}$ .

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condense. The extent of chromosome condensation and alignment on the metaphase plate is correlated with this interval (22). Because NEB is induced by active Cdk1, delayed NEB in CI embryos may be a direct consequence of delayed Cdk1 activation (Web fig. 2) (19).

CI is rescued when the egg is also infected with *Wolbachia*. This rescue may occur by delaying NEB of the female pronucleus to restore synchrony between the two pronuclei (9). To test this, we injected rhodamine-tubulin into rescue embryos, embryos derived from two infected parents, and timed NEB. Synchrony of NEB in the male and female pronuclei was significantly restored (Fig. 4). NEB of the male and female pronuclei occurred synchronously in 4 of 9 embryos and occurred between 31 and 60 s in the remaining 5. This further supports the model that *Wolbachia*-induced CI is the result of a timing mismatch between male and female pronuclei at the first mitotic division. When we used anti-PH3 to analyze entry into mitosis and chromosome condensation, we observed that male and female pronuclei were once again synchronous (Fig. 2C). Similar to control embryos, rescue embryos were positive for PH3 from late prophase through anaphase. Furthermore, we never observed em-

bryos in which only one pronucleus was positive for PH3. Thus, it is likely that NEB of male and female pronuclei were delayed in embryos derived from two infected parents.

Embryos derived from an uninfected male and an infected female (CI's reciprocal cross) develop normally. To test whether embryos from this cross are compatible because the male and female pronuclei are synchronous, we injected rhodamine-tubulin and timed NEB. NEB was synchronous in 9 of 10 embryos (Fig. 4), similar to control embryos. We think infected eggs are compatible with both infected and uninfected sperm because *Wolbachia* present in the egg can influence both the male and female pronuclei. Infected sperm are incompatible with uninfected eggs because sperm from infected males are modified but cannot modify the female pronucleus. *Wolbachia* modify sperm during spermatogenesis but then are shed from the mature sperm (23). As a result, only the sperm are affected, leading to asynchrony and CI.

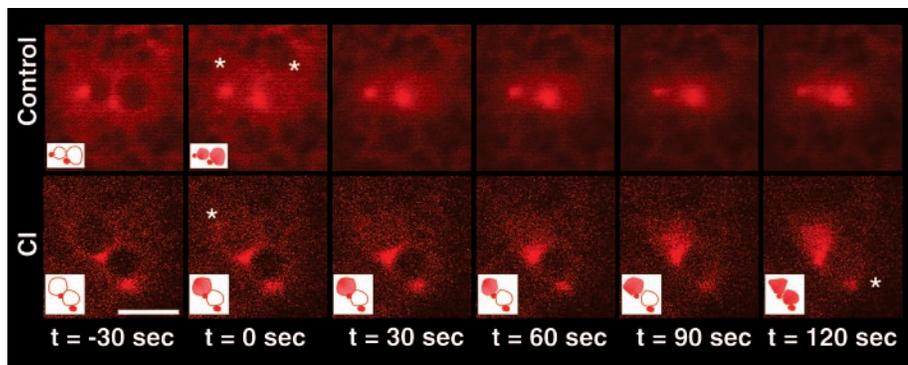
*Wolbachia* may target cell cycle regulators that time entry into mitosis (19). Timing of NEB is positively controlled by activation of the Cdk1/cyclin B complex and negatively controlled by checkpoints that inhibit its activation. *Wolbachia* may be functioning by

directly inhibiting Cdk1 or by activating a cell cycle checkpoint. Activation of Cdk1 is regulated by the relative nuclear import/export rates of cyclin B, and both *Wolbachia* and cyclin B concentrate at the centrosome during interphase (24–28). One possibility is that *Wolbachia* inhibit the ability of cyclin B to be imported into the nucleus. Alternatively, the *Wolbachia*-induced delay of NEB may result from activation of a cell cycle checkpoint in the male pronucleus. For example, significant delays in NEB are induced by inhibition of S phase (22, 29). Some bacteria species produce toxins that are potent cell cycle inhibitors (30, 31), which presents an intriguing alternative explanation for the *Wolbachia*-induced cell cycle delays observed in CI.

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32. Supported by grants from NSF (0091265) and NIH (GM16409). We thank M. Wolfner, K. Liu, K. Fredrick, D. States, S. Charlat, and J. Werren for thoughtful discussions and helpful comments.

4 February 2002; accepted 25 March 2002



**Fig. 3.** Relative timing of NEB was disrupted in CI embryos. NEB was assessed by time-lapsed confocal microscopy of 0- to 1-hour embryos injected with rhodamine-tubulin. When the nuclear envelope was intact, the nucleus appeared as a black circle surrounded by a ring of red (rhodamine-tubulin) ( $T = -30$  s). At NEB (indicated by \*), rhodamine-tubulin invaded the nucleus (control,  $T = 0$ ; CI,  $T = 0$  and  $T = 120$  s). (Insets) Schematized interpretation of NEB. This is a rapid event that requires analysis of living embryos. See movies at Science Online (19). Scale bar = 16  $\mu$ m.

**Fig. 4.** Relative timing of NEB in male and female pronuclei. Open symbols, uninfected; closed symbols, infected. NEB of the male and female pronuclei occurred synchronously (within 30 s of each other) in control embryos (white). NEB of male and female pronuclei occurred asynchronously ( $>30$  s apart) in CI embryos (red). Defects in NEB were suppressed in rescue embryos (black). NEB was synchronous in embryos derived from CI's reciprocal cross (gray).

