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Acknowledgments: We thank E. Avetissova for technical assistance, S. Mashko for materials, and members of the Nudler laboratory for valuable comments and discussion. This research was supported by the NIH Director's Pioneer Award, Biogerontology Research Foundation, and Dynasty Foundation (E.N.). Provisional patent application has been filed: U.S. Patent No. 61/438,524 "Methods for treating infections by targeting bacterial H_2S-producing enzymes." by K.S. and E.N.

Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6058/986/DC1
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15 June 2011; accepted 14 September 2011
10.1126/science.1209855

Wolbachia Enhance *Drosophila* Stem Cell Proliferation and Target the Germline Stem Cell Niche

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Wolbachia are widespread maternally transmitted intracellular bacteria that infect most insect species and are able to alter the reproduction of innumerous hosts. The cellular bases of these alterations remain largely unknown. Here, we report that *Drosophila mauritiana* infected with a native *Wolbachia w*Mau strain produces about four times more eggs than the noninfected counterpart. *Wolbachia* infection leads to an increase in the mitotic activity of germline stem cells (GSCs), as well as a decrease in programmed cell death in the germarium. Our results suggest that up-regulation of GSC division is mediated by a tropism of *Wolbachia* for the GSC niche, the cellular microenvironment that supports GSCs.

Wolbachia are maternally transmitted intracellular bacteria infecting a large number of invertebrates such as insects and parasitic worms (*1*). Many invertebrates that harbor these bacteria are either the vectors (for instance, mosquitoes) or the causative agent (for example, filarial nematodes) of devastating human infectious diseases. By understanding the biology at the interface between *Wolbachia* and their hosts, advances in the treatment of filarial diseases and the control of disease vectors are made possible (*2*–*7*). Furthermore, *Wolbachia* can dramatically alter host reproduction, affecting the evolutionary history of numerous invertebrates (*1*). Therefore, understanding how *Wolbachia* affect their hosts is an important ecological, evolutionary, and human health question.

To investigate the influence of *Wolbachia* on their hosts at the cellular level, we used the *Drosophila* gonad, a powerful experimental system. We have previously shown that in *Drosophila melanogaster*, *Wolbachia* target the somatic stem cell niche (SSCN) (Fig. 1A), the microenvironment that supports the somatic stem cells, in the female ovary (*8*). Further work shows that *Wolbachia* also target the somatic stem cell niche in the ovary of other insects (*9*, *10*). Here, we report two additional stem cell niches preferentially colonized (i.e., cell tropism) by *Wolbachia*: the female germline stem cell niche (GSCN) (Fig. 1A) and the hub, at the apical tip of the testis (discussed below). In a *D. mauritiana* stock infected with *Wolbachia w*Mau, we consistently noticed an intense accumulation of bacteria in the GSCN, the structure harboring the GSCs (infection frequency = 91 ± 5.7%, N = 958 germaria) (see *Wolbachia*, labeled green in Fig. 2, A and B, Fig. 3A, and fig. S1A). This GSCN accumulation was absent in *D. melanogaster* (GSCN infection frequency = 0%, N = 180 germaria) (see fig. S1, B compared to A). Electron microscopy (EM) and three-dimensional reconstruction of confocal images show that the vast majority of the cytoplasmic volume of the GSCN is occupied by *Wolbachia w*Mau [see Fig. 1B, the *Wolbachia* cells (a red asterisk indicates a single bacterial cell) occupy most of the GSCN (shown in green) compared with the noninfected control in fig. S1C; see also movie S1]. Because GSCN function is essential for stem cell maintenance and activity (*11*), we hypothesized that the high levels of infection in the niche would impair its associated stem cells to a certain degree. An easy readout of GSC activity is egg production, because every egg produced originates from the division of a stem cell associated with the GSCN (Fig. 1A′). The total number of eggs laid per *Wolbachia*-infected female was 3.5 times higher than that observed in noninfected flies (herein referred to as "W–"; the genetic background of the W– flies was homogenized by successive backcrossing to infected males, as shown in fig. S2). This experiment was repeated under different temperature, humidity, and age conditions [see supporting online material (SOM) methods and table S1] (*12*). Under these different conditions, infected flies (W+) still produced approximately fourfold more eggs than the noninfected females (Fig. 1C and table S1).

Given these levels of egg production, we reasoned that W+ ovaries contain GSCs that are more active. To test this possibility, we measured the frequency of GSC division in W+ and W– flies using three different markers for three distinct phases of the cell cycle. We performed the initial assessment with the use of an antibody to phospho-histone H3, which labels cells in mitosis (Fig. 2, A and C, and fig. S3G) (*12*). The labeling of GSCs in W+ flies was, on average, 2.7 (± 0.23)–fold higher than in W– flies (Fig. 2E and table S2). This increase could indicate either a higher GSC division in infected germaria or an arrest during the mitotic phase of the cell cycle.

We further investigated GSC proliferation using two additional markers: incorporation of the thymidine analog BrdU, an indicator of DNA synthesis during S phase (fig. S3, A, D, and G), and a particular fusome morphology characteristic of GSCs in G_2 (fig. S3, B, E, and H). The fusome is a germline-specific organelle that assumes the shape of an exclamation mark (!) during G_2 (*13*, *14*). Both markers corroborated a higher GSC proliferation rate in W+ (Fig. 2E). In nine independent experiments using three different methods, stem cell division in W+ flies was, on average, doubled (2.12 ± 0.66) (table S2). For

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all three methods, the increase in probability of GSC division in W+ was statistically significant (Fig. 2E, SOM methods, and table S2) (*12*). Although substantial, a twofold increment in GSC mitotic activity by itself does not suffice to explain the fourfold increase in egg production in infected flies. An additional cellular event that could alter egg production in a *Wolbachia*-dependent manner could be cell death in the ovary. Programmed cell death (PCD) is a known key regulator of egg production in *D. melanogaster* (*15*). Furthermore, previous studies in wasps and human neutrophils have shown that the presence of *Wolbachia* or *Wolbachia*-derived proteins, respectively, inhibits host apoptosis (*16*, *17*).

We quantified the influence of *Wolbachia* infection on two developmentally regulated PCD events that modulate egg production in *Drosophila*, the first in the germarium (Fig. 1A, left red arrow) and the second during the onset of vitellogenesis (Fig. 1A, right red arrow) (*15*, *18*). In the parasitic wasp *Asobara tabida*, removal of *Wolbachia* causes sterility through massive cell death in mid-oogenesis, at the previtellogenic stages (*16*). Therefore, we initially measured PCD at these stages. We found that the differences in PCD between W+ and W– previtellogenic egg chambers were highly variable and not significant regarding *Wolbachia*'s effects at this developmental point (fig. S4 and table S3) (*12*).

Accordingly, we measured the levels of PCD in the germarium. Using two different assays—DNA fragmentation in fixed tissue [terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL)] (Fig. 2, B and D) and visualization of dead cells via live imaging (Acridine orange) (fig. S3, C and F)—*Wolbachia* infection consistently decreased PCD in the germarium by approximately one-half as compared with noninfected flies (Fig. 2F and table S4) (*12*). *Wolbachia*-driven reduction of PCD in the germarium was statistically significant (Fig. 2 and table S4). Together, these results indicate that the increase in egg production in W+ *D. mauritiana* is due to both increased GSC mitosis and decreased PCD in the germarium.

Next, we examined the mechanistic foundation for *Wolbachia's* manipulation of GSC mitotic activity. Considering that GSCN regulates stem cell physiology (*19*), we designed an experiment to test whether levels of *Wolbachia* in the GSCN correlate with mitotic activity of the

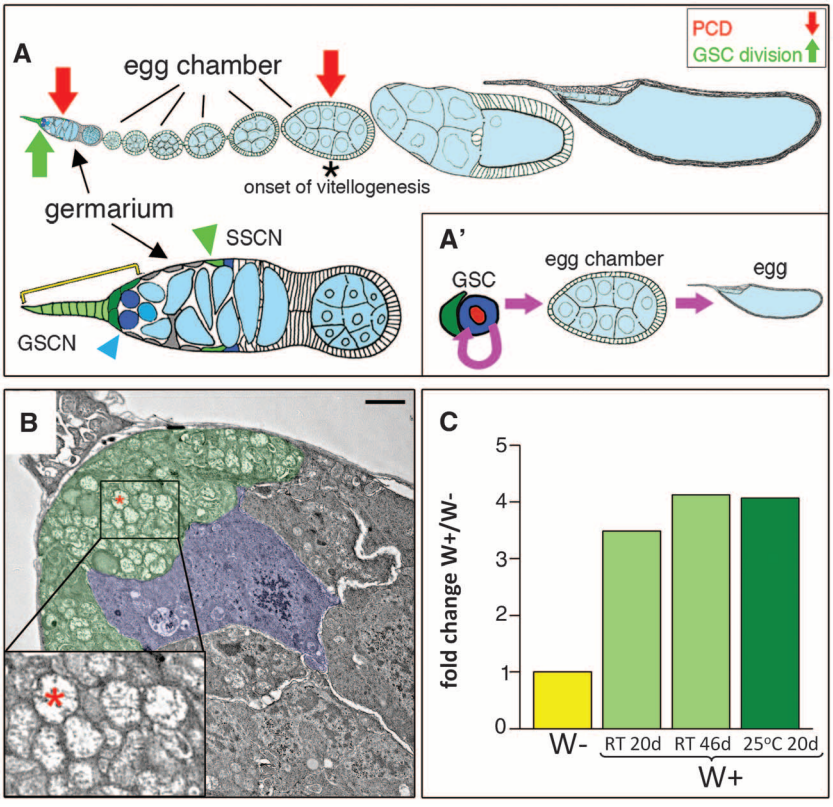

Fig. 1. *Wolbachia* target the GSCN, and infection increases egg production. (**A**) *Drosophila* ovariole with the germline shown in light blue and the somatic follicle cells in white. Egg chambers are formed in the germarium (left) and mature into the egg. The upward-pointing green arrow indicates GSC (dark blue) division, which positively affects egg production [see inset (**A'**): GSC divides asymmetrically, and one daughter cell exits the GSCN (green) and forms the egg's germline (light blue)]. The downward-pointing red arrows indicate developmental points where the onset of PCD reduces egg production, either in the germarium or in previtellogenic egg chambers. The black asterisk indicates the onset of vitellogenesis. (Lower left) A magnified view of the germarium shows both the SSCN (green arrowhead) and the GSCN (yellow bracket), formed by the terminal filament (light green) and the cap cells (dark green), which contact the GSCs (blue arrowhead). (**B**) Electron micrographs of a GSCN (green) and the GSC (blue) in infected *D. mauritiana*. Most of the cytoplasm of the cap cells (GSCN) is occupied by *Wolbachia* *w*Mau (red asterisk) (see also movie S1). Scale bar, 1 μm. (Inset) Magnified view of the GSCN. (**C**) Fold change of total amount of eggs laid per infected female (W+, green) under different conditions normalized to noninfected females (W–, yellow). Relative egg production was measured in triplicate for each condition: room temperature [(RT), 20 and 46 days (d), light green] or at 25°C (20 days, dark green). *Wolbachia* significantly induced fecundity gains at all conditions (Student's *t* test, $P_{RT\ 20\ days} = 6.5 \times 10^{-4}$, $P_{RT\ 46\ days} = 3.9 \times 10^{-4}$ and $P_{25°C\ 20\ days} = 1.7 \times 10^{-2}$) (table S1) (*12*).

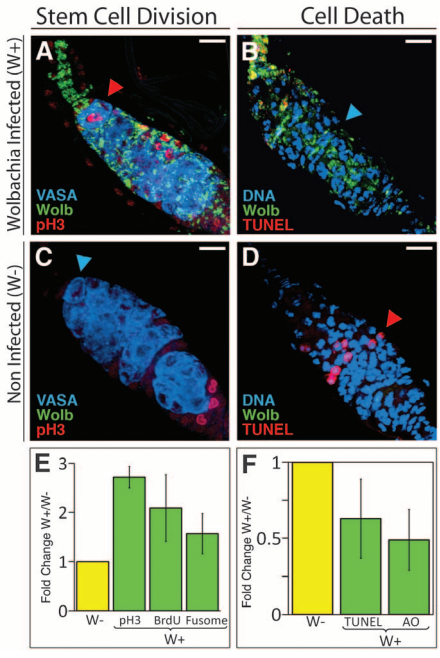

Fig. 2. *Wolbachia* infection increases GSC mitotic activity and suppresses PCD in the germarium. Representative confocal images of *D. mauritiana* germaria infected [W+, *Wolbachia* shown in green (**A** and **B**)] and noninfected [W–, (**C** and **D**)] are shown. Arrowheads indicate the presence (red arrowhead) or absence (blue arrowhead) of GSC division [pH3 (phospho-histone H3), red in (A) and (C)] or PCD [TUNEL, red in (B) and (D)]. Germline is labeled with anti-Vasa (blue). Scale bars, 10 μm. (**E** and **F**). Average fold difference for each marker indicated below the graphs, normalized to W– (mean of triplicates, 15 independent experiments total). Infection significantly affects GSC mitosis (E) and PCD (F) for all markers (logistic regression, $P_{pH3} = 5.4 \times 10^{-3}$, $N = 621$ germaria; $P_{BrdU} = 2.0 \times 10^{-2}$, $N = 1061$; $P_{Fusome} = 4.3 \times 10^{-3}$, $N = 695$; $P_{TUNEL} = 8.0 \times 10^{-3}$, $N = 802$; $P_{Acridine\ orange} = 1.2 \times 10^{-7}$, $N = 754$) (see also tables S2 and S4) (*12*). AO, Acridine orange; error bars indicate SD.

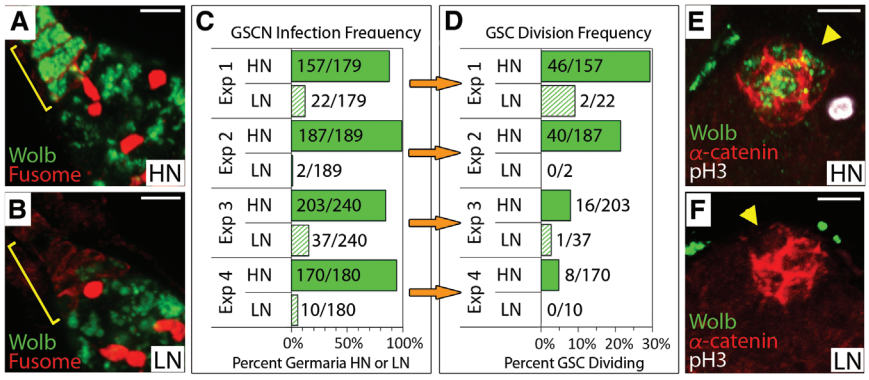

Fig. 3. High levels of *Wolbachia* at the GSCN up-regulate GSC mitosis. (**A** and **B**) Niches (yellow brackets) from infected flies are classified as highly infected [HN, (A)] or with low infection [LN, (B)]. Fusome staining (red) shows GSCs in the HN dividing ["!" morphology in (A)]. Scale bar, 5 μm. (**C**) Frequency of HN (solid green bars) and LN (hatched green bars) in four independent experiments. The numbers in each category and the total number of germaria analyzed are indicated for each experiment. (**D**) For each germarium counted, the frequency of GSC division was determined by either fusome morphology (Exp. 1 and 2) or BrdU incorporation (Exp. 3 and 4). HN significantly increases GSC mitosis (logistic regression, $P = 2.4 \times 10^{-2}$). (**E** and **F**) In infected testes of *D. mauritiana*, *Wolbachia* also target the stem cell niche (hubs, yellow arrowheads) at high [HN, (E)] and low levels [LN, (F)]. (F) pH3 staining (white) labels a dividing testis stem cell adjacent to an HN niche. Scale bars, 5 μm.

GSC (fig. S5). During this assay, we used only *Wolbachia*-infected flies. Even though in these W+ flies most of the GSCNs were highly infected (91 ± 6.5%, $N = 788$) (Fig. 3, A and C), there is a small population of niches that have either very low levels of or no *Wolbachia* present. These distinct types of niches were termed "LN" (low infection in the niche) (Fig. 3B), and their infected counterparts are referred to as "HN" (high infection in the niche) (Fig. 3A; see also fig. S6, A compared to B, and fig. S7). Because these distinct populations of GSCs are present inside the same infected flies, all of the environmental and systemic factors are exactly the same. In four independent experiments, the mitotic activity of GSCs residing in LN niches was substantially lower or absent in comparison with HN niches (Fig. 3, C and D). There is a statistically significant association of GSC mitosis with the high density of *Wolbachia* in the niche ($P = 2.4 \times 10^{-2}$) (table S5) (*12*). This observation favors a mechanism in which *Wolbachia*'s infection in the niche modulates stem cell activity, although it does not rule out a contribution from systemic or stem cell–intrinsic signals (see SOM text S1 and figs. S8 and S9).

We found that *Wolbachia wMau* also target the hub, a group of somatic cells that form the niche supporting germline and somatic stem cells of the testis (*20*). In males, both the targeting of the hub (64%, $N = 77$) (Fig. 3, E and F) as well as the up-regulation of GSC division did not occur to the same degree as in females (fig. S10 and table S6). It is possible that phenotypic consequences of niche tropism are diverse in males. *Wolbachia* and other maternally inherited endosymbionts can evolve drastically different germline-manipulation phenotypes between sexes (*21*).

The vast majority of insects have symbiotic associations with bacteria that are vertically transmitted through the egg cytoplasm (*22*). Because of maternal transmission, these host-bacteria partnerships evolve to favor the reproductive success of infected mothers (*1*, *23*–*25*). In the *Drosophila* genus, there are several reports of *Wolbachia*-induced changes in fecundity, including cases of rapid evolution of both partners, changing from a parasitic to mutualistic association in 20 years (*24*, *26*–*29*). There is little understanding of these dramatic and widespread interferences with host reproduction at the cellular and molecular level (*30*). Here, we have identified two cellular events that are manipulated by *Wolbachia*. The combination of *Wolbachia*-induced alterations of both PCD in the germarium and GSC mitosis results in higher egg production, which further promotes *Wolbachia* spreading through maternal transmission. These findings provide the cellular mechanisms for *Wolbachia*'s effects on host fecundity observed in this infected *D. mauritiana* strain over its noninfected counterpart (see SOM text S2).

Advancing our understanding of how endosymbionts subvert the cellular processes of insects will also be relevant to the growing efforts toward controlling human infectious diseases through symbiotic bacteria (*3*–*7*, *31*).

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Acknowledgments: We thank K. McCall, G. Cooper, D. Waxman, C. Bradham, and A. Boxer for valuable suggestions in the manuscript; E. Wieschaus, the McCall Lab, T. Blute, D. Gantz, and M. Bisher for help and support with PCD and EM experiments; E. Wieschaus, T. Schüpbach, R. Lehmann, P. Lasko, D. Stern, V. Orgogozo, and M. Ramos for fly stocks and reagents; members of the Frydman Lab for assistance and suggestions during the realization of this work; J. Li and D. Robson for help with MatLab software; and A. Mahowald for sharing his unpublished results and encouraging us to analyze *Wolbachia* in the testis. Finally, we would like to thank the anonymous reviewers for their helpful comments. This work was supported by funds from Boston Univ. and National Institute of Allergy and Infectious Diseases (grant 1K22AI74909-01A1 to H.M.F.). The data described in this paper are available in the SOM.

Supporting Online Material

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10 June 2011; accepted 7 October 2011
Published online 20 October 2011;
10.1126/science.1209609

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Science **334**, 990 (2011);
DOI: 10.1126/science.1209609