# A feedback loop between *Wolbachia* and the *Drosophila gurken* mRNP complex influences *Wolbachia* titer

**Laura R. Serbus[1,*], Amy Ferreccio[1], Mariya Zhukova[2], Chanel L. McMorris[1], Elena Kiseleva[2] and William Sullivan[1]**

[1]MCD Biology, University of California, 1156 High St, Santa Cruz, CA 95064, USA
[2]Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia
*Author for correspondence (lrserbus@ucsc.edu)

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## Summary

Although much is known about interactions between bacterial endosymbionts and their hosts, little is known concerning the host factors that influence endosymbiont titer. *Wolbachia* endosymbionts are globally dispersed throughout most insect species and are the causative agent in filarial nematode-mediated disease. Our investigation indicates that *gurken* (*grk*), a host gene encoding a crucial axis determinant, has a cumulative, dosage-sensitive impact on *Wolbachia* growth and proliferation during *Drosophila* oogenesis. This effect appears to be mediated by *grk* mRNA and its protein-binding partners Squid and Hrp48/Hrb27C, implicating the *grk* mRNA–protein (mRNP) complex as a rate-limiting host factor controlling *Wolbachia* titer. Furthermore, highly infected flies exhibit defects that match those occurring with disruption of *grk* mRNPs, such as nurse cell chromatin disruptions and malformation of chorionic appendages. These findings suggest a feedback loop in which *Wolbachia* interaction with the *grk* mRNP affects both *Wolbachia* titer and *grk* mRNP function.

**Key words:** *Wolbachia*, *Drosophila*, Oogenesis, *gurken*, mRNP

## Introduction

A wide range of organisms carry endosymbionts that provide resources that are necessary for host success. Because endosymbionts also rely upon host factors, both partners benefit from reliable endosymbiont transmission from host to offspring. One effective transmission strategy is for endosymbionts to interact with key developmental factors in the host, as is seen in embryos of the leaf hopper *Euscelis plebejus*. *Euscelis* endosymbionts associate with host posterior-patterning factors, localizing the symbionts in a ball to the embryo posterior (Sander, 1959; Sander, 1960). The localized symbionts help to induce formation of the mycetocyte tissue that ultimately positions the symbionts to invade developing eggs (Sander, 1968; Korner, 1976). Endosymbiotic *Wolbachia* bacteria use a conceptually similar strategy, associating with host germline determinants that promote inclusion of *Wolbachia* into maternal germline cells (Serbus and Sullivan, 2007).

Assurance of endosymbiont transmission from host to offspring relies upon maintenance of an appropriate level of the symbiont within the host. Reduced symbiont loads can result in a failure of symbiont transmission, whereas excessive symbiont loads can lead to host mortality. For example, a virulent strain of *Wolbachia* has been shown to replicate inappropriately in the adult nervous system, causing paralysis and early death (Min and Benzer, 1997). Although it is clear that host factors have a strong influence over *Wolbachia* titer (Boyle et al., 1993; Poinsot et al., 1998; McGraw et al., 2002; Veneti et al., 2004; Kondo et al., 2005; Serbus et al., 2008), little is known about the identity and function of these titer-influencing factors.

*Wolbachia* endosymbionts present a unique model for investigating the molecular underpinnings of host–symbiont interactions. *Wolbachia* are obligate intracellular bacteria carried by the majority of insect species, as well as some mites, crustaceans and filarial nematodes (Stouthamer et al., 1999; Serbus et al., 2008; Werren et al., 2008). *Wolbachia* are transmitted from female hosts to their offspring, in a manner that is analogous to mitochondrial inheritance. In some situations *Wolbachia* confer a selective advantage upon infected females by inducing parthenogenesis, feminization of males, male-killing or sperm–egg cytoplasmic incompatibility. *Wolbachia* are also now associated with neglected diseases carried by 150 million people. In these cases, *Wolbachia* endosymbionts of filarial nematodes are released into the human body, triggering an inflammatory response that causes African river blindness and probably contributes to the pathology of lymphatic filariasis (Saint Andre et al., 2002; Debrah et al., 2006; Debrah et al., 2007; Taylor et al., 2008; Debrah et al., 2009; Turner et al., 2009).

*Wolbachia* have the advantage that they can be studied in a well-established model system, *Drosophila* oogenesis. *Wolbachia* are carried naturally in this system, and *Drosophila* oogenesis has been studied intensively for many years as a paradigm for axis determination, providing a wealth of genetic, cellular and biochemical tools (King, 1970; Ashburner, 1989; Spradling, 1993; van Eeden and St Johnston, 1999; Riechmann and

Ephrussi, 2001). The *Drosophila* oocyte develops within the context of an egg chamber, consisting of an outer layer of somatic follicle cells surrounding two types of germline cells: 15 nurse cells and an oocyte, which are interconnected by cytoplasmic bridges known as ring canals (King, 1970). Egg chamber development proceeds through 14 morphologically distinct stages over a 3 day period.

*Wolbachia* localization and transmission is linked to core processes underlying *D. melanogaster* oogenesis (Ferree et al., 2005; Frydman et al., 2006; Serbus and Sullivan, 2007). *Wolbachia* initially concentrate at the oocyte anterior during early oogenesis (Ferree et al., 2005). This anterior enrichment depends upon microtubules and the minus-end-directed motor protein Dynein. *Wolbachia* are homogeneously distributed throughout the oocyte in mid-oogenesis, and then become enriched along the posterior cortex during mid to late oogenesis (Veneti et al., 2004; Serbus and Sullivan, 2007). This posterior concentration requires an active contribution by *Wolbachia*, as well as host microtubules, the plus-end-directed microtubule motor protein kinesin-1 and pole plasm, a complex mixture of components that specifies the posterior axis and germ cell fates in embryogenesis (Serbus and Sullivan, 2007). The posterior pole plasm and associated *Wolbachia* become enveloped by germ cells later in embryogenesis, promoting maternal transmission of *Wolbachia*.

Here, we examine the influence of host factors on intracellular *Wolbachia* titer. We have identified *grk*, a host gene encoding a crucial axis determinant, as a key component of an mRNP complex that regulates *Wolbachia* proliferation in *Drosophila* oogenesis. In instances where *Wolbachia* titer is high, we also detect phenotypes associated with impaired *grk* function. This indicates that *Wolbachia* can influence the function of this conserved morphogen and suggests a feedback mechanism that limits *Wolbachia* titer.

## Results

### Dosage of the host *grk* gene is correlated with intracellular *Wolbachia* titer

*Wolbachia* localization and transmission is promoted by interactions with host cytoskeleton and axis-determination factors in oogenesis (Ferree et al., 2005; Serbus and Sullivan, 2007). Therefore, we screened host factors that regulate transport, trafficking and developmental processes in oogenesis for their impact on *Wolbachia* biology using confocal microscopy. Mutant egg chambers were stained, imaged and examined for unconventional *Wolbachia* phenotypes including shifts in *Wolbachia* titer. Screening of 21 mutant strains revealed that oocytes mutant for *gurken* (*grk*) exhibited clearly reduced *Wolbachia* titer (Fig. 1A,B). The *grk* gene is known to synthesize a transforming growth factor alpha (TGFα) protein, which is crucial for antero–posterior and dorsal–ventral axis determination in oogenesis (Schupbach, 1987; Neuman-Silberberg and Schupbach, 1993; Roth and Schupbach, 1994; Gonzalez-Reyes et al., 1995; Roth et al., 1995; Neuman-Silberberg and Schupbach, 1996).

To quantify *Wolbachia* depletion in *grk* mutant oocytes, bacterial titer was scored by taking images from a single focal plate of a stage 10A oocyte stained with propidium iodide. Previous work demonstrated this procedure robustly stains *Wolbachia*, enabling rapid quantification (Ferree et al., 2005; Serbus and Sullivan, 2007). In our initial tests, stage 10A wild-type oocytes carried 456±70 (mean ± s.e.m.; $n=15$) *Wolbachia* within a single focal plane. By contrast, oocytes carrying the severe mutation $grk^{HK36}$ exhibited an average of 97±16 *Wolbachia* ($n=15$), about five-times less than in wild-type controls. To discern whether this was indeed due to *grk* or a second site mutation, other genetic disruptions of *grk* were also tested. Hemizygous oocytes carrying $grk^{HK36}$ and one copy of a chromosomal deficiency removing the *grk* coding region exhibited 81±7.5 *Wolbachia* ($n=15$). Similarly, mutants carrying the hypomorphic allele $grk^{DC9}$ yielded 85±11 *Wolbachia* per oocyte ($n=15$). The agreement of results from multiple *grk* disruptions suggests that the host *grk* gene affects intracellular *Wolbachia* titer.

To investigate whether *Wolbachia* are sensitive to the dosage of host *grk*, we next quantified *Wolbachia* in heterozygous and homozygous *grk* mutant oocytes (Fig. 1C). From this point forward, all strains were raised in parallel, rigorously standardized conditions (see the Materials and Methods). We also quantified all mutant oocytes in parallel with wild-type controls and normalized all values against the wild-type average to preserve maximum comparability between experiments. In this analysis, wild-type stage 10A oocytes carried 505±22.5 bacteria within a single focal plane ($n=107$). By contrast, oocytes heterozygous for $grk^{HK36}$ exhibited 304±32 *Wolbachia* ($n=30$), and $grk^{HK36}$ homozygous oocytes had 118±14 *Wolbachia* ($n=20$). Furthermore, $grk^{DC9}$ heterozygous oocytes showed 216±31 *Wolbachia* ($n=20$), and $grk^{DC9}$ homozygous oocytes revealed 119±11 *Wolbachia* ($n=18$). These results indicate a decrease in *Wolbachia* titer that is consistent with a reduction in *grk* function. To further test for dosage sensitivity, we overexpressed the *grk* gene as previously reduction (Ghiglione et al., 2002). This *grk* overexpression correlated with a significant increase in *Wolbachia* titer, with 689±62 bacteria visible in the oocyte ($n=37$). These data indicate that *Wolbachia* titer in the oocyte is dependent upon dosage of the host *grk* gene.

A change in oocyte titer could be due to an overall change in *Wolbachia* quantity throughout the egg chamber, or alternatively to a failure of *Wolbachia* import from adjacent nurse cells into the oocyte. To address this, *Wolbachia* were quantified in single focal planes of wild-type and *grk* mutant nurse cells (Fig. 2). At stage 10A, wild-type nurse cells had on average 33±2.0 bacteria each ($n=70$). However, $grk^{HK36}$ heterozygous nurse cells exhibited 19±1.8 *Wolbachia* ($n=30$) and 7.6±1.8 *Wolbachia* were detected in $grk^{HK36}$ homozygotes ($n=20$). Following a similar trend, nurse cells of $grk^{DC9}$ heterozygotes contained approximately 20±1.4 *Wolbachia* ($n=20$), and $grk^{DC9}$ homozygotes had 16±1.9 *Wolbachia* ($n=18$). Thus, the depletion of *Wolbachia* in the oocyte is not due to obvious retention of the bacteria within nurse cells. The combined oocyte and nurse cell data suggest that host *grk* contributes to general control of *Wolbachia* titer in late-stage egg chambers.

### *grk* has a cumulative impact on *Wolbachia* titer

To investigate whether *grk* also influences *Wolbachia* titer during the initial stages of oogenesis, we assayed titer in stage 2–3 egg chambers from wild-type and *grk* mutant flies (Fig. 3A,B). In oocytes, *grk* disruption correlated with 31% fewer *Wolbachia* than wild-type, with wild-type oocytes exhibiting 18±2.9 *Wolbachia* ($n=20$) compared with 12±1.7 *Wolbachia* for $grk^{HK36}$ oocytes ($n=20$) (Fig. 3C). Similarly, *grk* disruption in nurse cells resulted in a 40% drop in *Wolbachia* titer, with wild-

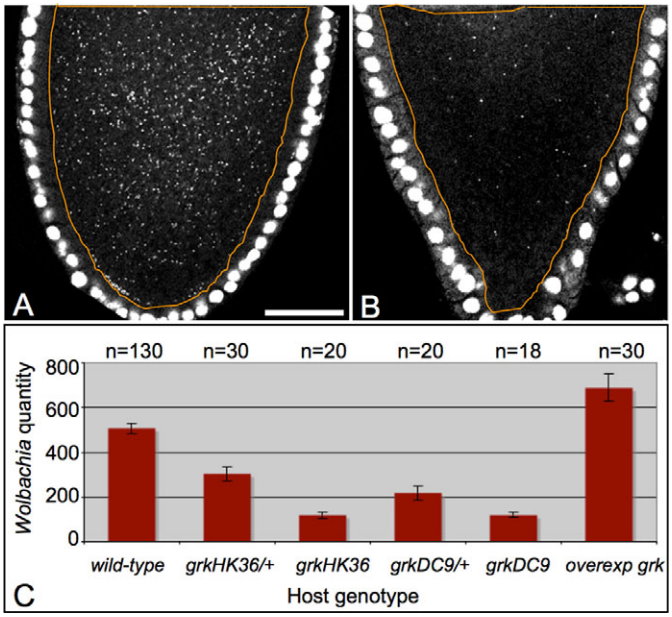


**Fig. 1. *Wolbachia* titer is affected by the host *grk* gene in stage 10A oocytes.** *Wolbachia*-infected oocytes were stained with propidium iodide, imaged by confocal microscopy, and analyzed. Posterior is down. Orange lines show oocyte boundaries. Puncta within oocytes represent *Wolbachia*. (**A**) Wild type. (**B**) $grk^{HK36}$. (**C**) Mean number (± s.e.m.) of *Wolbachia* puncta detected in a single focal plane of oocytes that are wild type, *grk* mutants or overexpress *grk*. Scale bar: 25 µm.

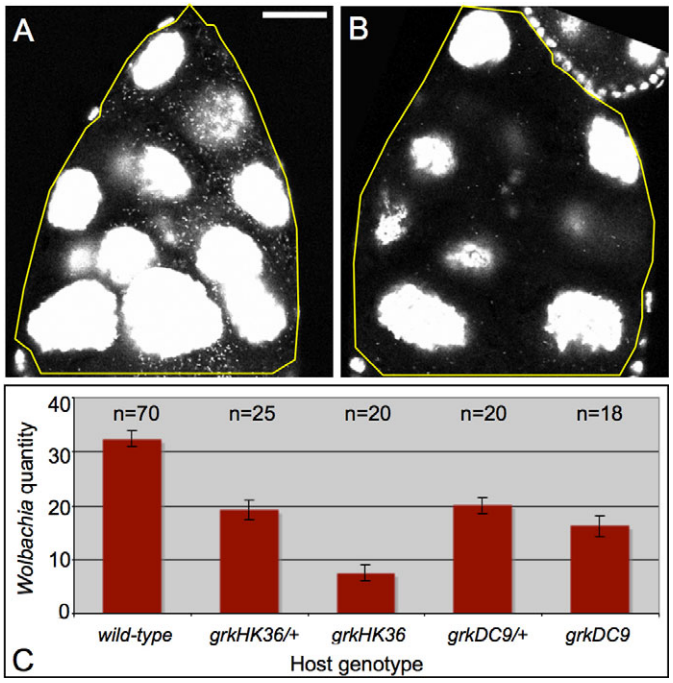


**Fig. 2. Host *grk* dosage affects *Wolbachia* titer in stage 10A nurse cells.** Confocal images of *Wolbachia*-infected nurse cells were acquired and analyzed. Posterior is down. (**A**) Wild type. (**B**) $grk^{HK36}$. Nurse cells are outlined in yellow. Large white areas are host nuclei. Small puncta represent *Wolbachia*. (**C**) Mean number (± s.e.m.) of *Wolbachia* puncta detected per nurse cell within a single focal plane of wild-type and *grk* mutant nurse cells. Scale bar: 25 µm.

type nurse cells exhibiting 11±1.7 *Wolbachia* on average ($n$=20) whereas $grk^{HK36}$ nurse cells displayed 6.9±0.61 bacteria ($n$=20) (Fig. 3D). Thus *grk* mutant egg chambers carry significantly fewer *Wolbachia* than control wild-type egg chambers, even early in oogenesis. This result also highlights how titer reduction in *grk* becomes magnified as oogenesis proceeds, with a 30–40% titer depletion at stage 2–3 becoming a 77% titer depletion by stage 10A (Fig. 1C, Fig. 2C). This indicates that host *grk* is important for elevation of the *Wolbachia* titer that normally occurs as oogenesis proceeds.

### *grk* has little impact on *Wolbachia* mortality

The significant impact of *grk* on *Wolbachia* titer raises the question of whether *grk* impacts the *Wolbachia* life cycle by affecting bacterial mortality or replication. To determine whether *grk* affects *Wolbachia* mortality, *Wolbachia* were examined in detail using quantitative electron microscopy in wild-type and *grk* nurse cells ($n$=1253 and $n$=808, respectively). Most of the bacteria appeared ovoid in wild-type and *grk* (91% and 83%, respectively; Fig. 4A). A sub-population of *Wolbachia* exhibited a bi-lobed morphology, appearing at a similar frequency in the wild type and $grk^{HK36}$ (2% vs 3%; Fig. 4B). A distinct group of bacteria were visible as doublets, with significantly fewer evident in wild-type than in *grk* mutant nurse cells (7% vs 14%, $P<0.05$; Fig. 4C,D). However, all bacteria examined in wild-type and *grk* nurse cells were encompassed by smooth, contiguous membrane typical of *Wolbachia* (Stouthamer et al., 1999; Serbus et al., 2008; Werren et al., 2008), unlike the whorled, fragmented appearance observed for *Wolbachia* killed by tetracycline (Ghedin et al., 2009). The bacterial cytoplasm also appeared homogenous, lacking the electron-dense accumulations visible in *Wolbachia* after heat shock or when heavily loaded with phage particles (Bordenstein et al., 2006; Zhukova et al., 2008). Thus, although there are some differences in *Wolbachia* morphology between the wild type and *grk*, there does not appear to be any obvious difference in *Wolbachia* mortality rates during oogenesis.

### *grk* influences *Wolbachia* titer mainly through a microtubule-independent mechanism

Previous studies demonstrated that disruption in the microtubule organization in the oocyte reduces *Wolbachia* titer (Ferree et al., 2005). *grk* has also been shown to exert a major influence over cytoskeletal organization (Clark et al., 1994; Gonzalez-Reyes et al., 1995; Roth et al., 1995). To test whether *grk* impact on titer is exerted through microtubules, the flies were fed with the microtubule-disrupting drug colcemid and *Wolbachia* titer assayed in stage 8–9 egg chambers (Fig. 5A,B) (Theurkauf et al., 1992; Serbus and Sullivan, 2007). In wild-type nurse cells, colcemid had no significant effect on *Wolbachia* titer, with untreated nurse cells exhibiting an average of 13±0.90 bacteria ($n$=18), and colcemid-treated conditions yielding 14±1.4 *Wolbachia* ($n$=17) (Fig. 5D). $grk^{HK36}$ nurse cells were similarly unaffected by colcemid, with untreated nurse cells displaying 9.5±0.56 *Wolbachia* on average ($n$=18), and colcemid-treated nurse cells showing 8.7±1.1 bacteria ($n$=17) (Fig. 5D). This indicates that *grk* impact on *Wolbachia* titer in nurse cells is independent of microtubules.

Consistent with previous work (Ferree et al., 2005), colcemid treatment of wild-type oocytes yielded a 32% depletion of *Wolbachia* titer, with untreated oocytes displaying approximately

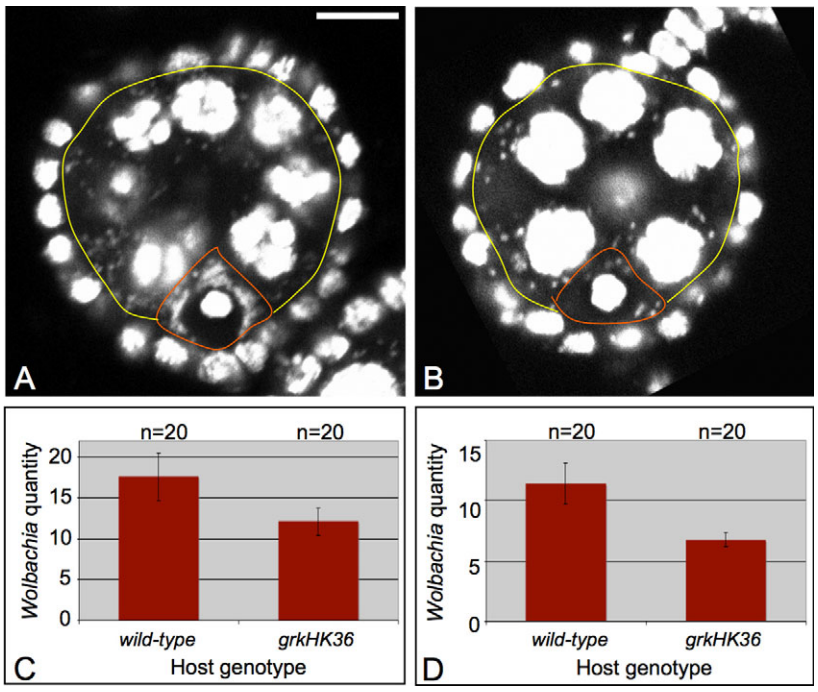


**Fig. 3. *Wolbachia* titer is affected by *grk* at stage 2–3 of oogenesis.** Oocyte is outlined in orange and nurse cells in yellow. (**A**) Wild type. (**B**) $grk^{HK36}$. (**C**) Mean *Wolbachia* quantity in a single oocyte focal plane. (**D**) Mean *Wolbachia* per nurse cell in the same focal plane. Data are means ± s.e.m. Scale bar: 10 μm.

117±16 *Wolbachia* ($n$=18), and colcemid-treated oocytes exhibiting 80±14 *Wolbachia* ($n$=17) (Fig. 5C). However, disrupting *grk* alone gave rise to a depletion of *Wolbachia* that is more severe than that seen in any of the wild-type conditions, with $grk^{HK36}$ oocytes displaying 56±7.5 *Wolbachia* ($n$=18). Furthermore, colcemid-treated $grk^{HK36}$ oocytes presented a much more severe reduction in *Wolbachia* titer, with oocytes exhibiting 22±3.2 *Wolbachia* on average ($n$=17) (Fig. 5C). These data indicate that although microtubules might have a role alongside *grk* on *Wolbachia* titer in oocytes, much of the contribution of *grk* is exerted by a microtubule-independent pathway, as observed in the nurse cells (Fig. 4D).

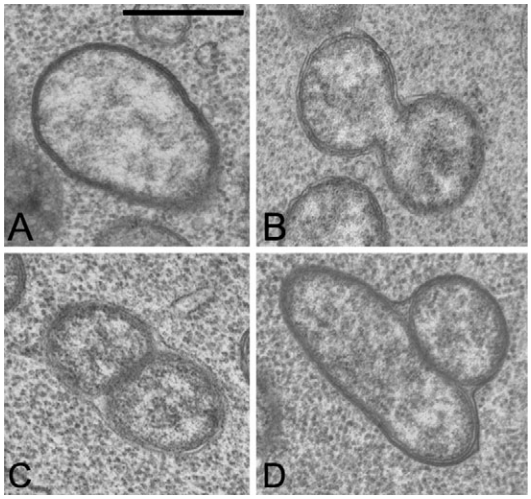


**Fig. 4. Electron microscopy images of *Wolbachia* morphology in wild-type and *grk* mutant egg chambers from stages 2–7 of oogenesis.** (**A**) Non-dividing *Wolbachia*. (**B**) Dividing, bi-lobed *Wolbachia*. (**C**,**D**) Doublet *Wolbachia*. Although there is no difference in *Wolbachia* morphology between wild-type and $grk^{HK36}$ oocytes, the latter exhibit a twofold increase in doublet *Wolbachia*. See also supplementary material Fig. S1. Scale bar: 0.5 μm.

### The *grk* mRNP complex affects *Wolbachia* titer

A microtubule-independent function for *grk* could be exerted through the mRNA or protein product of the *grk* gene. One way to differentiate between these possibilities is to compare the results from misexpression and overexpression of the Grk protein. The *grk* mRNA forms a complex with a collection of proteins that regulate its localization and translation, such as Squid (Sqd) and Hrb27C/Hrp48. Disrupting either of these factors derepresses the *grk* transcript, giving rise to ectopic Grk protein in the cytoplasm. This protein appears to be functional, as indicated by induction of ectopic dorsal patterning (Neuman-Silberberg and Schupbach, 1996; Norvell et al., 1999; Goodrich et al., 2004; Caceres and Nilson, 2009; Kugler and Lasko, 2009). To investigate whether *Wolbachia* titer responds similarly to Grk misexpression as it does to overexpression, we examined *Wolbachia* titer in *sqd* and *hrb27C* mutants. Compared with wild-type oocytes that exhibited an average of 505±39 *Wolbachia* in a single focal plane ($n$=36), *sqd* hemizygous mutants contained 316±34 *Wolbachia* ($n$=20) and oocytes homozygous for *hrb27C* had 166±22 ($n$=15) (Fig. 6A). These data suggest that, unlike Grk overexpression (Fig. 1C), Grk misexpression triggers a decrease in *Wolbachia* titer. These data are consistent with a role for the *grk* mRNP components in affecting *Wolbachia* titer.

One question raised by this analysis is whether ectopic Grk expressed in *sqd* and *hrb27C* mutants adversely affect *Wolbachia* by negatively influencing the microtubule cytoskeleton. To address that possibility, *Wolbachia* were also quantified in *sqd* and *hrb27C* nurse cells where ectopic Grk is not detected (Neuman-Silberberg and Schupbach, 1996; Goodrich et al., 2004; Caceres and Nilson, 2009) and microtubule disruption had no obvious impact on *Wolbachia* titer (Fig. 5D). In comparison with wild-type nurse cells carrying approximately 32±2.2 *Wolbachia*

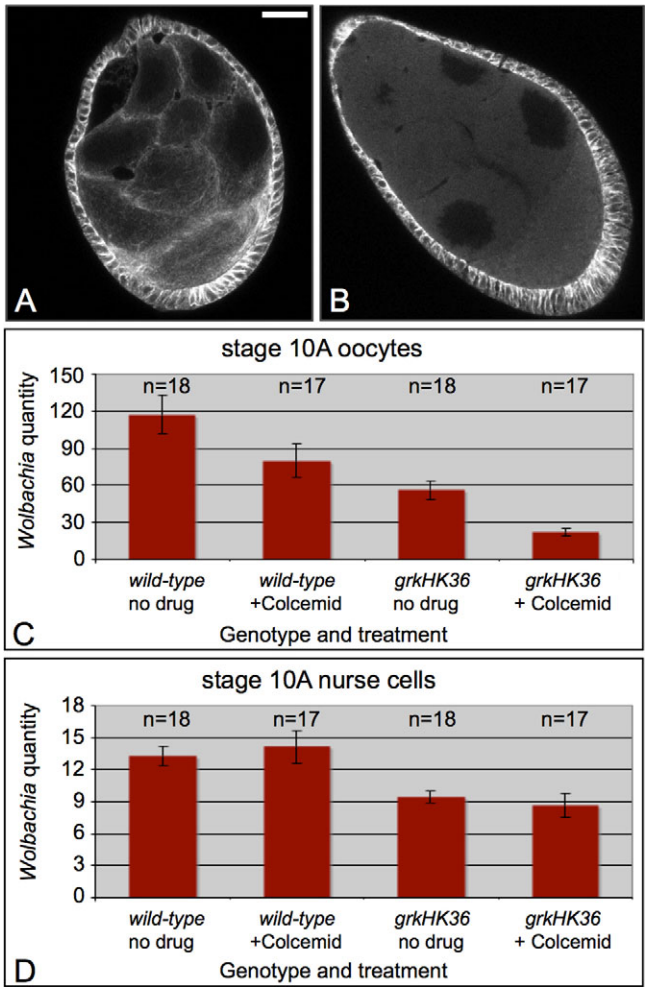


**Fig. 5. *grk* influences *Wolbachia* titer through a microtubule-independent mechanism.** (**A**,**B**) Untreated (A) and colcemid-treated (B) egg chambers immunostained for microtubules. (**C**,**D**) Quantification of *Wolbachia* for stage 8 oocytes (C) and nurse cells (D). Data are means ± s.e.m. Scale bar: 10 µm.

($n$=36), hemizygous *sqd* mutants exhibited 17±2.0 *Wolbachia* ($n$=20), and homozygous *hrb27C* oocytes had 13±2.4 *Wolbachia* per nurse cell ($n$=15) (Fig. 6B). These *Wolbachia* depletions in *sqd* and *hrb27C* mutant nurse cells are similar in magnitude to those seen in *sqd* and *hrb27C* mutant oocytes (compare Fig. 6A and B). The data implicate *grk* mRNP components in influencing *Wolbachia* titer.

### *Wolbachia* co-migrate with the *grk* mRNP component Sqd

A functional interaction between *Wolbachia* and *grk* mRNPs raises the question of whether physical interaction might occur between *Wolbachia* and *grk* mRNP components. To investigate this, we examined the relative localization of *Wolbachia* and GFP–Sqd in fixed egg chambers (Morin et al., 2001; Kelso et al., 2004). Quantitative analyses revealed that 39% of *Wolbachia* puncta appeared to be in contact with the GFP–Sqd signal in nurse cells ($n$=529 bacteria from three egg chambers; Fig. 7A–C, arrowheads).

To further test for an association of *Wolbachia* with Sqd, we performed time-lapse confocal imaging of GFP–Sqd and *Wolbachia* labeled with the vital dye Syto-82 ($n$=8 movies). The

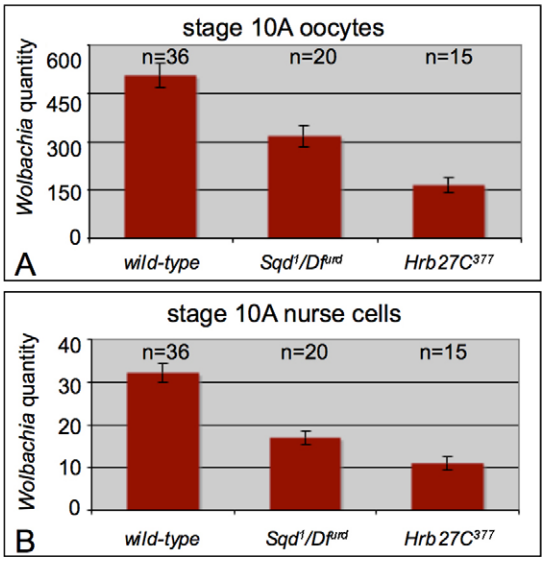


**Fig. 6. Mutations in other members of the *grk* mRNP complex also reduce *Wolbachia* titer.** (**A**,**B**) *Wolbachia* are quantified in stage 10A oocytes (A) and nurse cells (B). Data are means ± s.e.m.

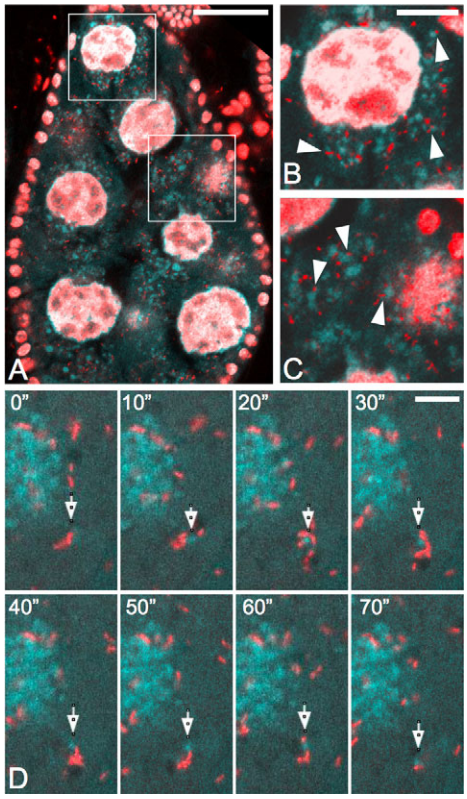


**Fig. 7. *Wolbachia* are localized in close proximity to Sqd.** (**A**) Nurse cells in a fixed stage 8 oocyte. (**B**,**C**) Magnified views of boxed areas. Arrowheads indicate *Wolbachia* in contact with GFP–Sqd. (**D**) Zoom of live imaging showing co-migrating *Wolbachia* (red) and GFP–Sqd (cyan) (arrows). Large red circles represent host DNA. Red puncta, *Wolbachia*; cyan, GFP–Sqd. See also supplementary material Movies 1 and 2. Scale bars: 25 µm (A), 10 µm (B,C), 5 µm (D).

movies indicated that *Wolbachia* and GFP–Sqd distant from the nurse cell nuclei were highly motile, and a subset of *Wolbachia* co-migrated with GFP–Sqd (Fig. 7D arrows; supplementary material Movie 1, arrows). The majority of *Wolbachia* proximal to the nurse cell nuclei appeared to be juxtaposed with the GFP–Sqd signal. The oscillating movements of these *Wolbachia* and GFP–Sqd occurred in tandem (supplementary material Movies 1, 2). These results are consistent with an association between *Wolbachia* and the *grk* mRNP component Sqd.

### High-titer *Wolbachia* infections can induce *grk*-related defects

If *Wolbachia* interact with *grk* mRNPs, it is possible that *Wolbachia* reciprocally affect *grk* mRNP function. One possible outcome from *grk* mRNP disruption is a change in the appearance of nurse cell nuclei. Nurse cell DNA normally exhibits a loose polytene appearance during stages 4–5 and adopts a more dispersed appearance for the remainder of oogenesis (King, 1970; Dej and Spradling, 1999). However, mutations in *sqd*, *hrb27C* and other *grk*-associated proteins result in the persistence of the loose polytene chromatin morphology well into late oogenesis (Storto and King, 1988; Keyes and Spradling, 1997; Goodrich et al., 2004; Hartl et al., 2008). To determine whether *Wolbachia* influence this function, nuclear morphology was assayed in *Wolbachia*-infected nurse cells. We focused on *D. simulans* transinfected with the *wMel Wolbachia* strain because the titer is particularly high in these stocks (Poinsot et al., 1998; Veneti et al., 2004; Serbus and Sullivan, 2007). Transinfected *D. simulans* nurse cells consistently exhibited severe defects in chromatin morphology, in contrast to uninfected *D. melanogaster*, *wMel*-infected *D. melanogaster*, uninfected *D. simulans* and *wRi*-infected *D. simulans* (Fig. 8A–F). The appearance of the *Wolbachia*-induced chromatin disruption matched the loose polytene structure observed in *hrb27C* mutants (Fig. 8E,F) (Goodrich et al., 2004). The chromatin morphology of *wMel*-infected *D. simulans* also reverted to normal after tetracycline treatment, indicating that the defects are *Wolbachia* dependent (data not shown). The observation that high-titer *Wolbachia* infection phenocopies *hrb27C* is consistent with a role for *Wolbachia* in disrupting *grk* mRNP component function.

To further investigate whether *Wolbachia* induce *grk*-related phenotypes, we also examined chorionic patterning. Alterations in Grk signaling commonly leads to dorsal–ventral defects indicated by enhancement or reduction of chorionic dorsal respiratory appendages (Kugler and Lasko, 2009). To test whether *Wolbachia* induce similar defects, chorions were examined from uninfected, *wRi*-infected and *wMel*-infected *D. simulans* flies. Unlike the other fly strains, transinfected *D. simulans* flies exhibited excess dorsal appendage material in 10% of eggs laid ($P<0.005$) (Fig. 8G). This result suggests that *Wolbachia* can affect the Grk signaling pathway.

## Discussion

The major findings of this study are that host *grk* has a cumulative, dosage-sensitive impact on *Wolbachia* titer. This impact does not appear to be related to the Grk protein, invoking a role for the *grk* mRNP. Accordingly, *Wolbachia* exhibit association with a *grk* mRNP protein, and disrupting known protein constituents of the *grk* mRNP affects *Wolbachia* titer analogously to *grk* disruptions. Highly infected flies also have defects analogous to *grk* mRNP disruptions, including defects in nurse cell chromatin structure and dorsal appendage formation. These findings suggest that *Wolbachia* interaction with the *grk* mRNP has a significant impact on both *Wolbachia* titer and *grk* mRNP function (Fig. 9).

One of the surprising outcomes from this study is the microtubule-independent impact of *grk* on *Wolbachia* titer. Disruptions of microtubules and cytoplasmic dynein have been shown to disrupt *Wolbachia* distribution and density in oogenesis (Ferree et al., 2005). One interpretation of this study is that *Wolbachia* are transported along microtubules into the oocyte where *Wolbachia* replicate preferentially at the oocyte anterior end. A role for *grk* in regulating *Wolbachia* titer initially appeared consistent with that scenario. Grk signaling is crucial for proper microtubule orientation in oogenesis, and *grk* mRNPs are known to be transported by the microtubule-based motor, cytoplasmic dynein. Thus *grk* could be argued to directly or indirectly affect *Wolbachia* transport toward a replication-promoting area of the oocyte. However, the results of this study indicate that the impact of *grk* on *Wolbachia* titer is independent of these models. *grk* has a comparable repressive effect on *Wolbachia* titer in both nurse cells and the oocyte

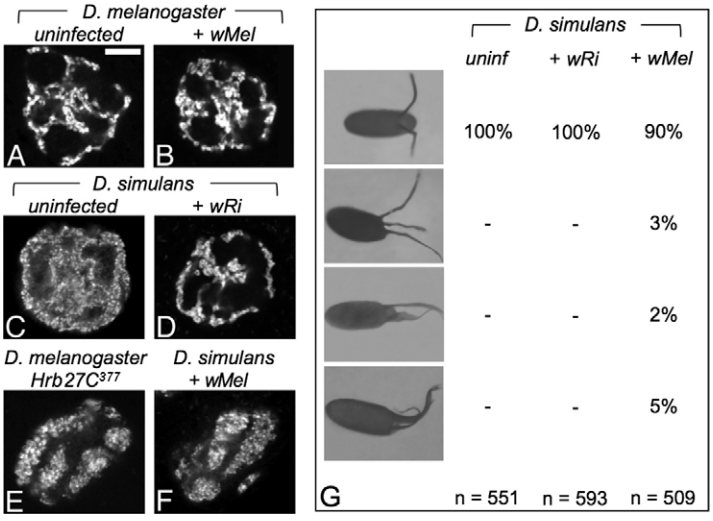


**Fig. 8. *wMel Wolbachia* induce defects in nurse DNA morphology and chorionic patterning.** Single focal planes of nurse cell nuclei are shown stained with propidium iodide. (**A**) Uninfected *D. melanogaster*. (**B**) *wMel* in *D. melanogaster*. (**C**) *hrb27C*$^{377}$ germline clone. (**D**) Uninfected *D. simulans*. (**E**) *wRi* in *D. simulans*. (**F**) *wMel* in *D. simulans*. (**G**) Chorion phenotypes exhibited by uninfected and *Wolbachia*-infected *D. simulans* flies. Scale bar: 5 μm.

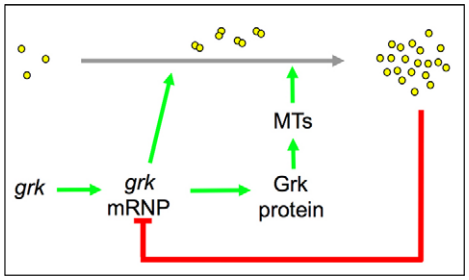


**Fig. 9. Model of interactions between *Wolbachia* and *grk* in oogenesis.** In this schematic, host *grk* facilitates *Wolbachia* growth and division, indicated by yellow circles. Grk protein might contribute to this through its influence over the microtubule cytoskeleton. The *grk* mRNP contributes to *Wolbachia* titer elevation by an independent mechanism. *Wolbachia* at high titer levels feed-back upon this regulatory loop by disrupting the function of *grk* mRNP components.

throughout oogenesis, although *grk* is primarily known to affect oocyte microtubules (Figs 1, 2) (Clark et al., 1994; Gonzalez-Reyes et al., 1995; Roth et al., 1995). The effects of *grk* on *Wolbachia* titer are detected before any known influence of *grk* on microtubules in oogenesis (Fig. 3). Furthermore, our colcemid tests indicate that the impact of *grk* on *Wolbachia* titer is largely independent of microtubules in both nurse cells and the oocyte (Fig. 5). This indicates that *grk* affects *Wolbachia* titer primarily through a different mechanism.

A microtubule-independent role for *grk* could be explained by a previously unrecognized function for *grk* mRNA or Grk protein. Our initial genetic tests did not differentiate between these possibilities because the *grk* mutants used disrupt both mRNA and protein (Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1996), and the *grk* overexpression tests should elevate both mRNA and protein loads (Fig. 1C) (Ghiglione et al., 2002). However, this issue is addressed by using well-established mutations in translational repressors *sqd* and *hrb27C*, which encode components of the *grk* mRNP complex that repress *grk* translation (Schupbach, 1987; Kelley, 1993; Neuman-Silberberg and Schupbach, 1994; Neuman-Silberberg and Schupbach, 1996; Goodrich et al., 2004; Caceres and Nilson, 2009). The reduction in *Wolbachia* titer seen in *sqd* and *hrb27C* mutants ultimately suggest that *Wolbachia* density does not correlate with Grk protein availability in the cytoplasm (Fig. 6). An alternative possibility is that the *grk* mRNP complex has a function in regulating *Wolbachia* titer. The appearance of *Wolbachia* associated with GFP–Sqd in fixed samples and live imaging are also consistent with a possible interaction between *Wolbachia* and mRNP components (Fig. 7, supplementary material Movies 1, 2).

One of the issues raised by this study is specificity of the *grk* effect because Sqd and Hrb27C are hnRNP proteins that are not exclusive to the *grk* mRNP complex. Thus, it is also possible that distinct mRNPs with a protein composition similar to *grk* mRNPs also contribute to *Wolbachia* titer control. However *osk* mRNP complexes are thought to share many components with *grk* mRNPs, including Sqd and Hrb27C (Kugler and Lasko, 2009), yet genetic disruptions of *osk* that reduce both mRNA and protein load (Rongo et al., 1995) did not induce a striking reduction in *Wolbachia* titer in our preliminary screen. Although this study does not rule out a role for other mRNPs, it suggests that the *grk*-related effects we observe on *Wolbachia* titer are not necessarily a general property of host mRNPs.

One of the remaining questions is how a *grk* mRNP exerts an influence on *Wolbachia* titer. Electron microscopy evidence shows no indication of a mortality-based effect. The significant increase in *Wolbachia* doublets detected in *grk* mutant nurse cells might be informative, however. Perhaps *grk* mutants prevent final abscission of the *Wolbachia* membrane during binary fission. Another interpretation is that upon completion of binary fission, the *Wolbachia* daughter cells remain trapped within the single original host vacuole, subjecting the bacteria to competition for limited nutrient resources. Either scenario would be consistent with a role for host *grk* in promoting *Wolbachia* growth and proliferation (Fig. 9). Future studies are needed to address how directly this defect might be attributable to the *grk* mRNP complex.

An association of *Wolbachia* with GFP–Sqd raises the possibility that *grk* mRNPs affect *Wolbachia* biochemistry or trafficking though a direct mechanism. It is also possible that the impact of *grk* mRNPs on *Wolbachia* is facilitated by intermediate factors. For example, Sqd has been shown to co-immunoprecipitate with the retinoblastoma Rb protein from *Drosophila* cell culture and ovarian extract (Ahlander and Bosco, 2009). Rb is known to bind and repress E2F family transcription factors (Zhu et al., 1994). Thus, *grk* mRNPs might affect Dp/E2F-based host transcriptional activation patterns that support *Wolbachia* trafficking and/or replication. Furthermore, Sqd binds Cup, a translational repressor that is required for localization of *grk* (Clouse et al., 2008). Cup has been shown to interact with Nup154, a member of a protein family that supports nuclear pore assembly and nuclear import processes (Grimaldi et al., 2007). This scenario provides another route by which *grk* mRNPs might affect availability of host products relevant to *Wolbachia* titer regulation.

One of the additional questions raised by this study is its applicability to neglected tropical diseases. It is known that *Wolbachia* endosymbionts of *Onchocerca volvulus*, *Wuchereria bancrofti* and *Brugia malayi* contribute significantly to African river blindness and lymphatic filariasis (Saint Andre et al., 2002; Debrah et al., 2006; Debrah et al., 2007; Taylor et al., 2008; Debrah et al., 2009; Turner et al., 2009). Because elimination of *Wolbachia* from these nematodes disrupts both the filarial host and manifestations of disease (Johnston and Taylor, 2007; Slatko et al., 2010), any insight into *Wolbachia* titer control is potentially useful. The recently sequenced *Brugia* genome does not appear to encode a *grk* gene, but it has possible homologs for *hrb27C* and *sqd*, as well as for the *grk* mRNA-binding proteins Bruno/Aret, Imp and Orb (Chang et al., 1999; Filardo and Ephrussi, 2003; Geng and Macdonald, 2006; Ghedin et al., 2007). Thus, a speculative possibility is that *Brugia* cells might also harbor *grk*-like mRNPs that exert an influence on *Wolbachia* titer.

This study raises the further question of whether intracellular pathogens could interact similarly with host mRNP components. Viruses such as hepatitis C have been shown to bind host mRNP proteins and use them to facilitate viral protein synthesis and viral replication (Kato et al., 1990; Han et al., 1991; Pestova et al., 1998; Kim et al., 2004). Although preliminary, there are some hints that pathogenic bacteria interact with host mRNPs as well. A number of genome-wide RNAi screens have been done in *Drosophila* tissue culture to assess the effect of host factors on

*Listeria*, *Mycobacterium*, *Chlamydia* and *Francisella* infection (Agaisse et al., 2005; Philips et al., 2005; Derre et al., 2007; Akimana et al., 2010). This work indicated that disruption of certain splicing or translation initiation factors correlated with reductions in intracellular *Listeria*, *Chlamydia* and *Francisella* infection levels (Agaisse et al., 2005; Derre et al., 2007; Akimana et al., 2010). The datasets also indicate that disruption of the *grk* mRNP component *hrb27C*, or *Brain Tumor*, a suppressor of *Hunchback* translation (Sonoda and Wharton, 1999), reduced *Francisella* and *Listeria* infection loads, respectively (Agaisse et al., 2005; Akimana et al., 2010). It will be of great interest to see whether future studies find *Wolbachia* interactions with *grk* mRNP components to be representative of a generalized titer-influencing mechanism shared by other intracellular symbionts and pathogens.

During oogenesis, *Wolbachia* are not only positioned where key developmental events occur, but also rely on the same transport mechanisms as many of the morphogens that control these events. For example, early in oogenesis, anterior localization of *Wolbachia* occurs at the same time and position as that of the patterning events that establish the anterior–posterior axis (Ferree et al., 2005). Later in oogenesis, both *Wolbachia* and host germline determinants rely on the motor protein kinesin-1 to concentrate at the posterior pole (Serbus and Sullivan, 2007). In both of these situations, despite relying on the same transport mechanisms and occupying the same position as the host patterning molecules, *Wolbachia* do not interfere with these essential developmental events. This suggests that *Wolbachia* achieve a balance in which titer is maximized without disrupting oocyte development. Support for this comes from our finding that *Wolbachia* with abnormally high titer produce defects in dorsal appendage formation. Because this event relies on the Grk signaling pathway, one possibility is that this occurs as a consequence of a disruptive association of *Wolbachia* with *grk* mRNP components (Fig. 9). This would create a selective pressure to establish more moderate *Wolbachia* levels within the host. Thus, the functional interaction between *Wolbachia* and *grk* provides a molecular example for how the interests of host and *Wolbachia* success can be achieved.

Other symbiotic organisms have previously been shown to direct morphogenetic processes in the host. *Vibrio fischerei* induce formation of the light-producing organ in squid (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1994) and *Rhizobium* induce root nodule formation in leguminous plants for nitrogen fixation (Crespi and Frugier, 2008; Oldroyd and Downie, 2008). Perhaps the interaction between *Wolbachia* and *grk* represents a step toward the evolution of symbiosis in which *Wolbachia* also become integral to regulation of host morphogenesis.

## Materials and Methods

### Fly strains

The initial screen for host factors affecting *Wolbachia* phenotypes was conducted by using the *w; Sp/Cyo; Sb/TM6B* stock to introduce *wMel Wolbachia* into the following genotypes: *Ar51F*$^{KG02753}$*/Df*$^{ED2426}$, *Chico*$^{1}$, *grk*$^{HK36}$ *cn bw, Moe*$^{1652}$*/Moe*$^{0415}$, *Nuf*$^{1}$*/Df*$^{ED4543}$, *Osk*$^{6}$*/Df*$^{(3R)p\text{-}XT103}$, *Osk*$^{54}$*/Df*$^{(3R)p\text{-}XT103}$, *Rab11*$^{J2D1}$*/Rab11*$^{93Bi}$, *Spir*$^{1}$, *Stau*$^{D3}$, *Stau*$^{D3}$*/Df*$^{(2R)Pcl7B}$, *Stau*$^{1}$*/Df*$^{(2R)Pcl7B}$, *Stau*$^{Rŷŷ}$*/Df*$^{(2R)Pcl7B}$, *Vas*$^{1}$*/Df(2L)osp*$^{29}$, *Adh*$^{UF}$*osp*$^{29}$*pr*$^{1}$*cn*$^{1}$, *Vls*$^{1}$, *Tud*$^{1}$, *yw{hs-FLP/+};{FRT}2A Klc*$^{1}$*/{FRT}2A{Ovo*$^{D1}$*}*, *yw {hs-FLP}/+;{FRT}2A Klc*$^{8ex94}$*/{FRT}2A{Ovo*$^{D1}$*}*, *yw {hs-FLP}/+;{FRT}42B Khc*$^{17}$*/{FRT}42B{Ovo*$^{D1}$*}*, *yw {hs-FLP}/+;{FRT}42B Khc*$^{23}$*/{FRT}42B{Ovo*$^{D1}$*}*, *yw {hs-FLP}/+;{FRT}42B Khc*$^{27}$*/{FRT}42B{Ovo*$^{D1}$*}*. The *grk*-focused studies used the following lines: *wMel*-infected *w; Sp/Cyo; Sb/TM6B* and uninfected strains *Oregon R*, *grk*$^{HK36}$ *cn bw/Cyo*, *grk*$^{DC9}$ *cn bw/Cyo*, *Df* $^{N22\text{-}14}$*/Cyo*, *w;{mat alpha tubulin: GAL4 VP16}V37*, *w;{UASp mb-grk-myc}/Cyo*, *yw;ry{sqd*$^{1}$ *ry*$^{+}$*} cv-c sbd/TM3 Sb e*, *Df*$^{urd}$ *e/TM3 Sb*, *hrb27C*$^{377}$*{FRT}40A/SM6A,{ovoD1}{FRT}40A/Dp bw S wg Ms/CyO*, *yw{hs-FLP};Sco/Cyo*, *w;sqd{GFP P02}*. For testing *Wolbachia* titer in *D. simulans*, we used a $w^{-}$ lab stock that was either uninfected, infected with the endogenous *wRi Wolbachia* strain, or transinfected with *wMel Wolbachia* (Poinsot et al., 1998).

### Tissue staining

All tissue fixation, colcemid treatments, propidium iodide staining and microtubule staining was done as described previously (Rothwell and Sullivan, 2000; Serbus et al., 2005; Serbus and Sullivan, 2007). Anti-Grk staining was done using rat anti-Grk antibody (Neuman-Silberberg and Schupbach, 1996) at 1:3000 in PBS with 0.1% Triton X-100 and goat anti-rat Alexa Fluor 488 (Invitrogen) at 1:500, with extensive rinsing. All samples were imaged on a Leica SP2 confocal microscope at 63× magnification.

### Live imaging

Dissected ovaries were incubated for 7 minutes in Syto-82 (Invitrogen) (Albertson et al., 2009) diluted into Shields and Sang M3 tissue culture medium (Sigma) at 1:250. The tissue was mounted on coverslips under 700 W halocarbon oil and left to equilibrate for a minimum of 1 hour. The Syto-82 dye initially labels dense perinuclear clouds of unclear origin. Examination of the egg chambers using TRITC epifluorescence settings triggers release of Syto-82 from the clouds into the cytoplasm. This gives rise to a uniform background in uninfected egg chambers, whereas infected egg chambers display a specific, punctate staining pattern that is consistent with *Wolbachia* size, motility and distribution as indicated by other studies (Ferree et al., 2005; Frydman et al., 2006; Serbus and Sullivan, 2007; Albertson et al., 2009; Casper-Lindley et al., 2011) (supplementary material Movies 1–3).

### Assessment of *Wolbachia* titer

*Wolbachia* were newly crossed into each mutant fly strain in all experiments from the infected strain *w;Sp/Cyo;Sb/TM6B* to ensure that no adaptation of either host or microbe had occurred. All crosses were done on standard soft medium (0.5% agar, 7% molasses, 6% cornmeal, and 0.8% killed yeast). Vials were seeded with 3–4 males and 5–7 virgin females that were 5 days old or less. Identical population density and female age distribution was used in all vials, and wild-type control crosses were run in parallel with the experimental ones. Crosses were kept at 25℃, with flies transferred onto new food every 2–3 days. Virgin female flies were collected during the first 3 days of eclosion only, then either used for the next round of crossing or for ovary staining at 5 days of age.

After propidium iodide staining, confocal microscopy was used to acquire *Z*-series images at 1.5 µm intervals. A minimum of six contiguous planes were imaged per specimen, with identical settings applied to both oocyte and nurse cells. Uniform intensity settings were also applied to control and experimental egg chambers within each run.

For quantification of *Wolbachia*, we determined that measurement of a single focal plane is generally representative of total *Wolbachia* titer present within egg chambers (supplementary material Table S1). To identify comparable focal planes for quantification from stage 4–5 egg chambers, we selected the plane where the widest cross-section of the oocyte was visible. For quantifying stage 10A egg chambers, we first established a reference point by identifying the *Z*-plane where follicle cells are visible as a smooth monolayer around the oocyte. Relative to this location, we identified the deepest possible focal plane where *Wolbachia* were clearly visible in all samples tested. After identifying the appropriate oocyte *Z*-plane, the corresponding nurse cell plane was also selected. The images were then imported into Photoshop for manual removal of host nuclei and extracellular debris from images. Images were thresholded to remove all background signal from images, allowing only *Wolbachia* signal to remain. The images were then inverted and imported into ImageJ, where *Wolbachia* puncta were quantified with the 'Analyze Particles' function.

For all mutant conditions tested, at least two experimental replicates were performed. A total of 15 or more egg chambers were assayed for each type of mutant in parallel with wild-type controls. To ensure maximal comparability of results between experimental runs, all data were normalized against wild-type control values.

### Electron microscopy

*Drosophila* ovaries were fixed by the previously described method (Terasaki et al., 2001; Zhukova et al., 2008). Fly ovaries were isolated in 0.1 M phosphate buffer (pH 7.4) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2.5 hours, then washed in the same buffer and post-fixed with 1% $OsO_4$ and 0.8% potassium ferrocyanide for 1 hour. Samples were washed with water and placed in a solution of 1% uranyl acetate in water for 12 hours at 4℃. Then, ovaries were dehydrated in an ethanol series and acetone and embedded in Agar 100 Resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM-100SX (JEOL) electron microscope. For quantitative electron microscopy analysis, 55 egg chambers from nine ovaries and 58 egg chambers

from 13 ovaries were assayed for wild-type and $grk^{HK36}$ flies, respectively. Bacteria were quantified on randomly chosen sections of nurse cells. Statistics were run using a chi-square test.

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