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

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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; Poinset 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 germine 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 Dynemin. 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 plane 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 grkΔK36 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 grkΔK36 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 grkΔCv 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 grkΔK36 exhibited 304±32 Wolbachia (n=30), and grkΔK36 homozygous oocytes had 118±14 Wolbachia (n=20). Furthermore, grkΔCv heterozygous oocytes showed 216±31 Wolbachia (n=20), and grkΔCv 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 (Giglione 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, grkΔK36 heterozygous nurse cells exhibited 19±1.8 Wolbachia (n=30) and 7.6±1.8 Wolbachia were detected in grkΔK36 homozygotes (n=20). Following a similar trend, nurse cells of grkΔCv heterozygotes contained approximately 20±1.4 Wolbachia (n=20), and grkΔCv 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 grkΔK36 oocytes (n=20) (Fig. 3C). Similarly, grk disruption in nurse cells resulted in a 40% drop in Wolbachia titer, with wild-
type nurse cells exhibiting 11±1.7 *Wolbachia* on average (*n*=20) whereas *grk*<sup>HK36</sup> 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*<sup>HK36</sup> (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*<sup>HK36</sup> 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
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*<sup>HK36</sup> oocytes displaying 56±7.5 *Wolbachia* (n=18). Furthermore, colcemid-treated *grk*<sup>HK36</sup> 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).

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*
(n = 36), hemizygous sqd mutants exhibited 17 ± 2.0 Wolbachia (n = 20), and homozygous hrh27C oocytes had 13 ± 2.4 Wolbachia per nurse cell (n = 15) (Fig. 6B). These Wolbachia depletions in sqd and hrh27C mutant nurse cells are similar in magnitude to those seen in sqd and hrh27C 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
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 (Poinot 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 lead to dorsal–ventral defects indicated by enhancement or reduction of choricline 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 grk-/- germeline 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.
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.
L. 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 tir inhibitor-mRNA 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 directly morphogenetic processes in the host. Vibrio fischeri 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, Sh/TM6B stock to introduce wMel Wolbachia into the following genotypes: AdhUFosp29pr1cn1;{hs-FLP}/+;{FRT}2A Klc8ex94/{FRT}2A{OvoD1}yw {hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}, w;Sp/Cyo; Sb/TM6B, w;{UASp mb-grk-myc}/Cyo, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw. The grk-focused studies used the following lines: w;Mel-infected w; Sp/Cyo, Sh/TM6B and uninfected strains Oregon R, grk{GFP P02}cn w;Cyo, grk{GFP P02}cn w;Cyo, Cy0{GFP P02}Cy0, w;mat alpha tubulin: GALA V16V37, w;{UKSp mb-grk-myc}/Cy0, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw. The grk-focused studies used the following lines: w;Mel-infected w; Sp/Cyo, Sh/TM6B and uninfected strains Oregon R, grk{GFP P02}cn w;Cyo, grk{GFP P02}cn w;Cyo, Cy0{GFP P02}Cy0, w;mat alpha tubulin: GALA V16V37, w;{UKSp mb-grk-myc}/Cy0, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw. The grk-focused studies used the following lines: w;Mel-infected w; Sp/Cyo, Sh/TM6B and uninfected strains Oregon R, grk{GFP P02}cn w;Cyo, grk{GFP P02}cn w;Cyo, Cy0{GFP P02}Cy0, w;mat alpha tubulin: GALA V16V37, w;{UKSp mb-grk-myc}/Cy0, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw, w;{hs-FLP}/+;{FRT}42B Khc17/{FRT}42B{OvoD1}yw.

Electron microscopy

Drosophila ovaries were fixed by the previously described method (Terasaki et al., 2001; Zakhova 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% OsO4 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°C. 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-1000SX (JEOL) electron microscope. For quantitative electron microscopy analysis, 55 egg chambers from nine ovaries and 58 egg chambers
from 13 ivories were assayed for wild-type and gurkH36 flies, respectively. Bacteria were quantified on randomly chosen sections of nurse cells. Statistics were run using a chi-square test.

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