Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue

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Summary

Wolbachia **are maternally inherited bacterial endosymbionts that occupy many but not all tissues of adult insects. During the initial mitotic divisions in** ***Drosophila*** **embryogenesis,** ***Wolbachia*** **exhibit a symmetric pattern of segregation.** ***Wolbachia*** **undergo microtubule-dependent and cell-cycle-regulated movement between centrosomes. Symmetric segregation occurs during late anaphase when** ***Wolbachia*** **cluster around duplicated and separating centrosomes. This centrosome association is microtubule-dependent and promotes an even** ***Wolbachia*** **distribution throughout the host embryo. By contrast, during the later embryonic and larval neuroblast divisions,** ***Wolbachia*** **segregate asymmetrically with the apical self-renewing neuroblast. During these polarized asymmetric neuroblast divisions,** ***Wolbachia*** **colocalize with the apical centrosome and apically localized Par complex. This localization depends on microtubules, but not the cortical actin-based cytoskeleton. We also found that** ***Wolbachia*** **concentrate in specific regions of the adult brain, which might be a direct consequence of the asymmetric** ***Wolbachia*** **segregation in the earlier neuroblast divisions. Finally, we demonstrate that the fidelity of asymmetric segregation to the self-renewing neuroblast is lower in the virulent Popcorn strain of** ***Wolbachia*****.**

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Introduction

Bacteria and viral host pathogens exhibit tissue-specific host tropisms. Much of this tropism is explained by routes of entry during infection and subsequent cell-to-cell migration (Ireton, 2007; Sieczkarski and Whittaker, 2005). Less well explored are mechanisms that regulate the tissue distribution of obligate intracellular bacteria that are inherited through the germline. Of particular interest is the segregation of intracellular pathogens in mitotically active host cells, as this might be an important mechanism to spread infection to specific tissue types during host development. *Wolbachia* is a bacterial endosymbiont that infects numerous insect species and is an effective system in which to identify the factors that control pathogen distribution in host tissue (Serbus et al., 2008; Werren et al., 2008). Although much research has focused on *Wolbachia* germline concentration and transmission, a number of studies have convincingly demonstrated that *Wolbachia* are present in a broad array of larval and adult somatic tissues. These include the head, thoracic muscles, midgut, Malpighian tubules (Dobson et al., 1999; McGraw et al., 2002), somatic cells associated with the testis, and ovaries (Clark et al., 2005; Clark et al., 2008; Frydman et al., 2006; McGraw et al., 2002; Riparbelli et al., 2007). Comparisons among several host species and *Wolbachia* strains demonstrate that factors intrinsic to both the host and *Wolbachia* control its tissue distribution and density (Dobson et al., 1999; Ijichi et al., 2002; Veneti et al., 2004). Popcorn (W_{Pop}), a virulent strain of *Wolbachia*, provides a particularly striking demonstration of the role of *Wolbachia*-specific factors as it over-replicates in adult neurons and muscle cells ultimately causing tissue degeneration and premature death (Min and Benzer, 1997). Recent studies demonstrate that when W_{Pop} is transferred from *Drosophila melanogaster* to *Aedes aegypti* (mosquito), overproliferation and early lethality are still observed (McMeniman et al., 2009). Conversely, experiments in which the W_{Mel} strain of *Wolbachia* overreplicates when transferred from *D. melanogaster* to *Drosophila simulans*, demonstrate that host factors also have an important role in *Wolbachia* density (Serbus and Sullivan, 2007; Veneti et al., 2004; Zabalou et al., 2008).

Insights into mechanisms of *Wolbachia* segregation during host mitosis have come from studying initial mitotic divisions in early *Drosophila* embryogenesis. After fertilization, the embryos undergo a series of rapid synchronous nuclear divisions before cellularizing during nuclear cycle 14. During nuclear cycles 10 to 13, the divisions occur on a plane just beneath the plasma membrane and thus are easily imaged. Cytological analysis of *Wolbachia*-infected embryos revealed that *Wolbachia* localize near the centrosomes throughout the cell cycle (Callaini et al., 1994; Kose and Karr, 1995; O'Neill and Karr, 1990), which was found to depend on microtubule asters but not actin (Callaini et al., 1994). During the syncytial mitotic divisions, the bacteria reside in equal numbers at each daughter centrosome, ensuring transmission to both daughter nuclei (Kose and Karr, 1995). This segregation pattern results in a broad *Wolbachia* distribution throughout the embryo by cellularization. If this pattern of segregation were to continue throughout host development, one would expect *Wolbachia* to be equally distributed

throughout all larval and adult tissues. However, *Wolbachia* are unevenly distributed in adult tissues (Dobson, 2003; Ijdo et al., 2007; McGraw et al., 2002).

In this study, we further identify the host cellular mechanisms that guide symmetric *Wolbachia* segregation during the syncytial divisions of early *Drosophila* embryogenesis. In addition, we identify potential cellular mechanisms that lead to the highly uneven and tissue-specific distributions of *Wolbachia* later in development. To address the first issue, we developed imaging techniques to analyze live *Wolbachia* movement during the syncytial divisions. These studies demonstrate that during syncytial mitosis, *Wolbachia* exhibit cell-cycle-dependent bidirectional movements along microtubules. This results in an exchange of *Wolbachia* between recently duplicated (sister) and neighboring (non-sister) centrosomes. During anaphase-telophase, when centrosomes duplicate and begin to separate, *Wolbachia* cluster tightly around the centrosomes and thus are evenly distributed between dividing sister centrosomes. This segregation pattern results in a broad *Wolbachia* distribution throughout the entire embryo.

To address the second issue of how uneven *Wolbachia* distribution in various tissues is achieved later in development, we focused on embryonic neurogenesis. In contrast to the syncytial cell cycles, neuroblast cells are highly polarized and undergo asymmetric cell divisions (Egger et al., 2008; Wu et al., 2008). Thus, it is of great interest to determine the segregation pattern of *Wolbachia* in this cell type. Embryonic neuroblasts are selected from a neuroectoderm layer and delaminate interiorly. Neuroblasts exhibit several aspects of asymmetry: they establish distinct apical-basal cortical protein domains, have an asymmetric mitotic spindle (the apical pole contains a larger centrosome and a more extensive astral microtubule network) and divide asymmetrically along an apical-basal axis to regenerate a large self-renewing neuroblast and a small ganglion mother cell (GMC) (Albertson and Doe, 2003; Kaltschmidt et al., 2000). The GMC undergoes an additional round of division to generate neurons and glia (Goodman and Doe, 1993). The neuroblast apical domain includes the evolutionarily conserved Par3-Par6-aPKC protein complex. After neuroblast delamination, this complex binds the protein Inscuteable, which is required to orient the mitotic spindle along the apical-basal axis (Kraut et al., 1996). This ensures that cell division is orthogonal to the apical-basal polarity determinants. Inscuteable recruits Pins and a heterotrimeric G-protein subunit Gαi, which is required for asymmetric spindle geometry, resulting in asymmetric daughter cells during the neuroblast division (Schaefer et al., 2001). These highly polarized cells are an excellent system to study mechanisms of *Wolbachia* segregation.

Although *Wolbachia* are symmetrically localized at sister centrosomes during the syncytial cortical divisions, we demonstrate that *Wolbachia* are asymmetrically localized at the apical pole of the polarized *Drosophila* embryonic epithelia cells and neuroblasts. *Wolbachia* colocalize with apical centrosomes and the apical cortical protein atypical protein kinase C (aPKC). We find that this apical localization is dependent on apical spindle pole microtubules, yet it is independent of extrinsic factors, cortical actin and cortically localized apical determinants. This segregation pattern results in an asymmetric *Wolbachia* distribution to the self-renewing neuroblast. Thus, *Wolbachia* is present in the CNS from early embryonic neuroblasts to mature neurons in the adult brain. In accord with *Wolbachia* maintenance in the embryonic and larval neuroblasts, *Wolbachia* localize to specific regions of the adult brain and might account for *Wolbachia* effects on behavior. Finally, we demonstrate that this segregation pattern is less stringent in Popcorn (W_{Pop}), which is a virulent *Wolbachia* strain.

Results

Wolbachia association with the MTOC results in a symmetric segregation pattern during the initial divisions of *Drosophila* embryogenesis

Wolbachia are associated with the centrosomes in the pre-cellularized, syncycial *Drosophila* embryo (Callaini et al., 1994; Kose and Karr, 1995; O'Neill and Karr, 1990). In the syncytial embryo, all blastoderm nuclei divide in synchrony through the first 13 mitotic cycles. During early interphase of cycle 14, membranes form between nuclei, dividing them into separate cells. During early gastrulation, cell clusters, known as mitotic domains, undergo synchronous mitosis (Foe, 1989). These cells exhibit symmetric distribution of cortical polarity proteins (such as Dlg, Scrib and aPKC), form equally sized and positioned spindle poles during division, and produce two equally sized daughter cells (Bilder et al., 2000).

To analyze *Wolbachia* dynamics throughout the syncytial cell cycles, we took advantage of the vital dye Syto-11. This fluorescent nucleic acid dye robustly labels *Wolbachia*. At long incubation times, Syto-11 labels both *Wolbachia* and host chromosomes. In fixed images, *Wolbachia* were readily identified as rod-shaped Syto-11-stained bacteria that were concentrated around centrosomes (Fig. 1). These Syto-11-positive clusters were only present in infected embryos, demonstrating that the dye is specific to *Wolbachia* and not to other organelles, such as mitochondria. When injected into live embryos and imaged immediately, Syto-11 predominantly labeled *Wolbachia* and not the host DNA (Fig. 2). Live images obtained with the Syto-11 stain were equivalent to images obtained from fixed analysis (data not shown), indicating that Syto-11 is not influencing the cell cycle or *Wolbachia* positioning and therefore provides a means of live analysis of *Wolbachia* dynamics throughout the syncytial cycles.

Embryos injected with Syto-11 and Rhodamine-tubulin were recorded during the cortical nuclear cycles (supplementary material Movie 1). During interphase, equal amounts of *Wolbachia*

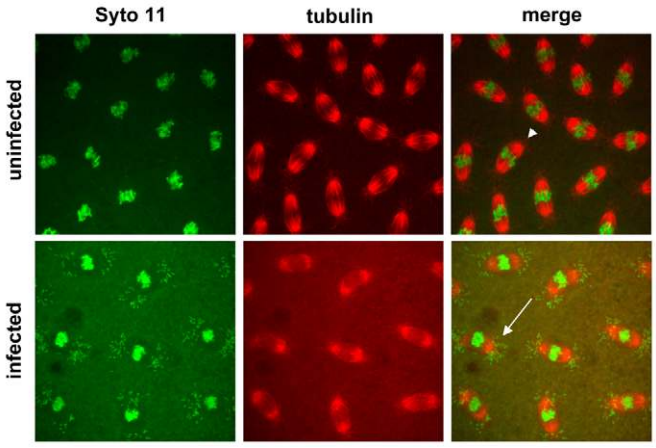

Fig. 1. Syto 11 labels *Wolbachia* and nuclear DNA in *Drosophila* embryos. Fixed uninfected and infected *D. simulans* embryos stained with Syto 11 (green) to label nucleic acid and α-tubulin (red) to label microtubules. *Wolbachia* (W_{Riv}) are present as rod-shaped particles (arrow) located at the poles of the mitotic spindle in infected embryos and are absent from uninfected embryos (arrowhead).

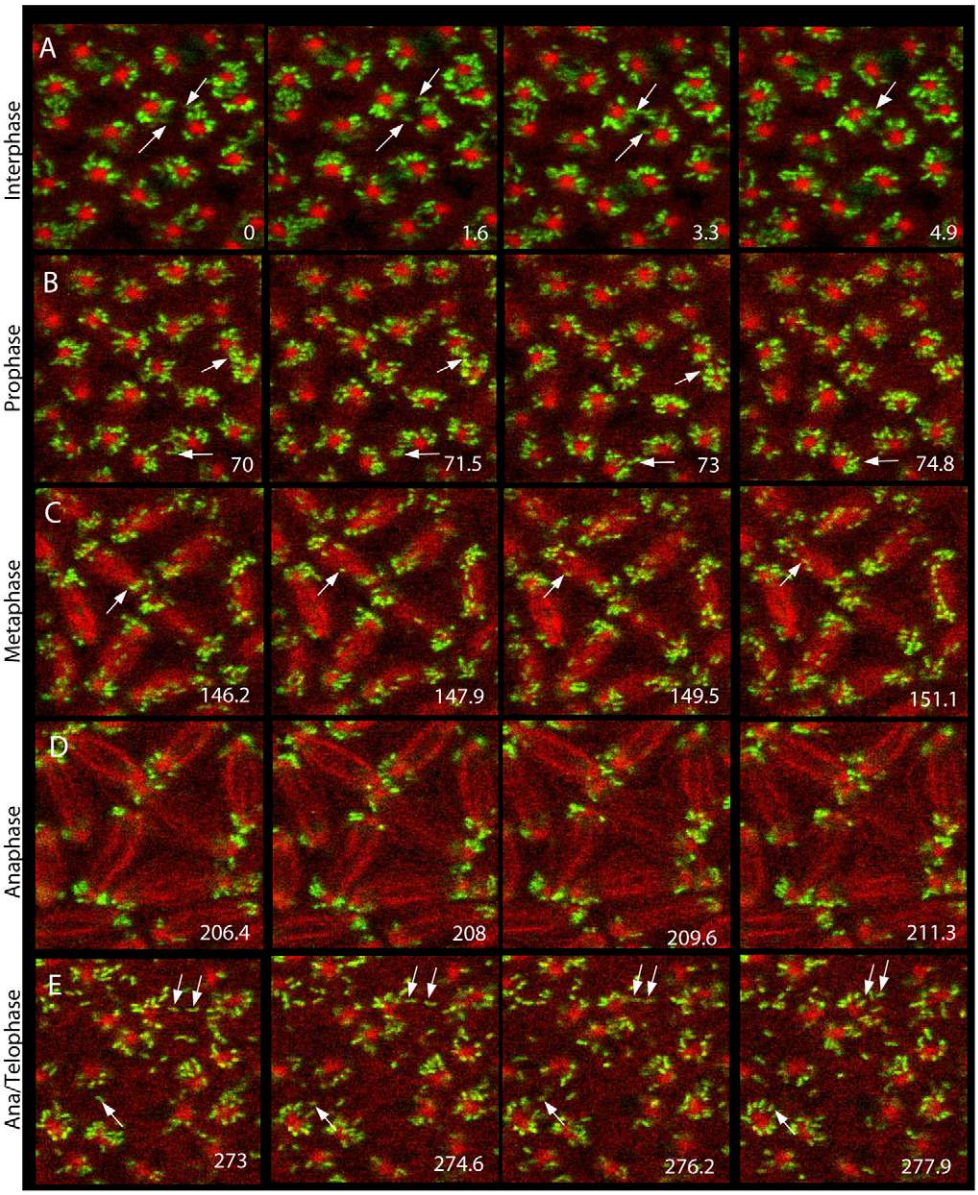

Fig. 2. Patterns of microtubule-based *Wolbachia* movement throughout the syncytial cell cycle. *Wolbachia* movement during consecutive 1.6 second intervals (indicated bottom right) are shown for different phases of the cell cycle (indicated on left). Embryos were injected with Syto-11 to label *Wolbachia* (green), and Rhodamine-tubulin to label microtubule structures (red). Moving *Wolbachia* are indicated by arrows. During late interphase (A), prophase (B) and metaphase (C), most *Wolbachia* movement occurs along microtubules between sister (newly duplicated and divided) centrosomes. Almost no *Wolbachia* movement is observed during anaphase (D), when *Wolbachia* form two clusters on the sides of each centrosome. (E) At late anaphase to early telophase, *Wolbachia* move along astral microtubules between neighboring, non-sister centrosomes. Panels are taken from the supplementary material Movie 1. Analysis was performed with W_{Riv} strain in *D. simulans*.

clustered around the separating sister centrosomes (Fig. 2A). A subset of bacteria moved rapidly between separating sister centrosomes (duplicated centrosomes associated with the same nucleus), presumably relying on the array of anti-parallel pole-to-pole microtubules (Fig. 2A, arrows). By contrast, few *Wolbachia* were observed moving between neighboring, non-sister, centrosomes (centrosomes associated with separate nuclei). This pattern of *Wolbachia* movement continued throughout prophase and metaphase (Fig. 2B,C, arrows). Upon entry into anaphase, *Wolbachia* movement ceased and no *Wolbachia* were found on the central spindle (Fig. 2D). During anaphase, *Wolbachia* formed two clusters at each spindle pole. The centrosomes duplicated during anaphase and existed as a pair at each pole. Thus, each *Wolbachia* cluster is probably associated with a member of the centrosome pair. During the late anaphase and early telophase, rapid *Wolbachia* movements resumed (Fig. 2E, arrows). In contrast to the earlier cell cycle stages, *Wolbachia* moved between sister centrosomes and neighboring non-sister centrosomes.

The ability of *Wolbachia* to move rapidly between sister and non-sister centrosomes facilitates a broad and equal *Wolbachia* distribution throughout the embryo. Consequently, *Wolbachia* have the potential to locate to many if not all different cell and tissue types as development proceeds. Another key factor in establishing this distribution is that during centrosome duplication at anaphase, *Wolbachia* are equally partitioned between separating sister centrosomes. To further understand the mechanisms of *Wolbachia* distribution, we explored the role of microtubules and actin in *Wolbachia* dynamics.

Wolbachia movement and symmetric distribution to syncytial mitotic products require microtubules

The close association between *Wolbachia* and microtubules suggests that *Wolbachia* movement and localization rely on microtubules. To test this, we injected embryos with both Syto-11 and Rhodamine-tubulin to label the *Wolbachia* and microtubules. We then injected the microtubule inhibitor Colchicine during late anaphase, when *Wolbachia* typically exhibit extensive trafficking between both sister

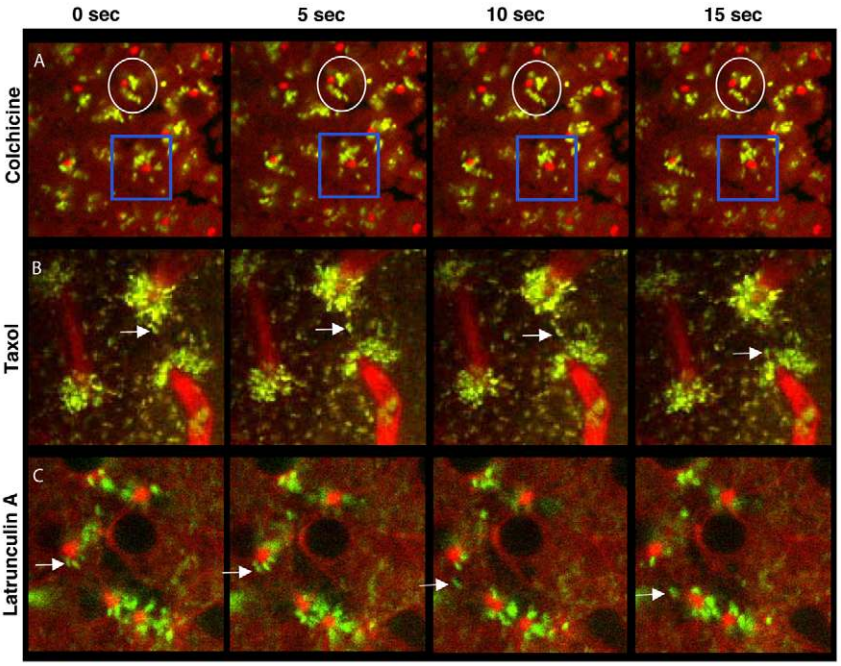

Fig. 3. *Wolbachia* movement depends on microtubules but not on actin filaments. Consecutive 5 second intervals of embryos injected with Syto-11 to label *Wolbachia* (green), with Rhodamine-tubulin to label microtubule-based structures (red) and with inhibitors (indicated to left of panels). (A) Colchicine arrests *Wolbachia* movement during anaphase. (B) Taxol arrests cells in metaphase and *Wolbachia* move only between neighboring non-sister centrosomes (arrows). (C) Latrunculin A has no obvious effect on *Wolbachia* movement during interphase. Colchicine panels are taken from supplementary material Movie 2, Taxol panels from supplementary material Movie 3 and Latrunculin A panels from supplementary material Movie 4. Time frame starts 200 seconds after injection in A and B. Analysis was performed with W_{Riv} strain in *D. simulans*.

and non-sister neighboring centrosomes (Fig. 3A, Fig. 4B; supplementary material Movie 2). *Wolbachia* trafficking between centrosomes immediately stopped in response to the Colchicine treatment (Fig. 3A). The efficacy of the Colchicine treatment was indicated by the lack of visible microtubule formation and the failure in centrosome separation during longer observations (Fig. 4B). In addition, the longer treatment showed that over a period of minutes, the close association of *Wolbachia* with centrosomes is lost, and clusters of *Wolbachia* become distributed throughout the cytoplasm encompassing the nuclei (Fig. 4B). This result shows that intact microtubules are required for *Wolbachia* movement between centrosomes and for maintaining *Wolbachia* centrosome association.

To determine whether *Wolbachia* movement and positioning require dynamic microtubules, we treated embryos with Taxol, a microtubule-stabilizing drug. Embryos were first injected with Syto-11 and Rhodamine-tubulin, followed by Taxol injection (supplementary material Movie 3). During prophase, *Wolbachia* movement in Taxol-treated embryos was similar to that in untreated embryos: *Wolbachia* moved between sister centrosomes and occasionally between neighboring centrosomes (data not shown). Taxol treatment caused cells to arrest at metaphase (Fig. 4C). During Taxol-induced metaphase arrest, *Wolbachia* movement occurred along astral microtubules between neighboring, non-sister centrosomes (Fig. 3B, arrows). Long-term Taxol-treatment also showed that *Wolbachia* remained closely associated with centrosomes (Fig. 4C), demonstrating that dynamic microtubules are not required for maintaining the *Wolbachia* concentration at the centrosomes. However, movement between sister centrosomes along the pole-to-pole axis was greatly reduced (Fig. 3B).

Wolbachia movement and *Wolbachia* centrosome positioning during syncytial divisions do not require actin

To determine whether actin is required for *Wolbachia* movement and positioning during the cortical syncytial divisions, *Wolbachia* and microtubule dynamics were monitored in real time after injecting Latrunculin A, a potent inhibitor of actin polymerization (Fig. 3C, Fig. 4D; supplementary material Movie 2). Latrunculin A compromises the cortical actin cytoskeleton, disrupts the interphase actin cap and metaphase furrow formation in syncytial embryos, resulting in failed centrosome separation and abnormal nuclear spacing (Cao et al., 2008; Spector et al., 1983).

Latrunculin A was injected during the anaphase-telophase transition, and *Wolbachia* dynamics were monitored during the following division cycle. *Wolbachia* movement between neighboring non-sister centrosomes was normal in Latrunculin-A-treated embryos (Fig. 3C, arrows). Longer observation of Latrunculin-A-treated embyos showed that, in contrast to the Colchicine treatment, *Wolbachia* generally maintained a close association with the centrosomes (Fig. 4D). Similar results were obtained after treatment with Cytochalasin D, another drug that disrupts actin filaments (data not shown). Taken together, these results demonstrate that actin filaments are not required for *Wolbachia* movement between the centrosomes or for *Wolbachia* positioning at the centrosomes.

Asymmetric distribution and segregation of *Wolbachia* to the self-renewing stem cell in the embryonic neuroblast divisions

The even *Wolbachia* partitioning among dividing nuclei in the syncytial embryo ensures that all tissues have the potential to inherit *Wolbachia*. However, the uneven distribution in adult tissues suggests that this distribution pattern changes during development. To understand the origins of uneven *Wolbachia* tissue distribution in adults, we examined *Wolbachia* later during the post-cellularized mitotic divisions at gastrulation (Fig. 5A). During metaphase, anaphase, early and late telophase, *Wolbachia* were clearly present at both poles. Consequently, as with the syncytial divisions, both daughter cells contained even numbers of *Wolbachia* (Fig. 5A, arrows).

In contrast to symmetric mitotic divisions of the syncytial and post-cellularization stages, embryonic neurogenesis involves highly polarized cells that undergo asymmetric mitotic divisions to produce daughter cells with different developmental fates (Egger et al., 2008). The apical centrosome enlarges and nucleates a large number of astral microtubules. Subsequently, apical astral microtubles are

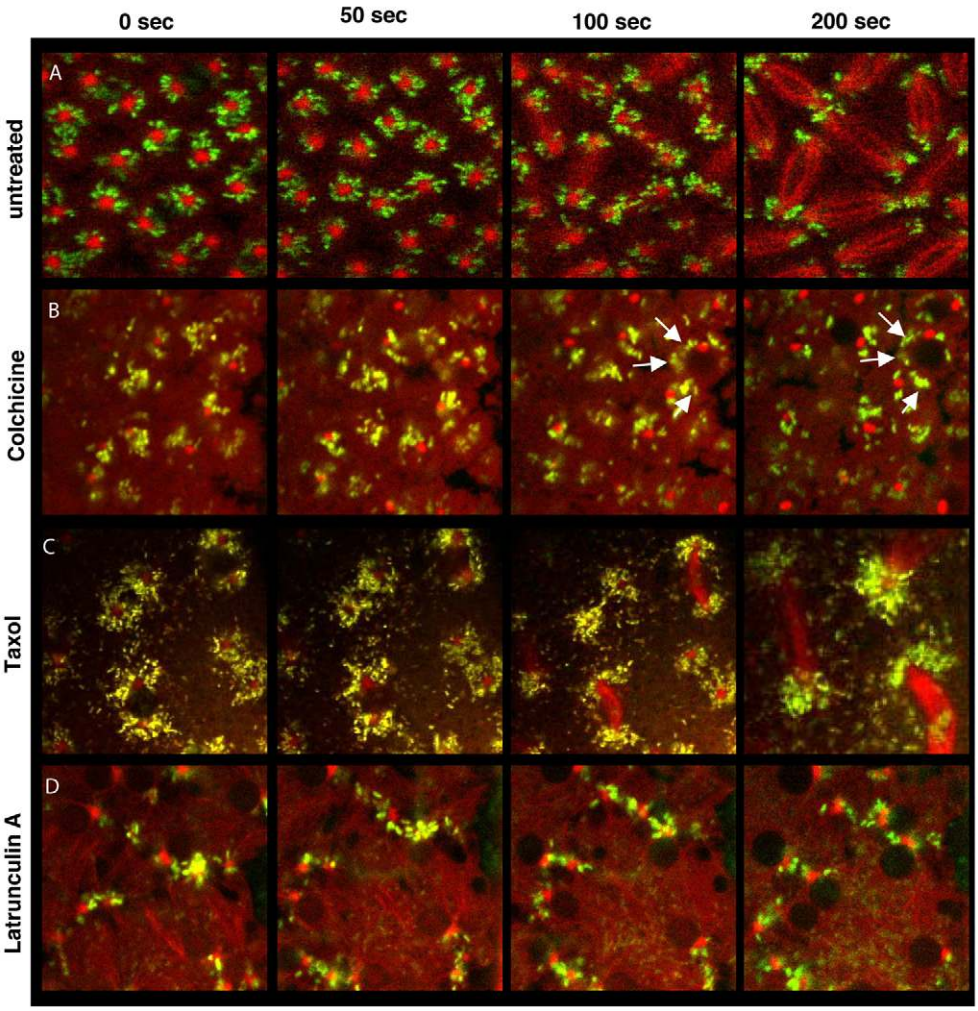

Fig. 4. *Wolbachia* association with centrosomes depends on microtubules. Consecutive 50 and 100 second intervals of the same embryos shown in Fig. 2 (untreated) and Fig. 3 (from the same supplementary Movies). (A) Untreated embryos with centrosome-associated *Wolbachia.* (B) Colchicine treatment at interphase results in loss of *Wolbachia* concentration around the centrosome and abnormal distribution around the entire nucleus (arrows). (C) Taxol treatment during interphase does not affect *Wolbachia* centrosome association. (D) Latrunculin A injection leads to unevenly spaced nuclei and prevents centrosome separation, yet does not affect *Wolbachia* localization to centrosomes. Analysis was performed with W_{Riv} strain in *D. simulans*.

longer and denser than basal astral microtubles. We discovered that *Wolbachia* exhibits a striking asymmetric segregation during these neuroblast divisions (Fig. 5B). For this analysis, we took advantage of *Wolbachia*-infected *D. simulans* lines originating from collections in Big Sur, California. One of these lines (named W_{Pinta}) exhibited a high concentration of *Wolbachia* in the embryonic neuroblasts and thus facilitated characterization of *Wolbachia* segregation in this cell type.

In contrast to the symmetric segregation patterns, *Wolbachia* were almost exclusively localized at the apical cortex during interphase in stage 11 neuroblasts. (Fig. 5B, arrows in first panel). This position was maintained as the neuroblast progresses into prophase and through telophase and cytokinesis (Fig. 5B). Consequently, nearly all *Wolbachia* were segregated to the apical daughter cell after cytokinesis. These images also show the size asymmetry during neuroblast division: the apical daughter cell was much larger than the basal daughter cell (Fig. 5B, right panel). After cell division, the apical cell self-renews as a neuroblast stem cell, whereas the basal cell forms the ganglion mother cell that will ultimately produce neurons and/or glial cells (Wu et al., 2008). Thus, asymmetric *Wolbachia* localization to the apical cell cortex is likely to ensure that bacteria will mostly remain in the self-renewing neuroblast during embryonic neurogenesis and infection will persist in neuronal stem cells through later development stages. By contrast, embryonic and larval neurons are likely to be less infected as a result of this segregation pattern.

To examine *Wolbachia* localization in dividing neuroblasts relative to spindle dynamics, we performed double immunofluorescence analysis using antibodies against the *Wolbachia* surface protein (Wsp) and against tubulin (Fig. 5C). Consistent with the propidium iodide staining results, *Wolbachia* specifically localized to the apical pole during interphase and maintained this apical association during metaphase and telophase. In addition, *Wolbachia* concentrated near the apical MTOC and apical astral microtubules (Fig. 5C, arrows).

We next examined whether asymmetric *Wolbachia* localization in dividing neuroblasts is maintained into late embryogenesis and larval developmental stages. Embryonic neuroblasts repeatedly divide and reduce in size. Neuroblasts then undergo a stage of quiescence before they are reactivated during larval stages. During the early larval stages, neuroblasts grow and asymmetrically divide to produce a self-renewing daughter neuroblast and a daughter that will give rise to a variety of specialized neural cells in the third instar larval brain (Ceron et al., 2001). We stained third-instar larval ventral ganglia and brain lobes, and examined *Wolbachia* distribution in dividing neuroblasts. *Wolbachia* showed nearly exclusive localization to the Scribble-enriched apical cortex throughout the entire cell cycle in both locations (Fig. 5D, insets, arrows). These results indicate that *Wolbachia* segregate asymmetrically to neuroblast stem cells throughout embryonic and larval neurogenesis.

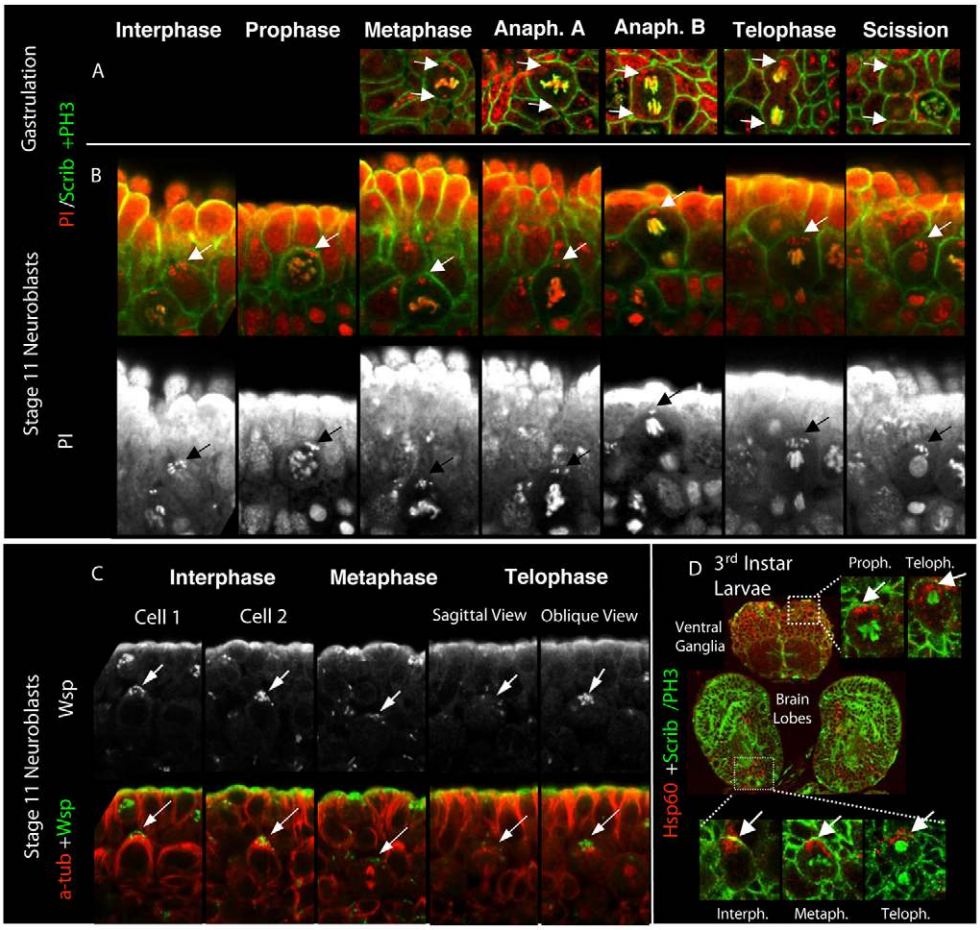

Fig. 5. *Wolbachia* localization in cells during *Drosophila* development. (A) *Wolbachia* (W_{Pinta} in *D. simulans*) distributes symmetrically in dividing daughter cells during gastrulation (arrows). (B) Neuroblasts from stage 11 embryos show asymmetric distribution and localize apically during host cell mitosis (arrows). *Wolbachia* are stained with propidium iodide (PI, red), the host cell cortex is marked with anti-scribble (green), and host-cell DNA is stained with anti-phospho-histone3 (PH3, green) in addition to PI staining (yellow in merged image). (C) *Wolbachia* specifically localize near the microtubule-organizing centers in neuroblasts (arrows). *Wolbachia* are labeled with anti-Wsp antibody (top row and green in bottom row), microtubules are marked with anti-α-tubulin (red). (D) *Wolbachia* are distributed asymmetrically in dividing neuroblasts in third-instar larval brains. Inset magnifications of dividing neuroblasts are shown from the ventral ganglia (top) and from the brain lobes (bottom) during different cell cycle stages as indicated. *Wolbachia* are labeled with anti-Hsp60 (red), host cell cortex is marked with anti-scribble (green), and host cell DNA with anti-PH3 (green). Antibodies against recombinant human heat-shock protein 60 (Hsp60) and Scribble were used to label *Wolbachia* and the neuroblast apical cortex, respectively.

Apical *Wolbachia* localization is independent of division axis in polarized epithelia cells

Neuroblasts and epithelia originate from the neuroectodermal layer at the embryo periphery (Kuchinke et al., 1998; Wodarz and Huttner, 2003). Similarly to neuroblasts, epithelial cells have a distinct cortical polarity, consisting of apical and basolateral domains (Hutterer et al., 2004; Suzuki and Ohno, 2006; Tepass et al., 2001). We found that in polarized epithelial cells, *Wolbachia* localized apically during interphase, similarly to neuroblasts (Fig. 6A-E, arrowheads). Interestingly, *Wolbachia* even localized apically during neuroblast delamination from the neuroectodermal layer (supplementary material Fig. S1). Unlike neuroblasts, however, epiblasts divided parallel (and not perpendicular) to the neuroectoderm, and they divided symmetrically based on cell size, spindle pole size and cortical protein localization. We examined *Wolbachia* position and segregation during mitosis in these polarized, but symmetrically dividing cells to understand the origin of asymmetric distribution. *Wolbachia* distribution was equal between the two spindle poles during symmetric epiblast divisions (Fig. 6F, arrowheads). Adjacent cells, a non-dividing polarized epithelial cell and two neuroblasts, all showed asymmetric apical *Wolbachia* localization (Fig. 6F, arrow). This result illustrates that epithelial cell asymmetry during interphase determines apical *Wolbachia* localization.

Wolbachia colocalizes with aPKC

Embryonic neuroblast polarity is influenced by both extrinsic epithelia signals and by cell-intrinsic factors such as cortical cell polarity and asymmetric spindle poles (Siegrist and Doe, 2006; Siegrist and Doe, 2007). The position of the apical protein domain is induced by the surrounding epithelia cells and generally aligned with the apical poles of the surrounding cells (Siegrist and Doe, 2006), but apical protein crescents are occasionally mispositioned toward the lateral cortex. Proteins of the highly conserved apical Par3 (Bazooka)-Par6-aPKC (Par) complex are interdependent for complex formation and maintenance at the apical cortex (Wu et al., 2008). During prophase, the apical Par protein complex recruits a second protein complex that includes heterotrimeric G proteins (Pins-Gαi complex) and ultimately regulates spindle orientation and geometry (Fuse et al., 2003; Wu et al., 2008). During metaphase, the Pins-Gαi complex can also be apically localized by microtubules (Siegrist and Doe, 2005; Siegrist and Doe, 2007).

We analyzed *Wolbachia* colocalization with the cortical protein aPKC as a marker for cell-intrinsic apical factors. For these experiments, we used the well-characterized and sequenced strains *Wolbachia Riverside* (W_{Riv}) in *D. simulans* (Salzberg et al., 2005), and *Wolbachia melanogaster* (W_{Mel}) in *D. melanogaster* (Wu et al., 2004). These are frequently used laboratory strains, and although there are fewer *Wolbachia* per cell than in W_{Pinta}, both strains also exhibit the asymmetric apical localization that was observed with the newly isolated W_{Pinta} strain (Fig. 6A-E). We labeled aPKC to identify the Par complex and showed that both W_{Riv} (Fig. 6A-C) and W_{Mel} (Fig. 6D-E) strongly colocalized with the aPKC crescent in the neuroblasts (arrows). The colocalization was particularly striking in Fig. 6C,E,

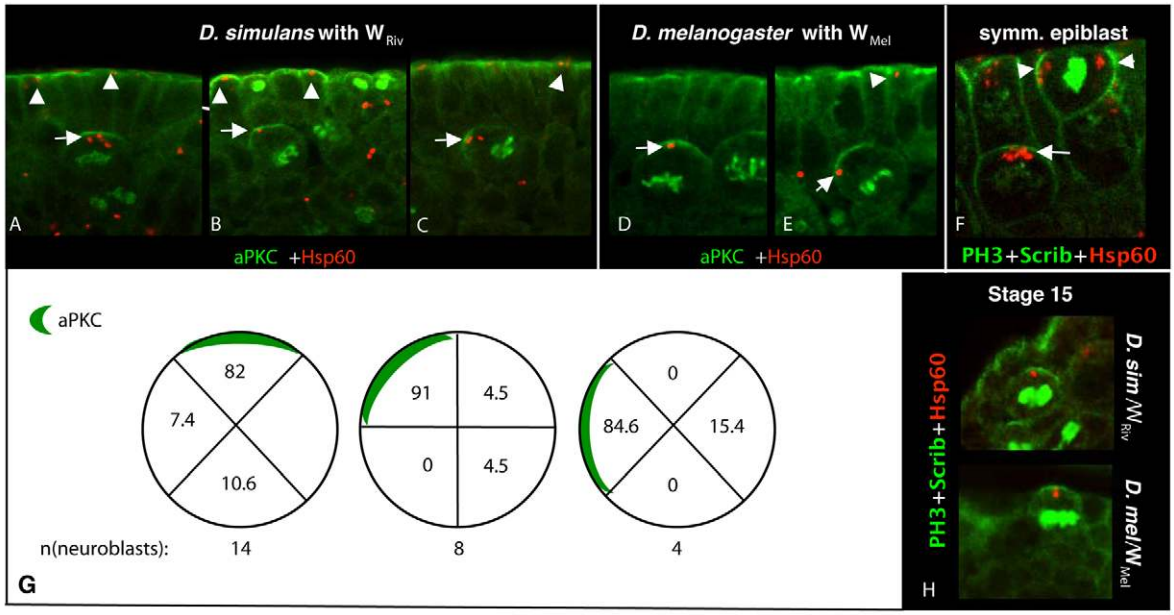

Fig. 6. *Wolbachia* localization in neuroblasts and epithelial cells (W_{Riv} in *D. simulans* and W_{Mel} in *D. melanogaster*). (A-E) *Wolbachia* localize to the aPKC-dense area in neuroblasts (arrows) and epithelial cells (arrowheads) in W_{Riv} in *D. simulans* (A-C) and in W_{Mel} in *D. melanogaster* (D,E). *Wolbachia* are stained with anti-Hsp60 (red), aPKC is labeled with anti-aPKC (green), and host cell DNA with anti-PH3 (green). (F) *Wolbachia* are distributed evenly between symmetrically dividing epiblasts (arrowheads) and apically localized in the neuroblast below (arrow). *Wolbachia* are labeled with anti-Hsp60 (red), dividing cells with anti-scribble (green), and host DNA with anti-PH3 (green). (G) Quantification of *Wolbachia* localization (%) in relation to aPKC distribution in neuroblasts of wild-type embryos with naturally differing neuroblast orientation. (H) *Wolbachia* localization near the aPKC crescent in Stage 15 neuroblast after dissociation from the epithelial layer. Staining as in panels A-E.

in which the aPKC crescent was mis-positioned laterally relative to the surrounding epithelia. In these instances, *Wolbachia* localized to the cortical region associated with the mislocalized aPKC crescent rather than to the cortical region that is apical relative to the surrounding epithelium.

Quantification of *Wolbachia* and aPKC colocalization in naturally occurring neuroblasts with correctly or mis-positioned apical proteins is shown in Fig. 6G. Roughly half of the neuroblasts (14 of 26) had apically localized aPKC with most (82%) of the *Wolbachia* cells localizing apically. About one third of the neuroblasts had the aPKC crest oriented 45 degrees off the apical-basal axis with respect to the surrounding epithelium. In those cells, almost all *Wolbachia* (91%) aligned with aPKC rather than with the 'true' apical position relative to surrounding cells. Few neuroblasts (4 of 26) had the aPKC crest oriented 90 degrees off the apical-basal axis. Even in those neuroblasts, most *Wolbachia* (84%) localized with the crest, and none with the 'true' apical position relative to the surrounding epithelium.

The data indicate that *Wolbachia* maintain strong colocalization with the aPKC crescent, even if the crescent is not aligned with true apical position, as determined by the surrounding cells. These results suggest that the extrinsic epithelia cells do not provide the primary cue for *Wolbachia* localization. Further support for the hypothesis that *Wolbachia* localization is predominantly determined by cell-intrinsic signals stems from the observation that W_{Mel} and W_{Riv} maintained colocalization with aPKC at late embryonic and larval stages, when neuroblasts have dissociated from the epithelial layer (Fig. 5D, Fig. 6H) and no longer receive extrinsic cues.

Taken together, the results indicate that *Wolbachia* localization is strongly influenced by cell polarity and that *Wolbachia* positioning is influenced by cell-intrinsic cues such as cortical protein domains and spindle pole microtubules.

Microtubules but not actin are essential for apical *Wolbachia* localization

The Par complex determines the apical-basal orientation as soon as neuroblasts delaminate from the neuroectoderm (Hutterer et al., 2004; Wu et al., 2008). Subsequent apical localization of the Pins-Gαi complex is either guided by signaling from the Par complex, or by apical microtubules (Siegrist and Doe, 2007). To determine whether apical *Wolbachia* localization in dividing neuroblasts depends on microtubules, we treated living embryos with Colcemid and assayed *Wolbachia* distribution. Propidium Iodide and anti-aPKC antibody were used to stain for *Wolbachia* and the apical cortex (Fig. 7A). Colcemid disrupted microtubule organization, as evidenced by a lack of a mitotic spindle in treated but not in control metaphase neuroblasts (Fig. 7A, bottom row). In mock-treated embryos, 90% of *Wolbachia* bacteria located to the apical cortex (Fig. 7A, top row schematics). After Colcemid treatment, cell orientation became randomized as the spindles depolymerized. After 30 and 60 minutes of treatment, an aPKC crescent was still defined, although the orientation relative to the surrounding tissue had rotated in some instances. After 30 minutes of Colcemid treatment, only 79% of *Wolbachia* remained apical with respect to aPKC localization. Basal and lateral localization increased from 9% to 21%. After a 60 minute treatment, the portion of *Wolbachia* maintained at the apical cortex was further reduced to 46% while basal-lateral localization increased to 54% (compared with 85% and 14%, for mock-treated embryos). These studies demonstrate that microtubules have an important role in *Wolbachia* localization to the apical crescent.

We next determined whether cortical actin has a role in localizing *Wolbachia* to the apical cortex. As opposed to microtubules, actin microfilaments are required to anchor asymmetrically distributed proteins (such as the Par complex) during neuroblast mitosis (Broadus and Doe, 1997). Neuroblasts were treated with the F-actin-

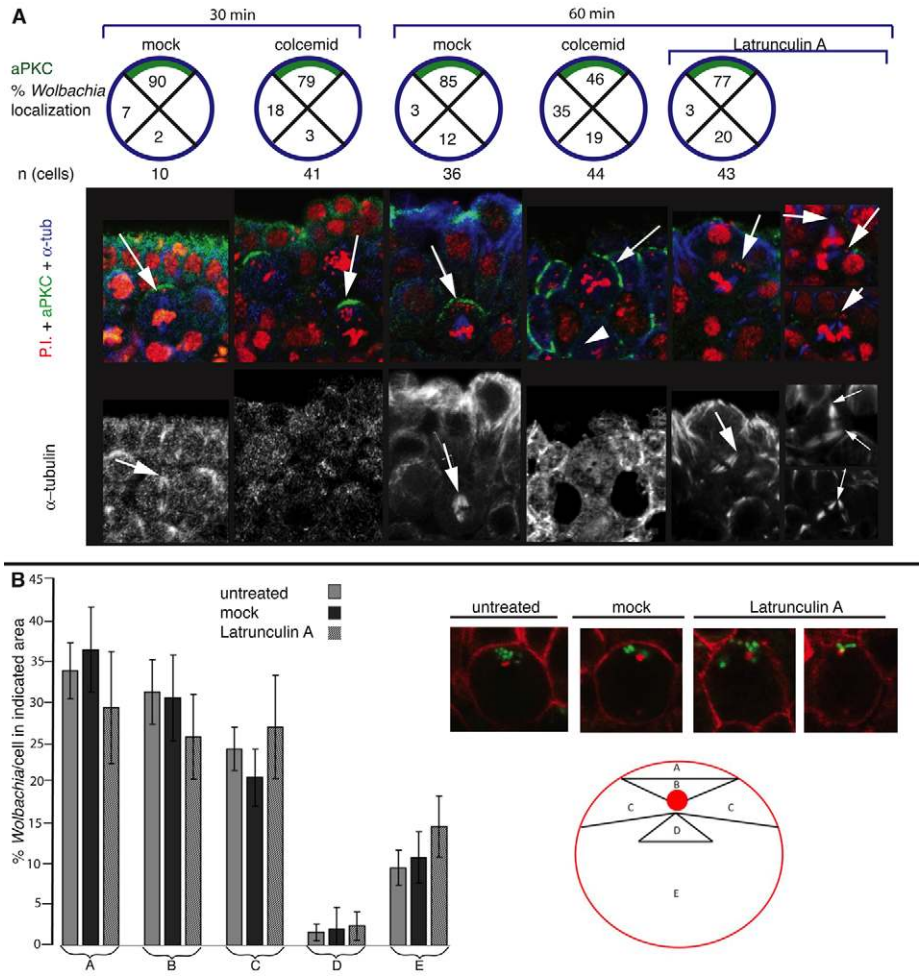

Fig. 7. *Wolbachia* localization depends on intact microtubules but not on actin filaments. (A) Top panel shows *Wolbachia* quantification (%) in neuroblasts in the indicated areas in relation to aPKC staining. Middle panels show representative images of the treatment indicated above the schematics. The lower panels show tubulin staining only of the same images as the middle panels. *Wolbachia* (red puncta) disperse from the apical cortex after microtubule disruption (Colcemid treatment) for 30 and 60 minutes, and no longer colocalize with the aPKC crescent (green crescent, arrow). Actin depolymerization (Latrunculin A treatment) does not disrupt microtubules, but causes spindle rotation and fused spindles (arrows in lower panels), and disperses aPKC (green) from the apical cortex. *Wolbachia* continue to localize to the spindle poles (arrows). *Wolbachia* are stained with propidium iodide (PI, red), aPKC is stained with anti-aPKC (green) and tubulin with anti-α-tubulin (blue and lower panels). Spindle poles are indicated by arrows in the mock and Latrunculin A treatments. (B) Quantification of *Wolbachia* localization with respect to the centrosome after no treatment, after mock treatment (DMSO in Schneider's medium) and after Latrunculin A treatment. *Wolbachia* (% ± s.e.) are quantified in the areas labeled with letters indicated on the schematic.

depolymerizing drug Latrunculin A, which has been shown to eliminate the cortical actin network and completely destabilize apical protein complexes (Barros et al., 2003). After Latrunculin A treatment for 60 minutes, aPKC was mislocalized and dissociated from the neuroblast cortex (Fig. 7A, right panels, lack of green crescent). Yet, in spite of aPKC dispersion, *Wolbachia* (Fig. 7A, arrows in merged panels) still localized to the MTOC of the spindles (Fig. 7A, arrows in bottom row). *Wolbachia* exhibited an apical localization in 77% of the neuroblasts compared with 85% for the controls. As expected, Latrunculin-A-treated cells failed to divide, resulting in cells with multiple nuclei, ectopic spindles poles and misoriented spindles (Fig. 7A, right α-tubulin panels). *Wolbachia* still localized to the spindle poles in these abnormal cells, even when aPKC was absent, and spindles were grossly misaligned with respect to surrounding cells.

Wolbachia localization around the MTOC was quantified in untreated, mock-treated and Latrunculin-A-treated neuroblasts (Fig. 7B). *Wolbachia* localization was scored relative to the MTOC, as apical in close cortex proximity (A), apical (B), basal (D), lateral (C), or randomly in cell cytoplasm (E). *Wolbachia* localization in either of the zones remained unchanged after Latrunculin A treatment, although the treatment resulted in multinucleate cells, loss of telophase, loss of epithelial columnar morphology and polarity (not shown). These data indicate that *Wolbachia* localization to the spindle pole does not require an intact cortical cytoskeleton, the presence of apically localized cortical proteins such as aPKC, or the alignment of astral microtubules with the apical cell pole. The results support a model in which the apical spindle pole is a key factor for asymmetric *Wolbachia* localization.

Asymmetric *Wolbachia* segregation depends on asymmetric neuroblast division

In asymmetric neuroblast division, the Par complex and the apical Pins-Gαi complex function in redundant pathways to induce asymmetric spindles and daughter cells (Cai et al., 2003; Wodarz, 2005) by suppressing basal spindle development (Fuse et al., 2003). Pins binds to Gαi in the heterotrimetic G-protein complex and causes the release of the Gβ13F subunit. Mutation of any of these proteins (Pins, Gαi, Gβ13F) causes defects in asymmetric protein localization (Insc, Numb, Miranda) and results in largely asymmetric neuroblast division (Fuse et al., 2003; Schaefer et al., 2001; Yu et al., 2003). Overexpression of Gαi, which binds and depletes the Gβγ pool, also leads to the loss of polarized localization of Gαi and Pins, Miranda, Numb, resulting mislocalized spindles, and terminating in neuroblast division into equally sized daughter cells (Schaefer et al., 2001). More specifically, Gαi overexpression leads to the formation of abundant astral microtubules at both centrosomes, rather than just at the apical centrosome as seen during wild-type neuroblast division (Yu et al., 2003).

We used GαI-overexpressing embryos (stage 11) to analyze whether *Wolbachia* apical localization in neuroblasts depends on Gβγ-dependent induction of asymmetric proteins and spindle

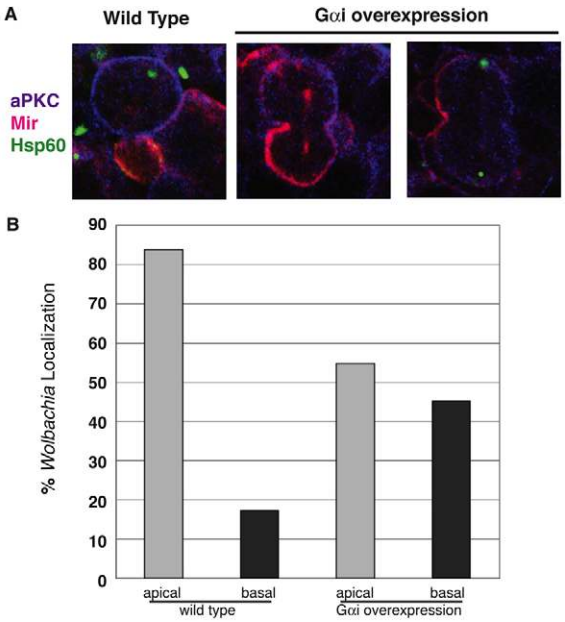

Fig. 8. Gαi overexpression in stage 11 embryos abolishes asymmetric division in neuroblasts and randomizes *Wolbachia* distribution. (A) Asymmetrically dividing neuroblast in the wild type and symmetrically dividing neuroblasts in Gαi-overexpressing embryos. Miranda (pink), a basally located cortical protein is localized properly in the wild type, but mislocalized to the mitotic spindle (middle panel) and to the apical and basal cortex (middle and right panels) in Gαi-overexpressing cells. *Wolbachia* localization (green) is apical in the wild type, and either absent (middle panel) or random (right panel) in Gαi-overexpressing cells. (B) Quantification of *Wolbachia* localization in asymmetrically dividing wild-type neuroblasts (n=23 cells) and in symmetrically dividing neuroblasts (n=18 cells).

poles. In accordance with Schaefer and colleagues (Schaefer et al., 2001), we observed that Miranda, a basal cortical protein, was no longer restricted to the basal cell in these neuroblasts (Fig. 8A). Analysis of *Wolbachia* localization showed a random distribution in dividing telophase neuroblasts (Fig. 8B). This result clearly indicates that *Wolbachia* localization to the apical centrosome depends on the induction of asymmetry in the neuroblast divisions.

Wolbachia strains differ in the extent to which they localize apically in embryonic epithelia cells and neuroblasts

We examined Wolbachia neuroblast localization patterns in W_{Riv}, W_{Mel} and W_{Pop}. The latter strain is known to overproliferate in adult brains and cause neurodegeneration (Min and Benzer, 1997). Similarly to W_{Riv} and W_{Mel}, W_{Pop} exhibited apical *Wolbachia* localization in epithelia cells of stage 9 and 10 embryos (Table 1, supplementary material Fig. S1). W_{Riv} showed the strongest *Wolbachia* localization to the apical domain (93%), whereas in W_{Mel} and W_{Pop}, two thirds of the bacteria were in the apical quarter (Table 1). While neuroblasts were delaminating from the epithelium, W_{Riv} maintained a strong apical localization, whereas W_{Pop} exhibited a weaker apical localization (Fig. S1). In interphase neuroblasts, all three strains exhibited apical *Wolbachia* localization, but W_{Pop} showed weaker retention (57%) in the apical region than W_{Riv} (95%) and W_{Mel} (71%) (Table 1, supplementary material Fig. S1). Quantification of *Wolbachia* revealed significantly more W_{Riv} per neuroblast in *D. simulans* (7.2 bacteria per cell) than W_{Mel} or W_{Pop} in *D. melanogaster* (1.6 and 2.4 bacteria per cell, respectively, Table 1). Taken together, these data highlight strain differences in the abundance and in the extent to which *Wolbachia* segregate asymmetrically in the neuroblasts.

Wolbachia localization in adult brain

To examine the consequences of asymmetric *Wolbachia* distribution during development, we determined abundance of *Wolbachia* the adult brain (Fig. 9). *Wolbachia* were detected by the DNA stain Syto11 and seen as small dots surrounding the Syto-11-stained host nuclei (Fig. 9B). We found that the *Wolbachia* titer differed significantly among brain regions (Fig. 9D). Images of W_{Riv} in *D. simulans* showed that *Wolbachia* density was highest in the central brain, containing subesophageal ganglia (Fig. 9A, bottom left panel), antennal lobes and the superior protocerebrum (Fig. 9A, right insets, bottom and middle panels). Bacteria densities were lower in the lamina (Fig. 9A, right inset, top panel) and eye (top inset). Very few bacteria were found in the optic lobe (Fig. 9A, left insets, top panel). *Wolbachia* strains W_{Mel} and W_{Pop} in *D. melanolgaster* also exhibited low titers in the optic lobe (Fig. 9A, left insets, middle and bottom panels), and were more abundant in the subesophageal ganglia (Fig. 9A, bottom insets, middle and right panels). Among the three strains, W_{Pop} exhibited the greatest concentration in the adult brain (Fig. 9A, bottom insets). W_{Pop} formed large aggregates in the brain and appeared to be more clumped than the other bacteria strains (Fig. 9C, arrows).

Discussion

Symmetric and asymmetric *Wolbachia* segregation patterns

The studies presented here define two distinct patterns of *Wolbachia* segregation during *Drosophila* development (Fig. 10). As has been previously reported (Callaini et al., 1994; Kose and

Table 1. Strain differences in apical *Wolbachia* localization in stage 9 and 10 embryo interphase neuroblasts and epithelia cells

	Wolbachia localization	W_{Riv} in *Drosophila simulans*	W_{Mel} in *Drosophila melanogaster*	W_{Pop} in *Drosophila melanogaster*
Interphase neuroblasts	100% in apical third	95	71	57
	<50% in apical third	5	13	19
	>50% in apical third	0	16	25
N (embryos)		4	5	5
n (cells)		119	45	53
Epithelial cells	All in apical quarter	93	58	59
	Apical and lateral	4	26	35
	Apical, lateral, basal	0	15	5
N (embryos)		3	4	4
n (cells)		162	79	79
No. of *Wolbachia* per infected cell		7.2	1.6	2.4

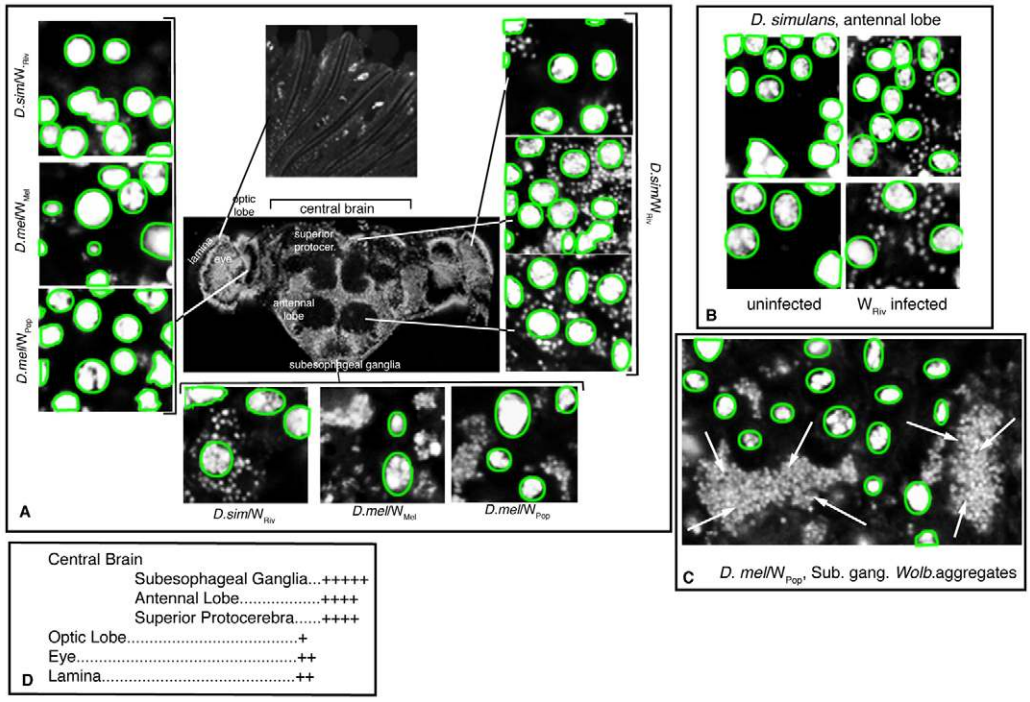

Fig. 9. *Wolbachia* are most abundant in the central brain. (A) Image of a male *Wolbachia*-infected adult brain, anterior view. Most bacteria can be seen in the subesophageal ganglia (bottom insets, hosts and bacteria strains are indicated), in the antennal lobe (right insets, bottom image) and in the superior protocerebrum (right insets, middle image). Fewer bacteria are found in the optic lobe in all three examined strains (left insets, hosts and bacteria strains are indicated), in the eye (right insets, top image) and in the lamina (top inset). Unless otherwise indicated, the images are W_{Riv} in *D. simulans*. (B) *Wolbachia* are visible as small puncta next to the host nuclei in *D. simulans*. (C) W_{Pop} form large aggregates in the subesophageal ganglia (arrows). Host nuclei are circled in green in A-C. (D) Subjective quantification of *Wolbachia* abundance in the different brain areas of a W_{Sim}-infected *D. simulans* fly.

Karr, 1995; O'Neill and Karr, 1990), we found that *Wolbachia* undergo a symmetric division pattern and segregate evenly throughout the syncytial divisions in the developing *Drosophila* embryo. However, later in embryogenesis and larval development we found a distinct and highly asymmetric segregation pattern, in which *Wolbachia* are preferentially inherited to only one of the two daughter cells. Both segregation patterns rely on a close association of *Wolbachia* with microtubules and centrosomes. Although the symmetric segregation broadly disseminated *Wolbachia* throughout all embryonic cell lineages, the later asymmetric segregation pattern in the post-cellularized embryo concentrated *Wolbachia* in specific cell lineages. This is in agreement with findings that *Wolbachia* is widely but unevenly distributed throughout the tissues of adult insects (Dobson, 2003; Ijichi et al., 2002; McGarry et al., 2004).

Cell-cycle regulation of *Wolbachia* movement

Live analysis revealed that during the syncytial division cycles, *Wolbachia* use microtubules to rapidly migrate between sister and non-sister centrosomes. From prophase through early anaphase, *Wolbachia* migrated on the pole-to-pole microtubules between sister centrosomes. Little movement was observed during late anaphase and early telophase. During the telophase-interphase transition, *Wolbachia* relied on astral microtubules to migrate between neighboring, non-sister centrosomes (Fig. 10). This extensive migration between centrosomes promotes a broad *Wolbachia* distribution throughout the embryo. Given the extensive movement of *Wolbachia* throughout much of the syncytial cycle, the stable and tight association with the centrosomes during early anaphase is particularly striking. Significantly, this coincides with centrosome duplication and separation. The stable association ensures that equal numbers of *Wolbachia* associate with the duplicated and separating sister centrosomes.

Previous work also demonstrated *Wolbachia* localization near the centrosome and suggested that this was the mechanism by which *Wolbachia* are widely distributed throughout the syncytial embryo (Callaini et al., 1994; Kose and Karr, 1995). Our studies confirm this finding and further demonstrate that shuttling between sister and non-sister centrosomes provides an additional distribution mechanism. One possible advantage of *Wolbachia* moving between both sister and non-sister centrosomes during the mitotic cycle is that this might facilitate a more rapid and broader bacteria distribution throughout the dividing nuclei of the entire embryo.

Microtubule-dependent *Wolbachia* movement

In accord with previous studies (Callaini et al., 1994; Kose and Karr, 1995), concentration of *Wolbachia* near the centrosomes required intact microtubules. Work in the *Drosophila* oocyte has demonstrated that microtubule-dependent *Wolbachia* movement during mid-oogenesis relies on the minus-end motor protein dynein for proper anterior positioning (Ferree et al., 2005). This raises the possibility that the concentration at the centrosome might be achieved by *Wolbachia* continuously engaging dynein, a minus-end-directed motor protein. It should be noted, however, that we also observe *Wolbachia* movement away from the MTOC. Work in the *Drosophila* oocyte during later stages of oogenesis has shown that *Wolbachia* also engage the plus-end motor protein kinesin to migrate to and concentrate at the posterior cortex (Serbus et al.,

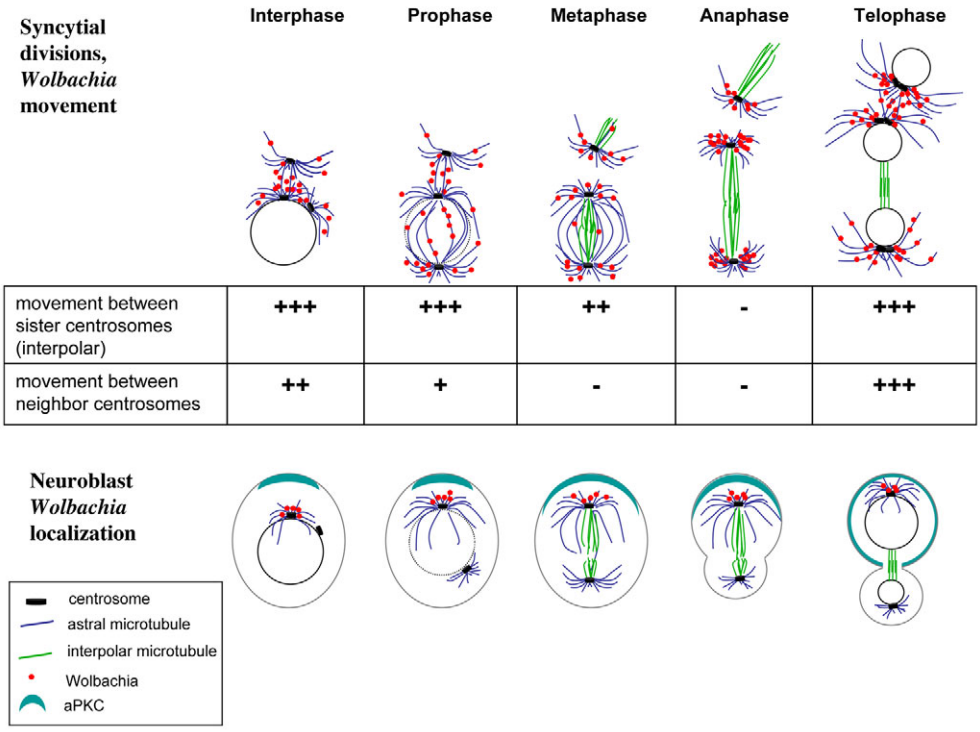

Fig. 10. Schematic of *Wolbachia* symmetric and asymmetric segregation patterns. During syncytial divisions (top), *Wolbachia* move along astral microtubules migrating between sister and non-sister centrosomes during interphase and prophase. During metaphase, less movement occurs between sister centrosomes, and no movement occurs between neighboring centrosomes. We propose that *Wolbachia* movement occurs predominantly on astral microtubules (depicted in blue). During anaphase, *Wolbachia* do not move, but maintain a close association with the separating centrosomes, resulting in a symmetric segregation pattern. *Wolbachia* movement resumes during telophase. During embryonic neuroblast divisions (bottom), *Wolbachia* are concentrated at the apical centrosome. This concentration is achieved primarily through an association with the robust astral microtubules arrays of apical centrosome. After centrosome duplication, the new centrosome moves basally, but does not establish robust astral microtubules. *Wolbachia* do not move to the basal centrosome. This results in an asymmetric segregation and maintenance of *Wolbachia* in the self-renewing neuroblast stem cell.

2008; Serbus and Sullivan, 2007). Therefore, the observed *Wolbachia* movement toward and away from the centrosomes might be the result of *Wolbachia* alternatively engaging dynein and kinesin. Surprisingly, Taxol treatment, which stabilizes microtubules, dramatically reduces *Wolbachia* movement on overlapping pole-to-pole microtubules, but not along astral microtubules connecting neighboring centrosomes. This raises the possibility that *Wolbachia* movement between sister centrosomes is different from that between neighboring non-sister centrosomes. Between sister centrosomes, *Wolbachia* might associate with the ends of dynamic pole-to-pole microtubules that are Taxol stabilized. *Wolbachia* might move using motors along astral microtubules, which continue to operate during Taxol treatment. This possibility is supported by studies suggesting that *Wolbachia* associate with microtubule plus-ends during spermatid elongation (Riparbelli and Callaini, 1998; Riparbelli et al., 2007), and via kinesin KLP67 on astral microtubules during early embryogenesis (Pereira et al., 1997). Another possibility is that *Wolbachia* only move on astral microtubules (see below).

The bacterium *Bradyrhizobium sp.* (Lupinus) also moves toward the spindle poles before cytokinesis (Fedorova et al., 2007) and segregates to both daughter cells during host cell mitosis. After mitosis, the bacteria move to the cortical region over the entire cell. The role of microtubules and motor proteins in *Bradyrhizobium* movement has not been determined and no other bacteria have been reported to segregate to the poles during host cell mitosis. It will be interesting to examine whether the mechanisms used by *Bradyrhizobium* and *Wolbachia* take advantage of the same host factors during their segregation into daughter cells.

Wolbachia localize apically in epithelia and neuroblast cells

After cellularization, *Wolbachia* are evenly distributed throughout the epithelial cell layer resulting from symmetric segregation during cell division in the *Drosophila* embryo. In epithelial cells, *Wolbachia* become apically located during interphase. When epithelial cells divide symmetrically (perpendicular to the apical-basal axis), *Wolbachia* redistribute to both of the spindle poles during metaphase, and segregate symmetrically. As neuroblasts delaminate from interphase epithelial cells, *Wolbachia* remained localized to the thin apical stalk that stretches into the epithelial layer (supplementary material Fig. S1), indicating that neuroblasts inherit apical *Wolbachia* from the neuroectoderm.

Asymmetric segregation of *Wolbachia* in neuroblast cells

In striking contrast to the symmetric *Wolbachia* segregation during syncytial divisions and gastrulation, and in symmetrically dividing epithelia, we discovered that *Wolbachia* exhibit a highly asymmetric segregation pattern in neuroblasts of embryos and larvae. After delamination from the epithelial layer, neuroblasts undergo a spindle rotation, resulting in the spindle being oriented along the apical-basal axis (Egger et al., 2008). Neuroblast cell division then results in segregation of both *Wolbachia* and apical determinants to the self-renewing apical daughter cell. The basal GMC daughter lacks *Wolbachia* and differentiates into larva neurons and glia cells. During the subsequent neuroblast divisions throughout embryo and larval stages, *Wolbachia* are continuously maintained at the apical cortex and segregate to the apical neuroblast daughter cell. Although *Wolbachia* strains vary in the extent to which they exhibit asymmetric segregation, all examined strains exhibit this pattern of segregation. Thus, in contrast to the distributive symmetric centrosome-based segregation pattern during the syncytial divisions, this highly asymmetric pattern of *Wolbachia* inheritance concentrates *Wolbachia* in specific neuroblast lineages. Presumably, this *Wolbachia* distribution results in an overall low infection rate of the larval and adult brain, but it ensures that at least a few glia cells in the adult brain, the ones resulting from the final differentiation of the neuroblast, are infected and reside in the adult CNS.

Possible mechanisms for *Wolbachia* localization

To undergo a switch from symmetric to asymmetric segregation, *Wolbachia* must either interact with different host factors as development proceeds, or with host factors that act differently during development. Either of these possibilities is likely, because studies of *Wolbachia* in oocytes have shown that the bacteria can engage different motor and cortical proteins during oocyte development (Ferree et al., 2005; Serbus and Sullivan, 2007).

A common theme of *Wolbachia* movement and position in the neuroblast and syncytial divisions is their colocalization with astral microtubules. It has been postulated previously that *Wolbachia* only locate to astral and not to spindle microtubules in syncytial embryos (Callaini et al., 1994; Kose and Karr, 1995). If, during syncytial divisions, astral microtubules reach from one pole to the opposite pole during prophase and metaphase but not during anaphase, it could explain the pole-to-pole *Wolbachia* movement during the early mitotic phases and the lack of movement between sister centrosomes in the later stages of mitosis. Similarly during Taxol treatment, when the distance between poles (sister centrosomes) increases (Figs 3B, 4C and 10), *Wolbachia* ceased movement between the sister centrosomes, but not between the closer neighboring centrosomes. During asymmetric neuroblast division, the basal centrosome builds fewer and shorter astral microtubules than the apical centrosome, and only after migration away from the apical centrosome (Rebollo et al., 2007; Rusan and Peifer, 2007; Wodarz, 2005; Yu et al., 2003). If *Wolbachia* only travel along astral microtubules toward the MTOC, the short basal microtubules might be insufficient to allow *Wolbachia* transport to the basal centrosome (Fig. 10).

In both interphase neuroblasts and epithelial cells, *Wolbachia* colocalize with the apical aPKC complex. However, our data show that *Wolbachia* and aPKC rely on different mechanisms for their apical localization: disruption of cortical actin results in severe aPKC mislocalization, but produces only minor disruptions in apical *Wolbachia* positioning. By contrast, apical *Wolbachia* localization in neuroblasts is dependent on intact microtubules. Treatment with microtubule inhibitors resulted in a significant, but not complete, loss of *Wolbachia* from the apical cortex, whereas most aPKC remained localized. One possibility is that in neuroblasts, *Wolbachia* interaction with the apical centrosome depends on compositional differences between the two centrosomes. For example, the basal centrosome has a reduced concentration of γ-tubulin and centrosomal proteins CP60 and CP190 from anaphase on (Kaltschmidt et al., 2000). The basal centrosome MTOC activity is also reduced and only accumulates pericentriolar material (PCM) at mitosis onset (Rebollo et al., 2007).

In asymmetric neuroblast divisions, astral microtubules are also involved in re-enforcing cortical polarity of the apical determinants Pins and Gαi via kinesin Khc-73 that binds to apically located Discs large (Dlg) (Siegrist and Doe, 2005). When microtubules are de-polymerized by Colchicine, cortical polarity is still established, but correct spindle alignment is only achieved in two thirds of the neuroblasts (Siegrist and Doe, 2005). If these microtubule-dependent apical determinants are involved in *Wolbachia* localization, this might explain why *Wolbachia* are largely, but not completely mislocalized after Colchicine treatment. Interestingly, Dlg is also localized to the apical margin of the lateral membrane in epithelial cells, where it induces apical localization of other proteins (Bilder et al., 2000) and might contribute to the apical *Wolbachia* localization in epithelia cells.

Functional significance of *Wolbachia* asymmetric segregation

The polarized localization and asymmetric segregation of *Wolbachia* in the embryonic neuroblast stem cells contrasts with the pattern of *Wolbachia* segregation observed in the female germline stem cells. Division of the germline stem cell (GSC) produces a self-renewing daughter and a daughter to differentiate into a mature oocyte (Fuller and Spradling, 2007). *Wolbachia* are found in the GSC and in the daughter cells that will develop into the mature oocyte (Serbus et al., 2008). The mechanism for *Wolbachia* distribution into both the stem and daughter cells, is not known. The functional significance of this segregation pattern is clear, because it enables *Wolbachia* to be stably transmitted into oocytes throughout the life of the insect. It is not clear whether male GSCs are infected with *Wolbachia*, but they are visible in early stage spermatocysts and in surrounding somatic tissues (Clark et al., 2003). During mitotic divisions during sperm development, *Wolbachia* are distributed unevenly in the cytoplasm, which results in cysts with both infected and uninfected cells (Clark et al., 2002; Clark et al., 2003; Riparbelli et al., 2007). The only common mechanism of *Wolbachia* localization during sperm development, oogenesis, and observations described in this paper, is that *Wolbachia* associates with astral microtubules during the meiotic prophase and telophase during sperm development. However, during male meiotic metaphase and anaphase, *Wolbachia* no longer associate with astral microtubules but instead locate to the spindle midzone (Riparbelli et al., 2007).

The functional significance of the highly asymmetric segregation of *Wolbachia* in the neuroblast stem cells is not immediately obvious. Possible insight comes from examining the fate of the neuroblasts. At the conclusion of embryogenesis, neuroblasts undergo a period of quiescence until reactivation at larval stages when they undergo asymmetric divisions similar to those in embryogenesis. Asymmetric divisions during larval development rely on the same apical-basal determinants, as described for the embryonic neuroblasts, with some minor differences (Slack et al., 2006). As with the embryonic neuroblasts, we found that *Wolbachia* exhibit an asymmetric segregation pattern such that they are maintained in the self-renewing larval neuroblast stem cells. These stem cells ultimately divide into daughter cells that develop into the adult central nervous system. Thus by maintaining an apical position in the embryonic and larval neuroblast stem cells, this ensures that at least some *Wolbachia* will eventually localize to some cells in the adult nervous system during the final neuroblast differentiation. After localization to the adult brain, *Wolbachia* appear to reproduce preferably in some areas of the brain. Larval brains are predicted to be less infected as a result of the asymmetric segregation pattern.

It is possible that the presence of *Wolbachia* in specific host brain regions alters insect behavior. Recent studies demonstrate that *Wolbachia* influences olfactory-cued locomotion (Peng et al., 2008) and mating behavior (Champion de Crespigny and Wedell, 2006; Gazla and Carracedo, 2009; Koukou et al., 2006). It is well known that distinct elements of the *Drosophila* brain govern certain behavior, especially well characterized are sex-specific behaviors such as courtship (Hall, 1979; Villella and Hall, 2008). Our images show that *Wolbachia* is not distributed evenly in different brain regions. The selective infection of the areas in the central brain might be related to the *Wolbachia*-induced effects on *Drosophila* behavior. Expression of behavior-related genes are found to be spatially and temporarily regulated in the developing brain (Lee et al., 2000). *Wolbachia* might influence these

expression patterns. The higher *Wolbachia* titer in specific adult brain areas is probably the result of targeting during development and increased *Wolbachia* reproduction in those regions, because the titers are significantly higher than those we observed in neuroblasts. This discrepancy is especially noticeable in the W_{Pop} strain in *D. melanogaster*, which has a lower titer than W_{Sim} in *D. riverside* throughout development, but overproliferates in the adult brain, as has been observed previously (Min and Benzer, 1997).

Strain variability

Although all three *Wolbachia* strains examined exhibited a pronounced apical localization in the embryonic neuroblasts, the stringency of apical localization varied among the strains. W_{Riv} exhibited the tightest apical concentration with 95% of infected cells having all *Wolbachia* in the apical third of interphase neuroblasts. W_{Pop} have the least-stringent localization with only 57% of the interphase neuroblasts exhibiting complete apical *Wolbachia* localization. Thus, in W_{Pop}, although most *Wolbachia* are maintained in the self-renewing neuroblast, significant numbers are also segregated to the differentiating neuronal daughter cells. This is likely to alter the tissue distribution of W_{Pop} relative to other *Wolbachia* strains. It is tempting to speculate that the less-stringent apical localization of W_{Pop} is partly responsible for the over-replication of *Wolbachia* in adult brains. One possibility is that replication of the apically localized cortical *Wolbachia* is strictly controlled, whereas these controls are not in place in the basally localized *Wolbachia*.

Materials and Methods

Fly strains

Drosophila stocks were maintained on standard molasses and cornmeal medium. The *D. simulans* strain with W_{Pinta} was collected at the University of California Big Creek Reserve (Big Sur, CA). *Drosophila melanogaster* with W_{Pop}, *D. simulans* with W_{Riv} and *D. melanogaster* with W_{Mel} are labstocks. UAS-Gai and UAS-GaiQ205L were generously provided by the Knoblich (IMBA, Vienna, Austria) and Hooper laboratories (University of Colorado, Boulder, CO) and expressed with a V32-Gal4 driver.

In vivo microscopy

Drosophila embryos were prepared for microinjection according to a standard protocol (Tram et al., 2001). Embryos were injected sequentially with Syto-11 (Molecular Probes, Eugene, OR) to visualize *Wolbachia* and Rhodamine-tubulin (Cytoskeleton, Denver, CO) to label microtubules. Syto-11 was diluted 1:10 with water, microfuged for 2 minutes at 4°C, and injected near the embryo cortex. Rhodamine-tubulin was injected undiluted into the embryo interior. Time-lapse microscopy was performed on a Leica DM IRB inverted microscope equipped with the TCS SP2 confocal system. For time-lapse analysis, images were taken every 1.6 seconds or every 5 seconds, as indicated.

Live inhibitor studies

For live examination of *Wolbachia* motility in syncytial embryos, inhibitors were injected into embryos approximately 5 minutes after the injection of Syto-11 and Rhodamine-tubulin. Colchicine was used at 1 mM in water, Taxol was used at 58.5 mM in fresh DMSO, Cytochalasin D was used at 1 mM, and Latrunculin A was used at 1 mM in fresh DMSO.

Fixation and antibody staining

Larval brains from third-instar larvae were dissected in PBS (with 0.1% Triton X-100) and fixed in PEM (100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$) with 4% paraformaldehyde for 20 minutes. 3- to 7-hour-old *Drosophila* embryos were collected and fixed in PEM with 4.5% paraformaldehyde and 50% heptane for 20 minutes. Embryos were de-vitellinized with 100% methanol as previously described (Rothwell and Sullivan, 2000). The following antibodies were used: anti-Hsp60 (1:200, Sigma) with secondary anti-mouse Alexa Fluor 488 (1:150; Molecular Probes). This antibody against recombinant human Hsp60 labels the *Wolbachia* homolog without crossreacting with *Drosophila* proteins (Hoerauf et al., 2000; McGraw et al., 2002; Taylor and Hoerauf, 1999) and stains better than the anti-Wsp antibody in some fixation processes. We also generated a rabbit polyclonal antibody against Wsp. This was achieved by cloning *wsp* from W_{Mel} *Wolbachia*, omitting the signal sequence. A Wsp-Gst fusion protein was expressed in *E.coli*. *E. coli* proteins were separated on a SDS gel, the Wsp-Gst fusion protein was cut out, and used for rabbit antibody production (Open Biosystems). The secondary antibody was anti-rabbit Alexa Fluor 488 (1:150, Molecular Probes). Anti-Scrib polyclonal antibody was raised against the C-terminal of Scrib fused to GST (Albertson and Doe, 2003) with secondary anti-mouse Cy5 (1:150, Molecular Probes); Rabbit anti-PH3 antibody (1:1000, Upstate Biotech, Waltham, MA) was used with secondary anti-mouse Cy5 (1:150, Molecular Probes); anti-aPKC (1:1000, SC biotech) was used with secondary anti-mouse Cy5 (1:150, Molecular Probes); anti-α-tubulin (1:150, Sigma) was used with secondary anti-mouse Alexa Fluor 488 (1:150, Molecular Probes). The Rabbit anti-Miranda antibody was used at 1:200 (Chris Doe, University of Oregon, Eugene, OR). All antibody staining was performed for at least 8 hours at 4°C. For propidium iodide (PI) staining, fixed larval brains were incubated in RNase (10 mg/ml PBS), rotated at 4°C overnight (or at 37°C for 3 hours) and mounted in mounting medium containing PI (10 μg/ml PI, 1× PBS, 70% glycerol in water). For Syto11 staining of adult brain, flies were dissected in PBS, transferred to a watch glass and incubated in Syto11 (Molecular Probes, 1:100 dilution in PBS) for 20 minutes in the dark. Non-fixed brains were directly transferred to a slide and imaged. Drug treatment for fixed-imaged embryos was performed as described previously (Knoblich et al., 1997). Bleach (50%)-dechorinated, washed embryos were incubated for 30 minutes in a 1:1 mixture of n-heptane and 200 μM Latrunculin A in Scheider's medium. Latrunculin A was dissolved in DMSO, which also served as the mock control. Embryos were then fixed in 5% paraformaldehyde for 20 minutes, and then devitellinized with methanol. Staining with antibodies was carried out overnight in PBST at 4°C. Colcemid (5 μg/ml) treatment during neurogenesis was performed similarly, and samples were incubated for the indicated times. Fixed embryos and larval brains were analyzed with the TCS SP2 confocal system on the Leica DM IRB inverted microscope.

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