Wolbachia rescue of several *Drosophila melanogaster bag of marbles* protein coding mutants

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Abstract

The *D. melanogaster* protein coding gene *bag of marbles* (*bam*) plays a key role in early male and female reproduction by forming complexes with partner proteins to promote differentiation in gametogenesis. Like another germline gene, *Sex lethal*, *bam* genetically interacts with the endosymbiont *Wolbachia*, as *Wolbachia* rescues the reduced fertility of a *bam* hypomorphic mutant. Here, we explored the specificity of the *bam*-*Wolbachia* interaction by generating 22 new *bam* mutants. We find *Wolbachia* rescues all six mutants of partially reduced fertility, but none of the four with severely reduced fertility. There is no specificity between the rescue and the known binding regions of *bam*, suggesting *Wolbachia* does not interact with one singular *bam* partner to rescue fertility. We further tested if *Wolbachia* interacts with *bam* in a non-specific way, by increasing *bam* levels or acting upstream in germline stem cells. However, a fertility assessment of a *bam* RNAi knockdown mutant reveals that *Wolbachia* rescue is specific to functionally mutant *bam* alleles and we find no obvious evidence of *Wolbachia* interaction with germline stem cells in *bam* mutants.

Author Summary

Reproduction in the *Drosophila melanogaster* fruit fly is dependent on the *bag of marbles* (*bam*) gene, which acts early in the process of generating eggs and sperm. Mutations to this gene negatively impact the fertility of the fly, causing it to be sterile or have less progeny. Interestingly, the bacteria *Wolbachia*, which resides within reproductive cells across a wide range of insects, partially restores the fertility of several *bam* mutants that have mutations affecting the resultant Bam protein that is produced. The protein function of Bam is further suggested to be important by the lack of fertility rescue for a fly that has a fertility defect due to low expression of a non-mutated Bam protein. Previous work makes similar conclusions about *Wolbachia* with another reproductive gene, *Sex lethal* (*Sxl*), highlighting the potential for rescue of fertility mutants to occur in a similar way across different genes. An understanding of the ways in which *Wolbachia* can affect host reproduction provides us with context with which to frame *Wolbachia*'s impact on host genes, such as *bam* and *Sxl*.

Introduction

The *bag of marbles* (*bam*) gene plays a critical role in female and male gametogenesis in *D. melanogaster*. In the stem cell niche of the ovaries and testes lies a collection of germline stem cells (GSCs) that asymmetrically divide to self-renew and produce differentiating daughter cells. In females, *bam* acts as a master regulator and its expression induces daughter cell differentiation [1]. In males, *bam* functions later in gametogenesis where it initiates terminal differentiation of the spermatogonia that eventually become sperm [1, 2, 3, 4].

In female *D. melanogaster*, *bam* is largely repressed in GSCs by signaling it receives from the stem cell niche [5, 6, 7]. Bam that escapes silencing in GSCs is antagonized by eukaryotic translation initiation factor 4A (eIF4A) [8]. When a GSC divides, a daughter cell is displaced from the stem cell niche, allowing *bam* to now be expressed. In the daughter cell, Bam functions to bring together various proteins that repress self-renewal and promote differentiation, leading to the establishment of a cystoblast.

In both male and female *D. melanogaster*, *bam* null mutants are completely sterile and have a tumorous cytological phenotype [1, 9]. A partial loss-of-function mutant (bam^{bw}) has reduced fertility in females and is sterile in males [10, 11]. In females, the fertility defect of the bam^{bw} mutant is rescued by the endosymbiont bacteria *Wolbachia pipientis* [11].

Wolbachia is estimated to inhabit 50% or more of insect species and is an obligate bacterium in filarial nematodes [12, 13, 14]. Across both arthropods and nematodes, *Wolbachia* manipulates reproduction to promote its own maternal transmission (reviewed in [15, 16]). For example, *Wolbachia* famously induces cytoplasmic incompatibility, in which a female's progeny are not viable if they do not harbor the same *Wolbachia* strain as the male parent. *Wolbachia* is also known to induce parthenogenesis, feminization, and male-killing of host progeny, all of

which promote its maternal transmission. Thus, the rescuing of the hypomorphic *bam* mutant aligns with the evolutionary interests of *Wolbachia.*

Wolbachia also rescues the sterility of hypomorphs of another GSC gene, *Sex lethal* (*Sxl*) [17]. Starr and Cline [17] proposed that the rescue is dependent on the point mutation induced in the *Sxl* mutant and not on the severity of the mutant, though they suggested that rescue cannot happen if *Sxl* protein levels are too low. Of note is that the *Sxl* mutants they studied were in different genetic backgrounds, which may have affected the results observed and the conclusions made. Such background effects were observed with *bam* in [18], where females with the same L255F *bam* mutation had strikingly different fertility across two different genetic backgrounds of *D. melanogaster*. Nevertheless, further research into one of the *Sxl* mutants, Sxl^{f4}, showed that the rescue is partially due to a restoration of GSCs by the *Wolbachia* protein toxic manipulator of oogenesis (TomO) [19].

Mutants of additional germline genes *meiotic P26* (*mei-P26*), *ovarian tumor* (*otu*), and *sans fille* (*snf*) are not rescued by *Wolbachia,* despite having similar severity of an egg-absence phenotype to some *Sxl* mutants [17]. These results suggest the *Wolbachia* interaction may be specific to a functional pathway in female *D. melanogaster*, possibly through a direct, physical interaction with a gene product like Sxl that is expressed in the germline.

In this study, we explore the *bam Wolbachia* interaction, building off the findings for the bam^{bw} hypomorphic mutant. The bam^{bw} hypomorphic allele is a L255F change in a gene region critical for *bam* function. Thus far, four functional regions of *bam* have been documented: The CAF40 binding motif (residues 13-36) [20], the Benign gonial cell neoplasm (Bgcn) binding region (residues 100-350) [21], the COP9 signalosome 4 (Csn4) binding region (residues 151-350) [21], and a ubiquitin binding region (residues 200-250) [22]. *Bam* also has a PEST domain

(residues 402-434), which marks the protein for rapid degradation [1]. *Bam*'s specific binding regions and their interactions with binding partners are illustrated in Figures 1a and 1b respectively.

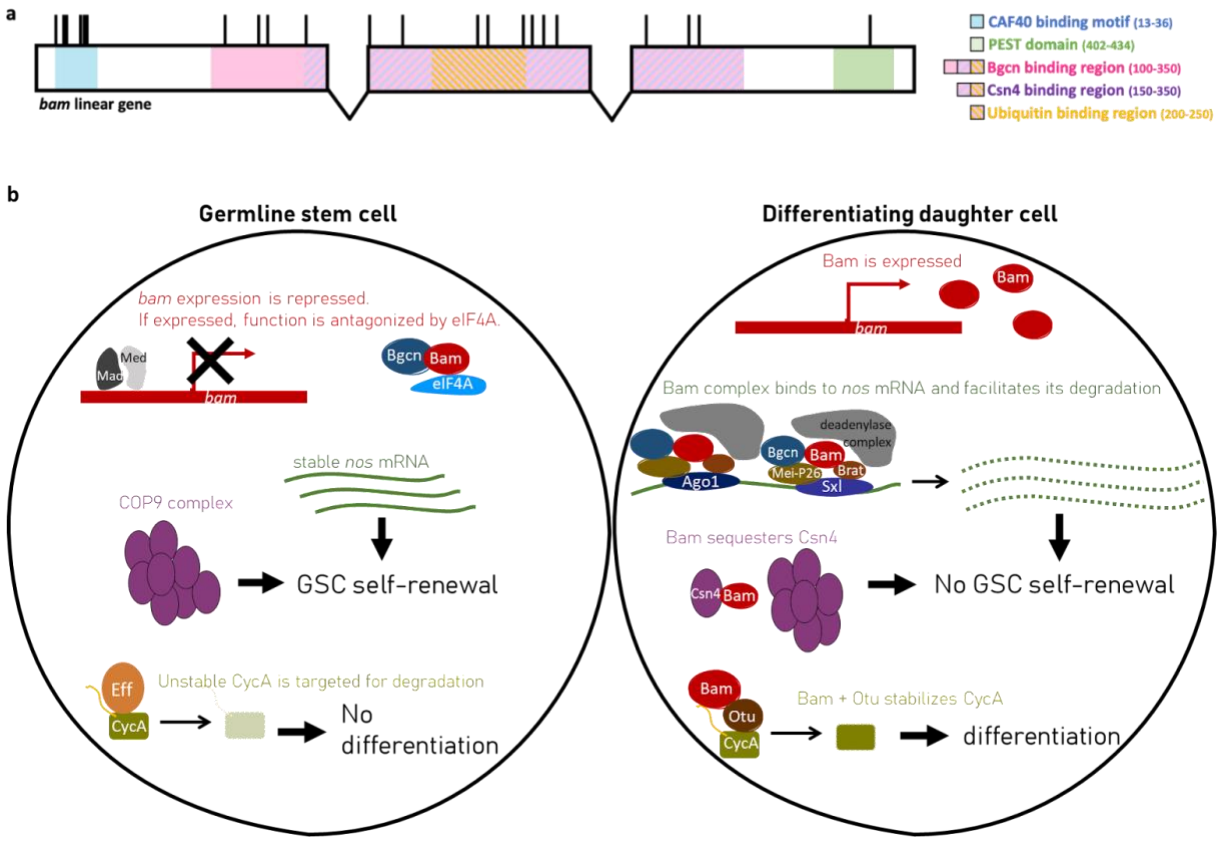


Figure 1. *Bam* is involved in several processes that regulate the differentiation of GSCs in female *D. melanogaster*. (a) Schematic of the five known binding or functional regions of *bam* are colored across the coding sequence exons of the gene. Each black vertical bar represents the location of a new mutant generated with alanine replacements in this study. (b) In GSCs (left), Bam is repressed and antagonized by eIF4A. In the differentiating daughter cells (right), Bam is expressed and coordinates other molecules to inhibit the self-renewal pathway or promote the differentiation pathway. Bam has been shown to genetically and physically interact with additional molecules that are not shown.

To repress GSC self-renewal, Bam forms a complex with Bgcn, Meiotic-P26 (Mei-P26), Brain tumor (Brat), a deadenylase, and either Argonaute-1 (Ago1) or Sxl to destabilize *nanos* (*nos*) mRNA [23, 24, 25]. The destabilization of *nos* mRNA may occur through Bam's interaction with the CAF40 which is part of the CCR4-NOT deadenylase complex [20, 26]. Thus, in GSCs, *nos* mRNA is translated to promote GSC self-renewal; in differentiating daughter cells, *nos* mRNA is destabilized by the Bam complex, inhibiting *nos* translation and the self-renewal pathway.

Bam also represses the self-renewal pathway through its interaction with Csn4 [21]. In GSCs, the COP9 complex is composed of nine Csn subunits. When Bam is expressed in differentiating daughter cells, it breaks up the complex by sequestering Csn4, thereby inhibiting the GSC self-renewal pathway.

Additionally, Bam promotes differentiation by forming a complex with Otu to deubiquitinate Cyclin A (CycA) [22]. In GSCs, CycA is degraded through a process involving the ubiquitin-conjugating enzyme Effete [27]. In differentiating GSC daughter cells, it is proposed that Bam binds to both Otu and to proteins such as CycA, which have ubiquitin chains that mark them for degradation. When in a complex with Bam, Otu deubiquitinates CycA, stabilizing CycA levels and thus promoting GSC daughter cell differentiation.

Here, we have generated 22 new *bam* mutants and analyzed the genetic interaction between these mutants and *Wolbachia* to better understand the extent of the *bam*-*Wolbachia* interaction. We are partly motivated by the observation of rapidly diversifying amino acids at *bam* across the *Drosophila* phylogeny [28, 29, 30]. We have previously hypothesized that *Wolbachia* could contribute to this rapid evolution observed at *bam* [11, 29, 30, 31]. Intriguingly, recent results for *Sxl* could be consistent with a parallel hypothesis for that gene, as the excess

nonsynonymous substitutions observed in *Sxl* cluster near the locations of the alleles that genetically interact with *Wolbachia* [32].

In this study, we find that *Wolbachia* genetically interacts with alanine point mutants throughout the *bam* gene, suggesting that *Wolbachia* rescue of *bam* mutants is not specific to any single binding/functional region of *bam*. We find no evidence for two hypotheses of how *Wolbachia* could rescue *bam* in a non-specific manner: There is no effect of *Wolbachia* on a hypomorph-like *bam* knockdown mutant, nor is there an effect of *Wolbachia* on the frequency of GSC mitosis that occurs upstream of *bam*.

Materials and Methods

Design of putative *bam* hypomorph alleles

22 *bam* mutant alleles were designed with a Venus fluorescent tag (Fig. 1a), described further below. The known Bam binding residues in the CAF40 binding motif were targeted for replacement [20], producing independent alleles with mutations of L17A, M24A, and L28A. The remaining mutant alleles were designed through an alanine scanning approach [33] that targeted the known functional and/or binding regions of *bam.* Strings of charged residues were changed to alanine by choosing an alanine codon with similar usage bias in *D. melanogaster* as that of the original residue [34]. Additionally, we designed an allele to recreate the bam^{bw} L255F mutation as a transgene with Venus tag. All new alleles are listed in Supplemental file S1, with the original and mutated codons.

Generation of transgenic flies

Transgenic fly lines carrying distinct putative hypomorphic *bam* alleles and a control transgenic fly line were generated using PhiC-mediated integration [35]. Approximately 1.5 kb upstream and 700 bp downstream regions of *bam* were cloned from genomic DNA of a *D. melanogaster bam::Venus* line [36]. This construct is similar to that of the *D. melanogaster bam* YFP line from [11] but contains a 36bp glycine-glycine-serine linker sequence between the 3' end of the *bam* coding sequence and the Venus tag to allow for proper folding of both proteins. The *bam::Venus* sequence was cloned into a pMiniT 2.0 vector, using the NEB PCR cloning kit. Mutant alleles were subsequently made in the established *bam::Venus* pMiniT 2.0 plasmid using the NEB Q5 Site-Directed Mutagenesis Kit, with primers designed online using NEBase Changer (Supplemental table S1) [37]. Briefly, mutagenic primers were used to amplify the *bam::Venus* pMiniT 2.0 plasmid using with a PCR cycle of 98°C for 30 seconds; 25 cycles of 98°C for 10 seconds, 57-72°C annealing for 30 seconds, 72°C for 3.5 minutes; and 72°C for 2 minutes. PCR products were treated with kinase, ligase, and DpnI before being transformed into competent cells. The resultant plasmids were subcloned into the pCasper\attB vector, a gift from Dan Barbash that was used by [11] for PhiC transgenesis. Plasmids were verified by sequencing.

Plasmids were injected into embryos of a *w*, *nos*-int; P{CaryP}attP40 *D. melanogaster* line by GenetiVision. G_0 flies were mated with a w^{1118} isogenic line and F_1 progeny were screened for successful transgenesis by selecting for those with mini-white eye color. Mutant lines were identified by Sanger sequencing.

All transgenic flies were assessed for function in a *bam* null background. Thus, the genotype that was assessed in all experiments was *w*; [w^{+mc}, *bam::Venus*]/+; $bam^{\Delta 59}/bam^{\Delta 86}$, where *bam::Venus* represents the control *bam* allele or one of the 22 mutant alleles. For the remainder of this paper, "transgenic females" refers to flies of this genotype. *Wolbachia* status of

the assessed flies (uninfected or *Wolbachia* infected) was established by maternal inheritance when crossing in the *bam* null alleles.

Fertility assays of female transgenic flies

Virgin transgenic females and virgin CantonS males were collected over 48 hours and aged two days. At least 30 individual crosses of one female and two males were set up on yeast-glucose food (minimum of 30, maximum of 88 crosses were set per genotype tested). All crosses were kept in an incubator at 25°C with a 12-hour light/dark cycle. Parents were removed on the seventh day. Progeny were counted every other day over seven days, starting on the day of first eclosion. If the female parent died by the time of removal, data for that cross was discarded. Five independent fertility assays were conducted to cover all 22 mutants, with a transgenic control (*w*; [w^{+mc}, *bam::Venus*]/+; $bam^{\Delta 59}/bam^{\Delta 86}$) present in each assay.

The total progeny after seven days was summed and estimation statistics [38] were performed to determine first, if a fertility defect was present for a mutant, and second, if there was a *Wolbachia* rescue effect. Estimation statistics and plots were generated using the dabestr package in R (v0.3.0). While this offers an alternative statistical test that allows us to easily visualize effect size, we also report p-values for ease and convention. P-values were calculated through the associated website as results of a two-sided permutation t-test [39], as they were not provided in the R package.

Cytological observations of the ovaries of female transgenic flies

For cytology of transgenic females with fertility defects and control females, flies were aged 2-3 days and then dissected and prepared for confocal microscopy as in [11, 30]. Ovaries were dissected in ice-cold 1x PBS and fixed in 4% paraformaldehyde (Electron Microscopy

Sciences) for 15 minutes. Tissue was rinsed 3x and washed 4x, respectively, with PBST (1X PBS, 0.1% Triton-X 100), blocked in PBTA (1X PBS, 0.1% Triton-X100, 3% BSA) (Alfa Aesar), and then incubated with primary antibodies overnight. The rinse and wash steps were repeated, followed by an overnight incubation with a goat anti-serum to minimize fluorescent background binding, and then a 1.5-hour incubation with secondary antibodies. The rinse and wash steps were repeated and tissue was mounted in ProLong Diamond AntiFade (Invitrogen).

The primary antibodies that were used were anti-Vasa (anti-rat, 1:20, Developmental Studies Hybridoma Bank (DSHB)), anti-Hts-1B1 (anti-mouse, 1:40, DSHB), and anti-GFP (anti-rabbit, 1:200, Invitrogen Cat No: G10362). Secondary antibodies that were used were Alexa488 (goat anti-rat, Invitrogen Cat No: A-11006), Alexa568 (goat anti-mouse, Invitrogen Cat No: A-11031), and Alexa633 (goat anti-rabbit, Invitrogen Cat No: A-21070), all at 1:500.

A Zeiss i880 confocal microscope was used for all imaging with 405, 488, and 568nm laser lines at 63X (Plan-Apochromat 1.4 NA, oil) (Cornell BRC Imaging Core Facility). Images were edited using Fiji [40].

RNAi knockdown of *bam*

We performed reciprocal crosses between a *Wolbachia* infected *nos*-Gal4 and an uninfected UAS-*bam*^{HMS00029} line (a TriP *bam* shRNA line) (Bloomington Drosophila Stock Center lines 4442 and 33631, respectively) to generate the *bam* knockdown lines.

Bam expression was evaluated by RT-qPCR. RNA of four samples consisting of three 1-2 day old female flies each were prepared using the NEB Monarch Total RNA Miniprep Kit. RT-qPCR was performed with 10μL reactions (5μL Luna Universal One-Step Reaction Mix (2X), 0.5μL Luna WarmStart RT Enzyme Mix (20X), 0.4μL forward primer, 0.4μL reverse

primer, 2μL template RNA, 1.7μL water), using three technical replicates and a standard curve. Reverse transcription was performed at 55°C for 10 minutes, followed by initial denaturation at 95°C for 1 minute, and then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Primers included 5'GCGCAATCGAAACGGAAACT3' and 5'GAGTAGCGGTGCTCCAGATC3' for *bam* and 5'CCGCTTCAAGGGACAGTATC3' and 5'CAATCTCCTTGCGCTTCTTG3' for the reference gene *Ribosomal protein L32* (*Rpl32*) (*Rpl32* primers are from [41]). Relative expression to a wildtype *bam* control was done by the method of [42].

A fertility assay was performed as described above, but at 29°C to increase the activity of the Gal4-UAS system. Estimation statistics [38] were used to compare the mean fertility of the uninfected and infected *bam* knockdown mutants.

Additionally, an assay for nurse-cell-positive egg chambers was performed, as has been previously done for a supplemental assessment of *bam*'s function [11, 18]. Briefly, ovaries of 1-2 day old flies were fixed in 4% paraformaldehyde, rinsed and washed with PBST as described above, and then mounted in ProLong Glass with NucBlue (ThermoFisher). Ovaries were imaged on a Zeiss i880 confocal microscope at 40X and 63X (Plan-Apochromat 1.4 NA, oil), using a 405nm laser line (Cornell BRC Imaging Core Facility). Egg chambers with any identifiable large, polyploid nuclei of nurse cells indicated an event of successful Bam function and were counted as nurse-cell-positive egg chambers. Statistically significant results were determined with the Wilcoxon signed rank test.

Analysis of GSC mitosis frequency

Nurse cell assays were performed as described above to confirm that the L255F bam mutation that we created as a transgene produced a partial *bam* mutant phenotype and *Wolbachia* rescue like that of the original bam^{bw} hypomorph.

Then, we assessed GSC mitosis frequency in the transgenic control (*w*; [w^{+mc}, *bam::Venus*]/+; $bam^{\Delta 59}/bam^{\Delta 86}$) and the transgenic L255F mutant (*w*; [w^{+mc}, bam^{L255F}*::Venus*]/+; $bam^{\Delta 59}/bam^{\Delta 86}$) by comparing the number of actively dividing GSCs indicated by mitosis marker pH3 to the number of non-dividing GSCs. Flies were aged 2-3 days at 25°C, at which time ovaries were dissected, immunostained and prepared for confocal microscopy, and imaged as described above.

Primary antibodies that were used were anti-Vasa (anti-rat, 1:20, DSHB), anti-GFP (anti-rabbit, 1:200, Invitrogen Cat No: G10362), and anti-pH3 (anti-rabbit, 1:200, Millipore Sigma Cat No: 06-570). The same secondary antibodies listed above (Alexa488, Alexa568, and Alexa633) were used at 1:500.

For each genotype, a Fisher's exact test was used to determine if there were more mitotically active GSCs in the *Wolbachia* infected germariums than in the uninfected germariums.

Results

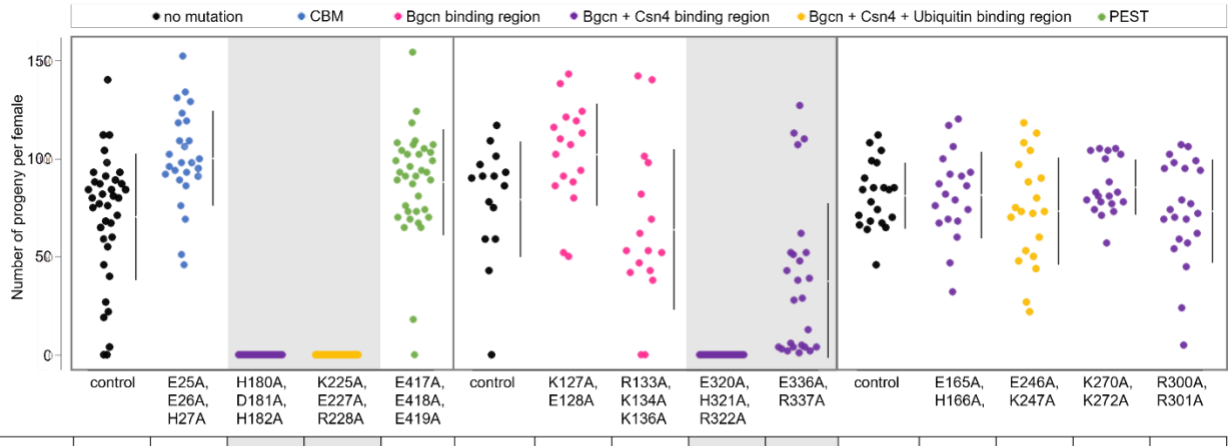
22 mutants were generated, ten with *bam* female fertility defects

22 novel mutants were generated as *bam* transgenes with alanine replacements and a Venus fluorescent tag. They were distributed across the five functional domains/regions of the *bam* gene (Fig 1a). Each mutant was assayed for fertility defects and results were compared to a control transgenic line of a wildtype *bam* gene tagged with Venus. Mutants were characterized

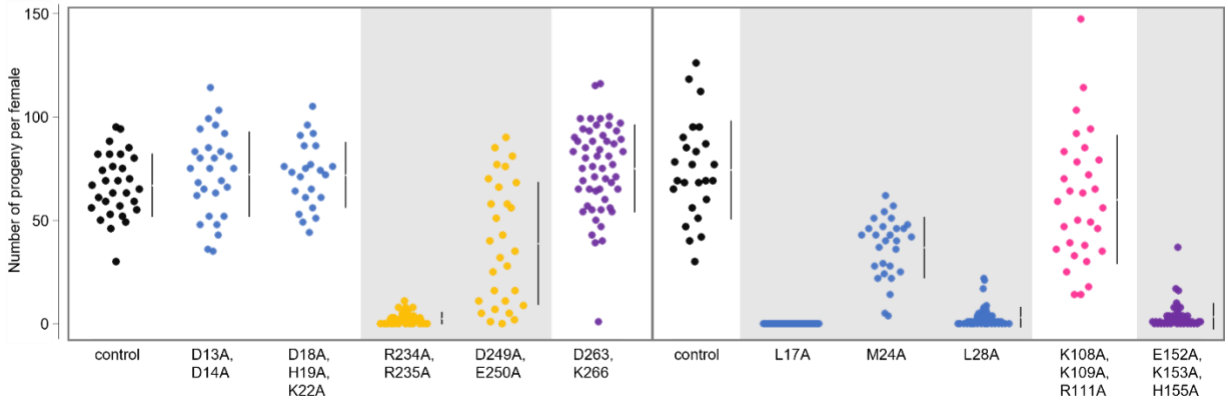
by comparing the average number of progeny produced by females with and without *Wolbachia* infection.

Of the 22 *bam* alanine-scan mutants generated, ten had fertility defects in uninfected females (Fig 2, Supplemental fig S1, Supplemental table S2). These included all three of the mutants that targeted known binding residues in the CAF40 binding motif of *bam*, as well as mutants that targeted residues in the Bgcn, Csn4, and ubiquitin binding regions. There were four null-like fertility mutants that produced no progeny when uninfected (transgenic Venus-tagged bam^{L17A}, $bam^{H180A, D181A, H182A}$, $bam^{K225A, E227A, R228A}$, and $bam^{E320A, H321A, R322A}$). There were six hypomorph-like fertility mutants that produced less progeny on average than the control when uninfected, which included three that exhibited severe fertility defects (transgenic Venus-tagged bam^{L28A}, $bam^{E152A, K153A, H155A}$, and $bam^{R234A, R235A}$) and three that exhibited mild fertility defects (transgenic Venus-tagged bam^{M24A}, $bam^{D249A, E250A}$, and $bam^{E336A, R337A}$). The mean effect size between the number of progeny of the mutants with fertility defects compared to the control ranged from a minimum of -28.2 to a maximum of -74.3 (Fig 2, Supplemental fig S1).

That these new *bam* mutations that produce fertility defects are distributed throughout the gene demonstrates that multiple regions of the gene and protein are critical for *bam* function. Additionally, the variation in severity of fertility defects for mutations within one binding or functional region shows that these phenotypes are allele specific.



mean	70.270	100.231	0	0	87.917	79.267	102.000	63.875	0	37.880	81.095	81.369	73.250	85.421	73.273
p-value		0	0	0	0.01		0.03	0.24	0	0.001		0.96	0.27	0.38	0.27
effect size		30	-70.3	-70.3	17.6		22.7	-15.4	-79.3	-41.4		0.27	-7.85	4.33	-7.82



mean	92.538	72.214	71.913	2.706	38.690	75.132	74.280	0	36.893	3.200	59.933	3.667
p-value		0.26	0.18	0	0	0.07		0	0	0	0.06	0
effect size		5.35	5.05	-64.2	-28.2	8.27		-74.3	-37.4	-71.1	-14.3	-70.6

Figure 2. Fertility of transgenic mutants compared to the respective *bam::Venus* transgenic control. Data is colored according to the documented functional region that the mutation is located in. Below the jitter plots are the mean, two-sided permutation t-test p-value, and the average effect size between the mutant and the respective control. Gray panels indicate mutants with fertility defects (negative average effect size and $p<0.05$).

We also assayed the ovarian cytology of the mutants with fertility defects. Our transgenic *bam::Venus* control had no tumorous germaria and proper Bam expression (Fig 3a-b). In parallel with the novel mutants we generated, we recreated the bam^{bw} hypomorphic mutation as a transgenic bam^{L255F}*::Venus* mutant. As has been previously characterized for this L255F *bam* mutation in a *bam* null background, we see a tumorous germarium with some cells containing a spectrosome (Fig 3c) [8, 10, 21]. We also found that the overproliferating GSC-like cells in this hypomorph expressed Bam, and the lack of Bam expression in the anterior most GSCs suggested that there was no ectopic expression of Bam or loss of GSCs.

The ten new *bam* fertility mutants displayed cytological tumorous phenotypes like that observed in the bam^{L255F}*::Venus* mutant, with the severities largely consistent with the severity of their fertility defects (Fig 3d-f, Supplemental fig S2). When uninfected, the null-like fertility mutants consistently had more severe tumorous germariums and severe tumorous stage one egg chambers, like that in Fig 3d and 3e. The hypomorph-like fertility mutants had more mild tumorous defects, like that in Fig 3f, and more wildtype egg chambers. Of note, the bam^{M24A}*::Venus* mutant, which had a mild fertility defect, had no tumorous germariums (Supplemental fig S2e). The fertility counts of females of this line are consistent with the cytological phenotype, as all but three flies had upwards of 20 progeny over the course of the fertility assay. All mutants, including the null-like fertility mutants, stained successfully with anti-GFP, indicating the presence of Bam::Venus protein.

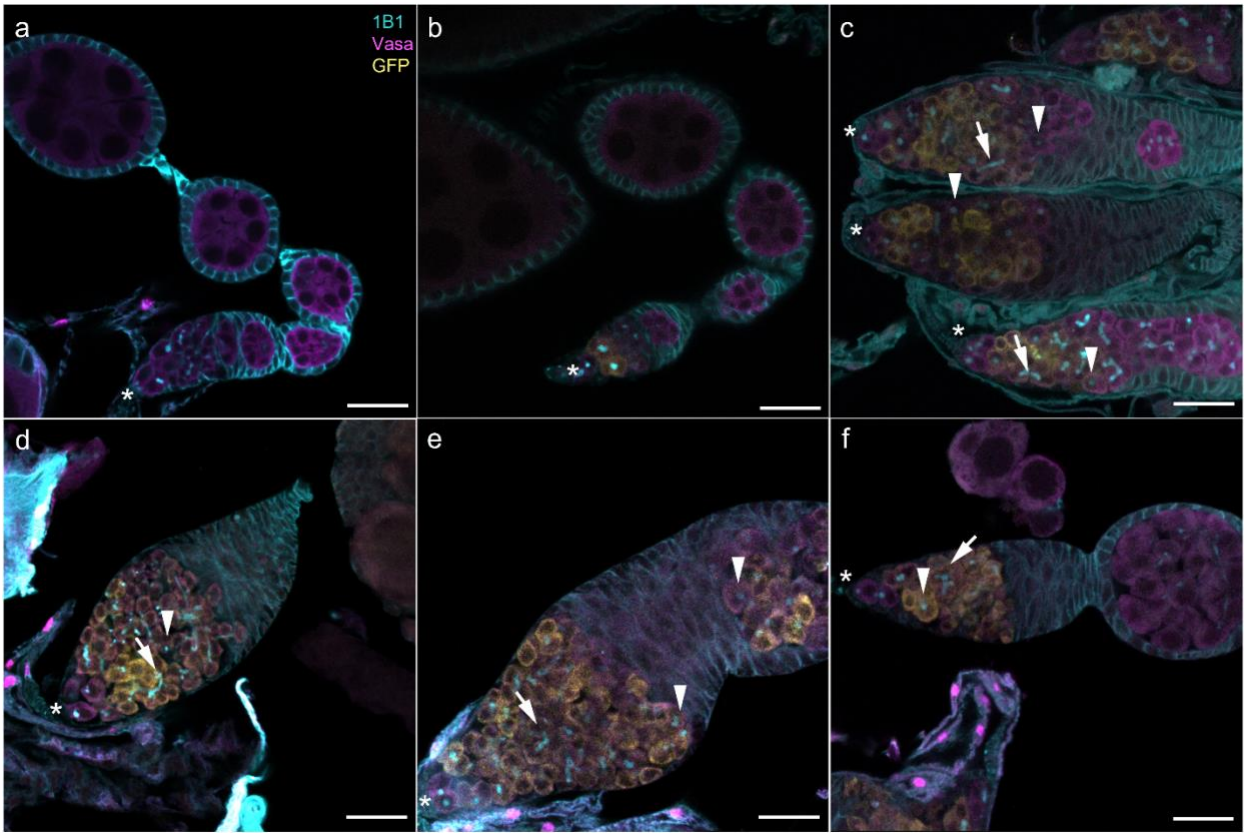


Figure 3. Wildtype and transgenic *D. melanogaster* ovarioles stained with anti-Hts-1B1 (cyan), anti-Vasa (magenta), and anti-GFP (yellow). (a) CantonS wildtype control. (b) Transgenic *bam::Venus* control. (c) Transgenic *bam*^{L255F}*::Venus* mutant. (d-f) Representative images of cytological defects observed in the transgenic *bam* mutants with fertility defects, with arrowheads indicating spectrosomes, arrows indicating branched fusomes, and asterisks indicating the apical tip of the germarium where GSCs are identifiable as cells not expressing Bam::Venus. (d) Mutant E152A, K153A, H155A. (e) Mutant K255A, E227A, R228A. (f) Mutant E336A, R337A. Scale bar for all images is 20μM.

The twelve *bam* mutants that did not have fertility defects when uninfected were also distributed across the *bam* gene (Fig 2, Supplemental fig S1, Supplemental table S2). These were the transgenic Venus tagged *bam*$^{D13A, D14A}$, *bam*$^{D18A, D19A, K22A}$, *bam*$^{E25A, E26A, H27A}$, *bam*$^{K108A, K109A, R111A}$, *bam*$^{K127A, E128A}$, *bam*$^{R133A, K134A, K136A}$, *bam*$^{E165A, H166A}$, *bam*$^{E246A, K247A}$, *bam*$^{D263A, K266A}$, *bam*$^{K270A, K272A}$, *bam*$^{R300A, R301A}$, and *bam*$^{E417A, E418A, E419A}$. Three of these mutants (*bam*$^{E25A, E26A, H27A}$, *bam*$^{E417A, E418A, E419A}$, and *bam*$^{K127A, E128A}$) had greater fertility on average than their respective control ($p<0.05$). Mutants without fertility defects include those with replacements at highly conserved amino acid sites and replacements at poorly conserved sites (Supplemental file S1). These results identify residues that are not critical to *bam*'s function in differentiation of GSC daughter cells in *D. melanogaster* oogenesis, at least in laboratory conditions.

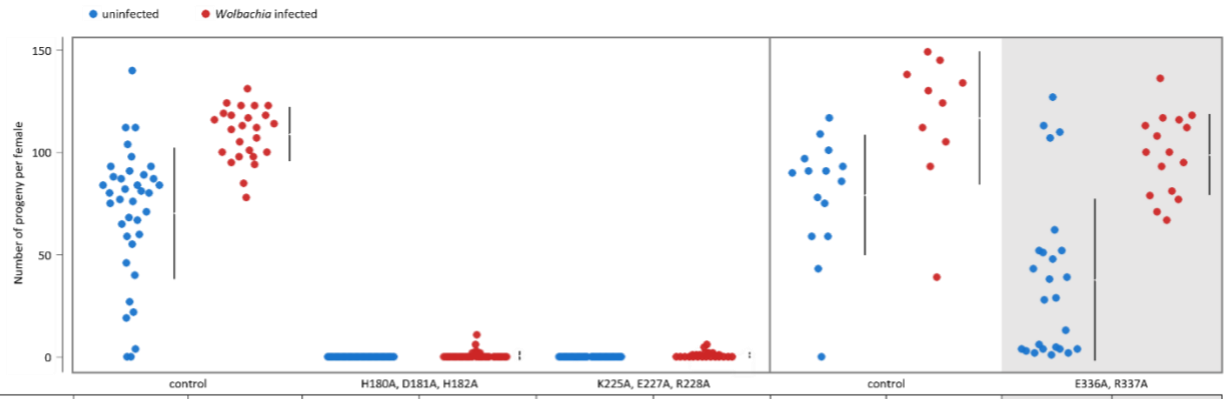
Wolbachia rescues all of the female hypomorph-like fertility mutants

To determine if *Wolbachia* rescues the new *bam* mutants with fertility defects, we compared fertility of uninfected and infected transgenic mutants. The transgenic *bam*::Venus control showed a slight increase in fertility with *Wolbachia* infection across each independent fertility assay ($p<0.05$, Fig 4, Supplemental table S2). Thus, for a mutant to be classified with *Wolbachia* rescue of fertility, the increase in mean effect size had to be greater than that observed in the control.

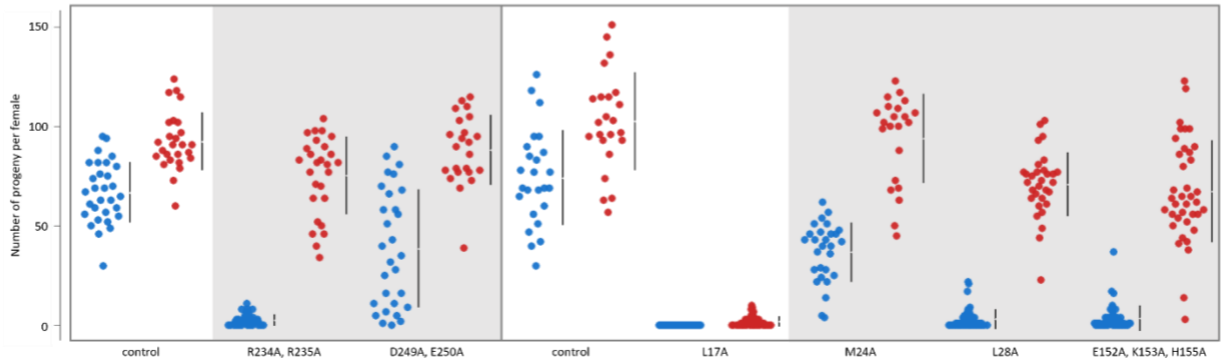
Of the four null-like fertility mutants that produced no progeny when uninfected, three produced some progeny when infected with *Wolbachia* (transgenic Venus-tagged *bam*^{L17A}, *bam*$^{H180A, D181A, H182A}$, and *bam*$^{E320A, H321A, R322A}$) ($p<0.05$); the fourth null-like mutant (transgenic Venus-tagged *bam*$^{E320A, H32A, R322A}$) did not produce progeny regardless of infection status (Fig 4, Supplemental table S2). The slight production of progeny in the three null-like fertility mutants

with *Wolbachia* is consistent with *Wolbachia* infection increasing fertility of transgenic flies but cannot be classified as *Wolbachia* rescue here because of the small effect size.

All six hypomorph-like mutants that had reduced, but not zero, fertility compared to the control when uninfected and had an increase in average fertility with *Wolbachia* infection, with a mean effect size greater than that of the control ($p<0.05$, Fig 4, Supplemental table S2). The three mutants with severe fertility defects (transgenic Venus-tagged bam^{L28A}, $bam^{E152A, K153A, H155A}$, and $bam^{R234A, R235A}$) saw an approximate 20-fold increase in the average number of progeny per female when infected with *Wolbachia*. The three mutants with mild fertility defects (transgenic Venus-tagged bam^{M24A}, $bam^{D249A, E250A}$, and $bam^{E336A, R337A}$) saw an approximate 2.5-fold increase in the average number of progeny per female when infected with *Wolbachia*, bringing them to a fertility level similar to that of the infected control.



	control	H180A, D181A, H182A	K225A, E227A, R228A	control	E336A, R337A
mean	70.270 / 108.920	0 / 0.771	0 / 0.920	79.267 / 116.900	37.880 / 98.938
p-value	0	0.03	0	0.01	0
effect size	38.6	0.771	0.92	37.6	61.1



	control	R234A, R235A	D249A, E250A	control	L17A	M24A	L28A	E152A, K153A, H155A
mean	66.867 / 92.538	2.706 / 75.481	38.690 / 88.174	74.280 / 102.609	0 / 1.960	36.892 / 94.091	3.200 / 71.000	3.667 / 67.342
p-value	0	0	0	0.0	0	0	0	0
effect size	25.7	72.8	49.5	28.3	1.96	57.2	67.8	63.7

Figure 4. Fertility of transgenic control and transgenic *bam* mutants with fertility defects as uninfected and infected with *Wolbachia.* Two-sided permutation t-test p-values and the average effect size between the uninfected (blue) and infected with *Wolbachia* (red) data are shown below each data set. Mutant E320A, H321A, R322A was completely sterile with and without *Wolbachia* and thus is not shown. Gray panels highlight the mutants that are classified as having *Wolbachia* rescue due having an average effect size greater than that observed in the respective control and $p<0.05$.

Overall, we find that *Wolbachia* rescues the reduced fertility of the hypomorph-like fertility mutants across all the documented binding regions of *bam* and throughout the predicted 3D protein structure (Fig 5) [43, 44]. These results show that the *Wolbachia* rescue is not specific to the L255F *bam* mutation and that *Wolbachia* interacts with *bam* in a manner that is not specific to the known binding regions of the gene.

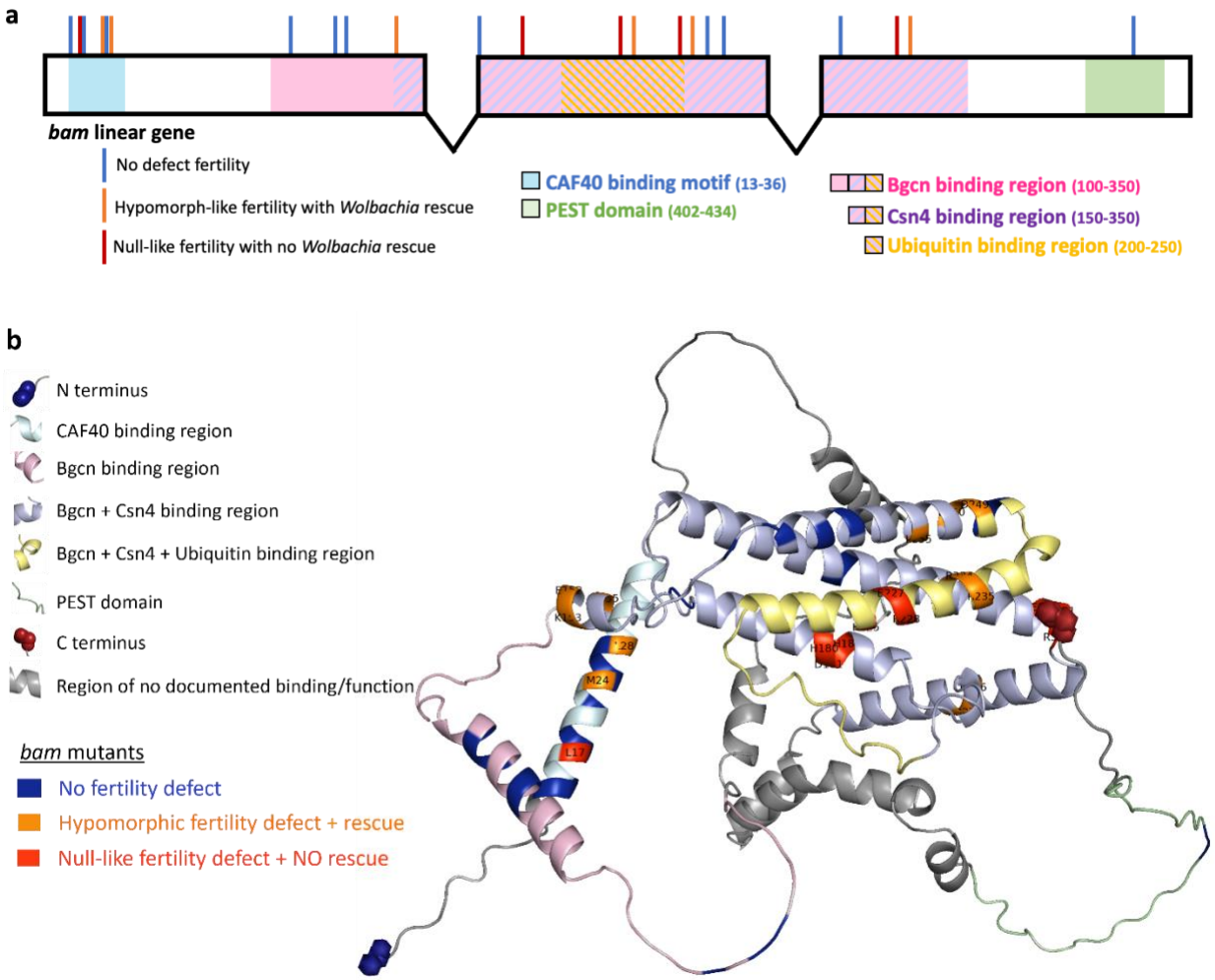


Figure 5. The locations of the mutations generated and their effect on fertility and *Wolbachia* rescue across the documented binding and functional regions of *bam*. Shown in (a) a linear schematic of the gene and (b) the predicted protein structure.

We plotted the mean progeny for all transgenic females (both uninfected and *Wolbachia* infected) to easily compare the above fertility results across all mutants (Fig 6). When comparing the mean fertility for each mutant between the uninfected and *Wolbachia* infected lines, we find that the data falls roughly along a logarithmic curve, suggesting there is a relationship between the severity of uninfected mutants and the increase in fertility seen with *Wolbachia* infection. However, we cannot discount the possibility of the increase in fertility being due to an allele-specific interaction with *Wolbachia*.

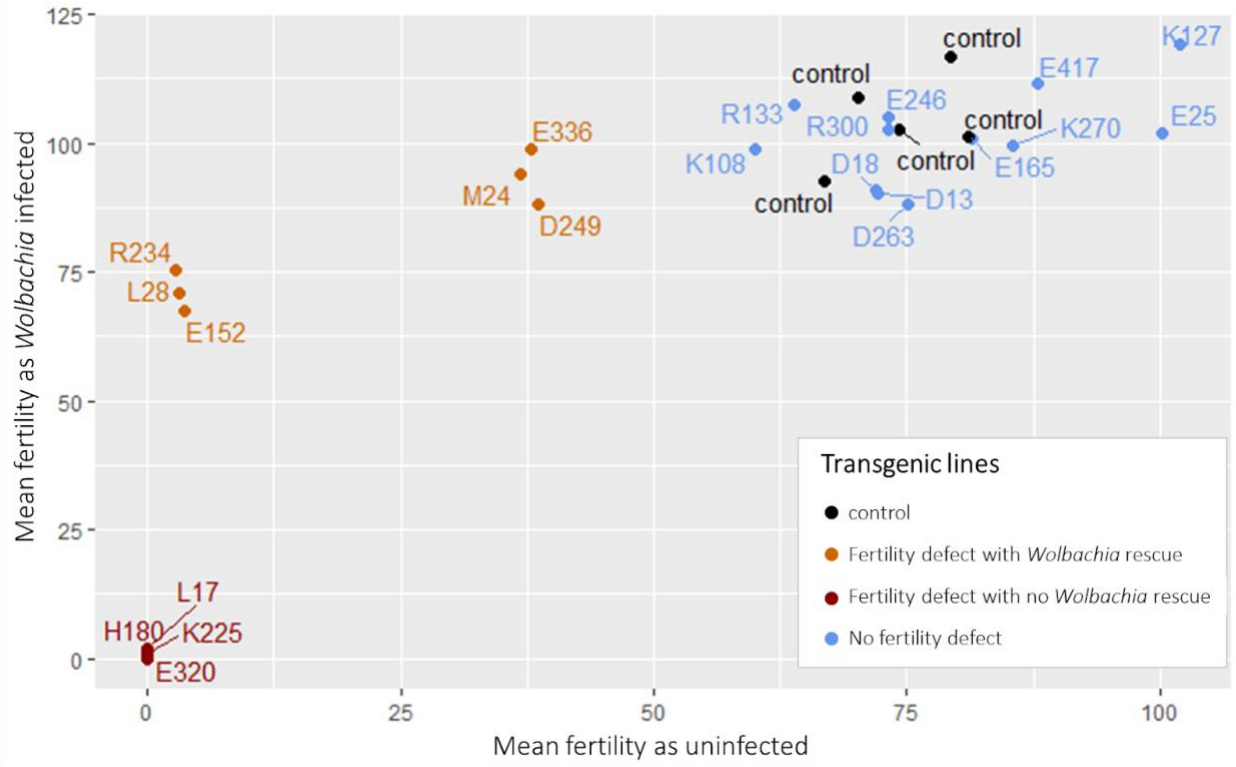


Figure 6. Mean fertility comparison of all transgenic lines generated with and without *Wolbachia.* The control lines are transgenic *bam::Venus* (black) and the transgenic mutants are labeled by the first residue that was changed to alanine (orange for mutants with fertility defects with *Wolbachia* rescue, red for mutants with fertility defects without *Wolbachia* rescue, and blue for mutants without fertility defects). Figure shows data across five independent fertility assays with five different controls.

GSC mitosis in a *bam* hypomorphic mutant is not affected by *Wolbachia*

To determine if the increase in fertility of *bam* mutants is due to an increase in asymmetric division by GSCs, we quantified GSC mitosis in the *bam::Venus* control and the bam^{L255F}*::Venus* mutant. The bam^{L255F} transgenic hypomorph recapitulates the severity of the endogenous bam^{bw} hypomorph, though shows slightly reduced rescue compared to the bam^{bw} hypomorph (Supplemental fig S3). This difference is likely due to the differences in the genetic backgrounds of these two otherwise identical mutations.

Cells in the apical tip of the germarium that expressed the pH3 mitosis marker but not the Venus-tagged Bam protein were identified as GSCs actively undergoing mitosis (Supplemental fig S4). The rate of GSC mitosis was not different between *Wolbachia* infected and uninfected flies within both the transgenic control and the transgenic hypomorph (p=1.0) (Table 1). Overall, the bam^{L255F}*::Venus* transgenic hypomorph had fewer GSC mitotic events compared to the transgenic control, but it was not statistically significant (p=0.259 with *Wolbachia* and p=0.448 without *Wolbachia*).

	Transgenic control		Transgenic *bam* hypomorph	
	uninfected	*Wolbachia* infected	uninfected	*Wolbachia* infected
# of germariums with pH3 positive GSCs	5	9	2	4
# of germariums without pH3 positive GSCs	111	173	105	173

Table 1. Number of mitotically active (pH3 positive) and quiescent GSCs. GSC counts are shown for the transgenic control (*bam::Venus*) and a transgenic hypomorph (bam^{L255F}*::Venus*) when uninfected and infected with *Wolbachia.*

***Wolbachia* does not rescue a *bam* knockdown phenotype**

To determine if *Wolbachia* can rescue the phenotype of a reduced expression, but functional Bam protein in females, we tested for the rescue of a *bam* RNAi knockdown mutant by performing a reciprocal cross using the UAS-Gal4 system. We assessed both the number of egg chambers that have a nurse cell present (nurse-cell-positive egg chambers) per ovary in wildtype and *bam* knockdown mutants, as in [11, 18], as well as the fertility of the knockdown mutants.

We find that the *bam* knockdown mutants have fewer nurse-cell-positive egg chambers per ovary than the wildtype lines regardless of *Wolbachia* status ($p<0.05$) (Fig 8a, Supplemental fig S5). *Bam* knockdown mutants with *Wolbachia* had fewer nurse-cell-positive egg chambers on average than *bam* knockdown mutants without *Wolbachia* ($p<0.05$) but had similar levels of fertility over a period of seven days ($p = 0.69$, Fig 8b). This difference is likely due to the narrow window of time captured in the nurse-cell-positive assay compared to the fertility assay. These results show that *Wolbachia* does not rescue the tumorous mutant phenotype or the fertility of a *bam* knockdown mutant.

Additionally, there is no increase in *bam* expression in the knockdown mutant with *Wolbachia* compared to the uninfected knockdown mutant (Fig 8c). This shows that *Wolbachia* does not increase the expression levels of *bam*, consistent with the lack of rescue that we observe. All together, these results suggest that *Wolbachia* rescue is specific to partial-loss-of-function protein coding *bam* mutants.

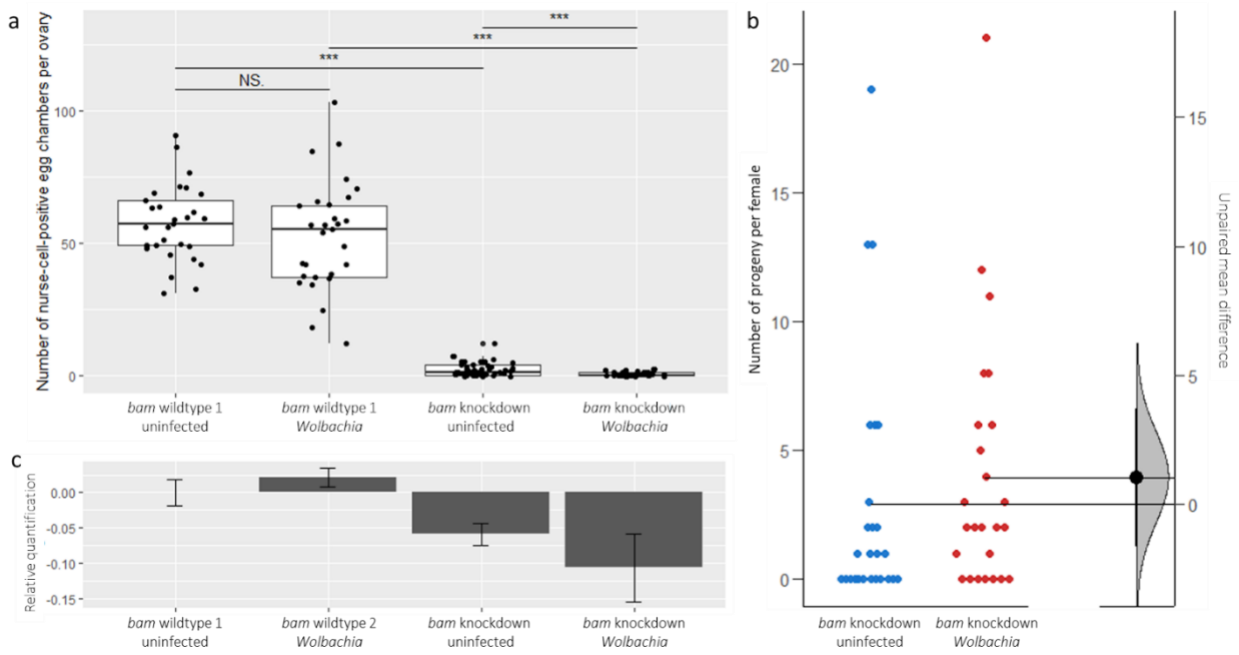


Figure 7. *Bam* knockdown down by RNAi in the UAS-Gal4 system. *Bam* wildtype 1 and 2 are the UAS and Gal4 stocks, respectively; *bam* knockdowns are the nos-Gal4/+; UAS-*bam*^{HMS00029}/+ reciprocal F1 progeny of the stocks. (a) The number of nurse-cell-positive egg chambers per ovary. *** = significant at p<0.001; NS = not significant. (b) Fertility of the *bam* knockdown mutants as uninfected and *Wolbachia* infected. Jitter plots (left) show the raw data as dots with horizontal lines representing the mean values of each dataset. The mean difference between uninfected and infected lines are plotted as a bootstrap sampling distribution with a 95% confidence interval (right). (c) *Bam* expression on a log2 scale relative to *bam* wildtype 1. Each bar represents the dataset of four samples consisting of three female flies, and shows the mean value with standard error.

Discussion

Bam is one of a few genes that has been shown to genetically interact with *Wolbachia* in *D. melanogaster*. In female and male adults, *bam*'s molecular role is to bring together other proteins into complexes, which then carry out functions vital to the maintenance or progression of gametogenesis. *Bam*'s central and extensive role in early reproduction motivates a question about the specificity of the *bam Wolbachia* interaction.

We targeted *bam*'s role of protein binding and minimized affecting the overall conformation of the Bam protein by replacing charged residues with alanine throughout *bam*'s documented binding regions [33]. We find no specificity to the presence/absence of rescue or the magnitude of fertility rescue for the different functional regions of the linear *bam* sequence or with the AlphaFold-predicted Bam protein structure. The strongest evidence for this comes from our finding that *Wolbachia* rescues fertility defects of the known CAF40 binding residues (bam^{M24A} and bam^{L28A}), as well as fertility defects of several mutations not involved in CAF40 binding. This suggests that *Wolbachia* does not rescue the specific interaction between Bam and a single one of its known protein partners in *bam* fertility mutants.

While it is possible that the rescued mutants' amino acid sites are involved in a direct interaction between Bam and an unmapped binding partner (e.g., Otu [22]), we tested two alternative hypotheses for how *Wolbachia* could be rescuing *bam* fertility mutants. The first proposes that *Wolbachia* induces a fertility rescue by affecting *bam*'s expression. It has previously been shown that there is no difference in *bam* expression in the bam^{bw} hypomorph as uninfected and infected with *Wolbachia* [11]. Here, we tested the ability of *Wolbachia* to rescue the mutant phenotype of a wildtype *bam* gene that is reduced in expression via RNAi. Importantly, we have a knockdown mutant that displays the hypomorph phenotype of tumorous

germariums with some wildtype egg chambers. If the knockdown phenotype is too severe, *Wolbachia* may have no effect or only a minimal effect as evidenced by the null-like fertility mutants generated in this study (transgenic Venus-tagged bam^{L17A}, $bam^{H180A, D181A, H182A}$, $bam^{K225A, E227A, R228A}$, and $bam^{E320A, H321A, R322A}$) and *Wolbachia*'s lack of interaction with the *bam* null mutant documented previously [11]. With the hypomorph-like-phenotype *bam* knockdown mutant (*nos*-Gal4/+; UAS-$bam^{HMS00029}$/+), we find that *Wolbachia* does not rescue the number of nurse-cell-positive egg chambers or affect the fertility of female flies; it also does not increase the expression level of *bam*.

The second hypothesis proposes that *Wolbachia* increases the rate of GSC mitosis, which increases the output of reproduction. Previous studies have shown *Wolbachia* to interact with GSCs, including research in *D. mauritiana,* where the increased fertility of *Wolbachia* infected females is attributed to the increased rate of GSC mitosis [45]. Other evidence of *Wolbachia*'s interaction with GSCs includes research on the Sxl^{f4} *D. melanogaster* mutant that has shown that *Wolbachia* restores the number of GSCs that were lost due to ectopic *bam* expression [19] and the necessity for *Wolbachia* for GSC maintenance in filarial nematodes [46].

We find that *Wolbachia* does not increase the rate of GSC mitosis in the bam^{L255F}*::Venus* hypomorph, and we do not observe any obvious ectopic Bam expression in the GSC niche in any of our transgenic *bam* mutants. These results are consistent with what we see with the *bam* knockdown results, as an increase in GSC mitosis rate would likely be reflected in an increase in progeny in the *Wolbachia* infected *bam* knockdown females.

Thus, while it is possible for there to be a subtle *Wolbachia*-GSC interaction that we have not yet assessed, collectively, our results suggest that a large component of the *Wolbachia bam* interaction is specific to mutant phenotypes that are the result of a disruption of *bam* function.

Biochemical assays are needed to firmly conclude that the *Wolbachia bam* interaction is specific to *bam* functions, but it is notable that this proposal is akin to that suggested for *Wolbachia's* interaction with *Sxl* [47]. The observations that *Wolbachia* cannot rescue the reduced egg count of a *snf* mutant, which has no *Sxl* expression in the germline, and a $Sxl^{f5,fHa}$ mutant, which has a transposon insertion in the second intron [17] parallels the *bam* knockdown results of our study.

It is possible that the fertility rescue of *bam* mutants occurs through the same (unknown) mechanism by which the rescue of *Sxl* mutants occurs. This would be a process beyond the documented restoration of GSC number in Sxl^{f4} mutants by *Wolbachia* factor TomO, as it was found that TomO is not sufficient to restore fertility of the Sxl^{f4} mutants [19].

Bam and Sxl have been proposed to partner together for two distinct functions. Together, with Bgcn, Mei-P26, Brat, Ago1, and a deadenylase complex, they repress the translation of the stem cell maintenance factor *nos* to determine GSC daughter cell fate [24, 25]. In this sense, *Wolbachia* could increase stability of host protein complexes, through, for example, use of molecular chaperones to help fold mutant Bam or Sxl proteins. It has also been suggested that Bam and Sxl function together to maintain female sexual identity in the adult germline [48], as failure to regulate *nos* does not cause the tumorous phenotype observed in *Sxl* and *bam* mutants [24]. Here, *Wolbachia* could restore proper female sexual identity to the germline by repressing testes specific gene expression.

Alternatively, it is possible that *Wolbachia* interacts with *bam* and *Sxl* independently. Bam has a number of other genetic and physical interacting partners that offer functional pathways for which *Wolbachia* could be interacting with. For example, Bam promotes differentiation of GSC daughter cells by facilitating deubiquitination of CycA with Otu [22]. Most recently, Bam itself has been shown to interact with E3 ligase Cullin-2, which ubiquitinates

Bam and leads to Bam's controlled expression [49]. Critical ubiquitin binding residues in Bam have been identified which offer another opportunity to assess the extent of *Wolbachia*'s interaction.

While more research is needed to determine if the *Wolbachia* interaction is specific to Bam-Sxl complexes or if *Wolbachia* interacts with *bam* independently of *Sxl*, our study suggests that *Wolbachia* could play a role by stabilizing the complexes or restoring binding affinity between Bam and its partners to restore *bam* function in *bam* protein-coding mutants.

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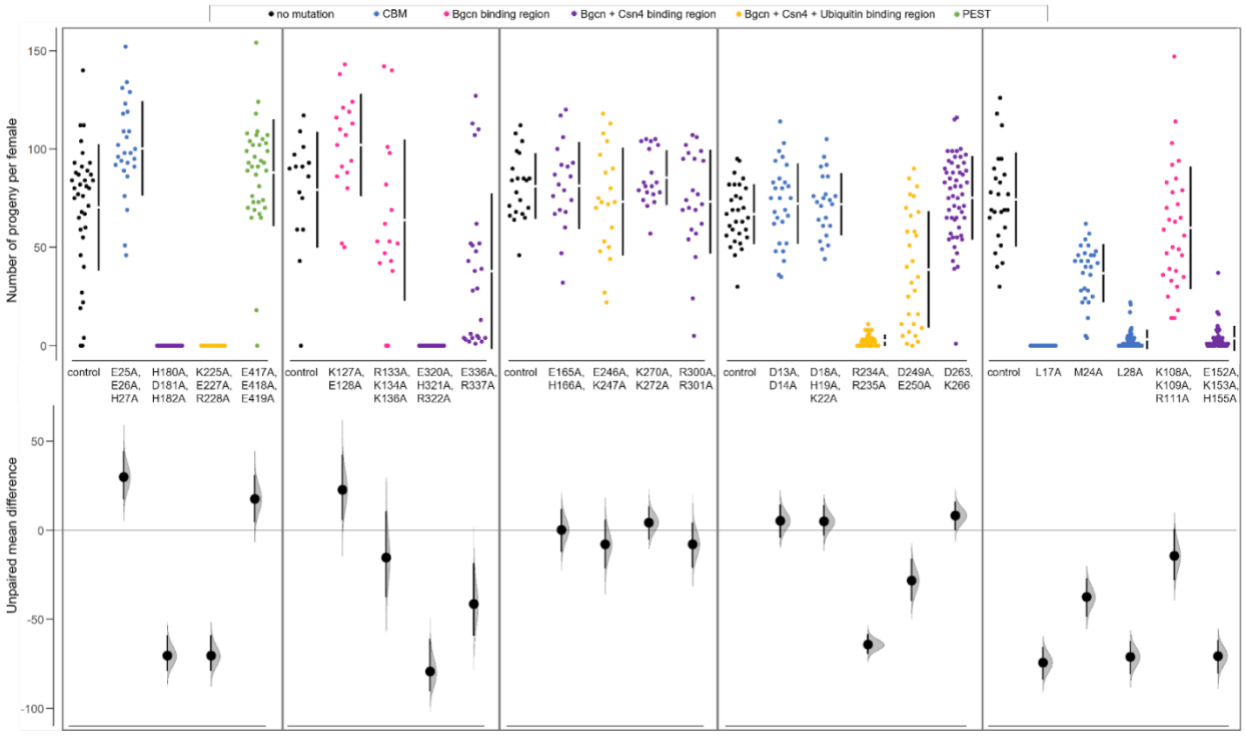
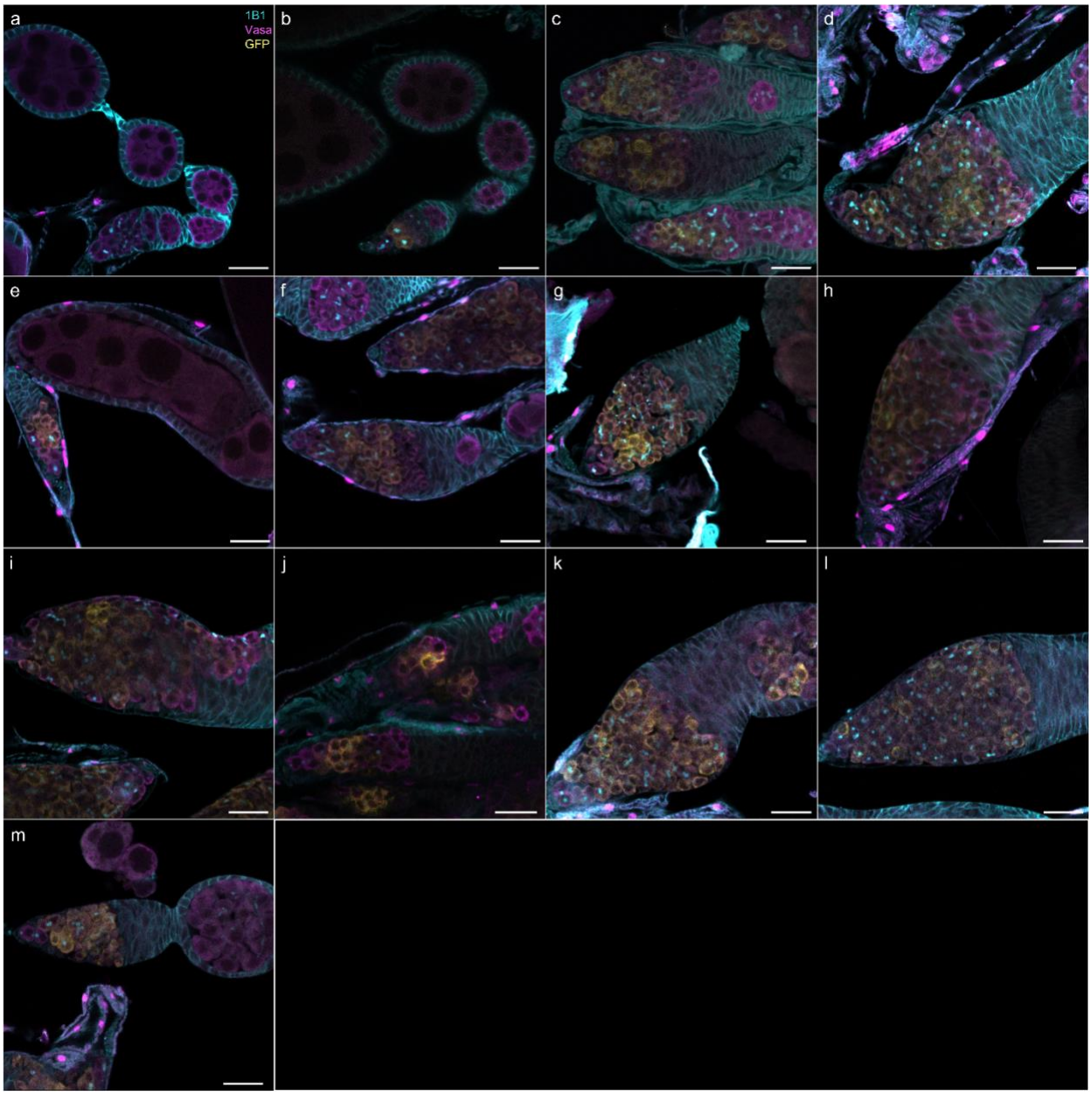
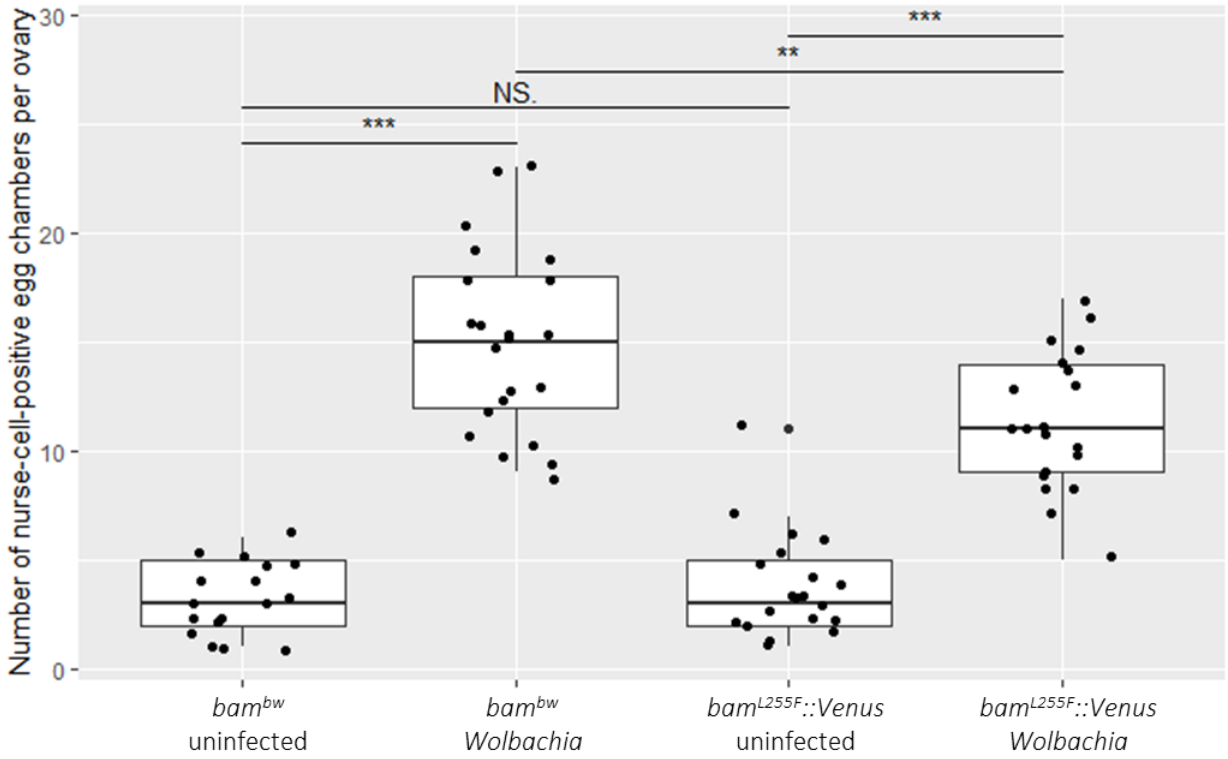
SUPPPLEMENTAL FIGURES AND TABLES



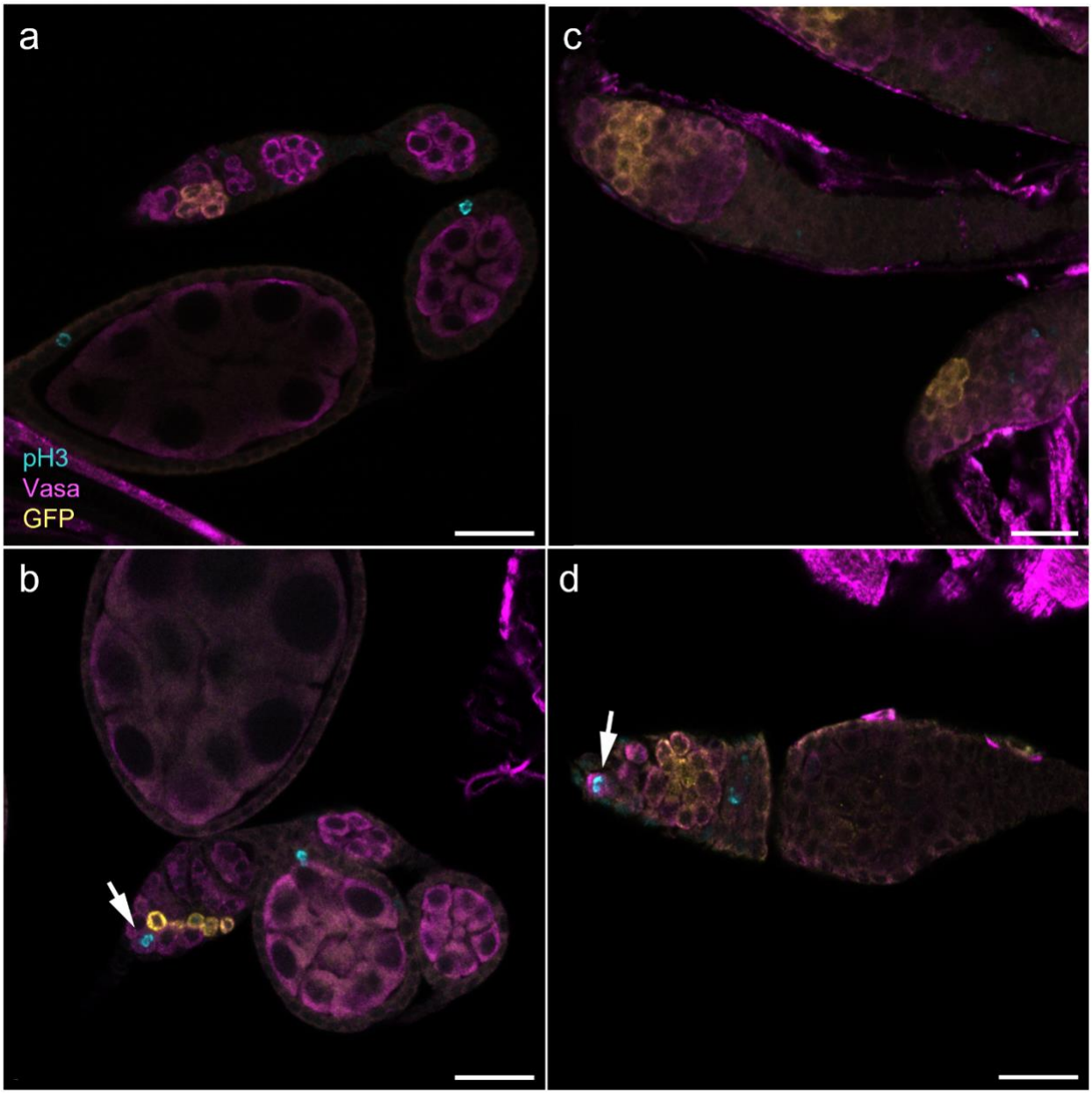
Supplemental figure S1. Full estimation plots for fertility data of transgenic mutants compared to the *bam::Venus* transgenic control. Fertility assays were done in five independent rounds. Below the jitter plots are the resampled bootstrap sampling distributions, with the mean differences represented by the black dots and the 95% confidence intervals represented by the vertical lines.



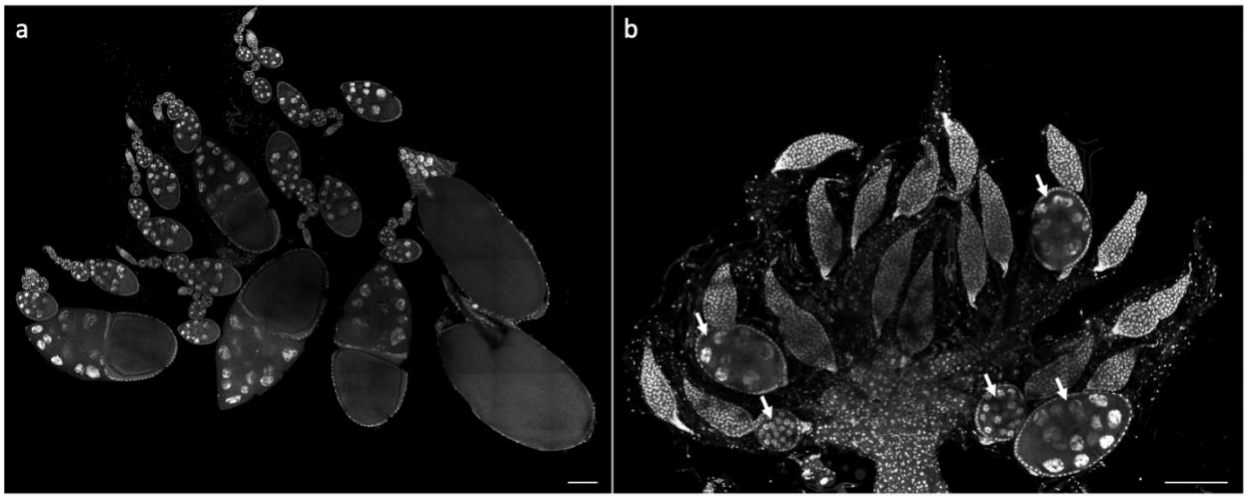
Supplemental figure S2. Germariums of controls and all transgenic *bam* mutants with fertility defects when uninfected with *Wolbachia*. Stained with anti-Hts-1B1 (cyan), anti-Vasa (magenta), and anti-GFP (yellow). (a) CantonS, (b) *bam::Venus* control, (c) bam^{L255F}*::Venus*, (d) bam^{L17A}*::Venus*, (e) bam^{M24A}*::Venus*, (f) bam^{L28A}*::Venus*, (g) $bam^{E152A,\ K153A,\ H155A}$*::Venus*, (h) $bam^{H180A,\ D181A,\ H182A:}$*::Venus*, (i) $bam^{R234A,\ R235A}$*::Venus*, (j) $bam^{D249A,\ E250A}$*::Venus*, (k) $bam^{K255A,\ E227A,\ R228A}$*::Venus*, (l) $bam^{E320A,\ H321A,\ R322A}$*::Venus*, (m) $bam^{E336A,\ R337A}$*::Venus*. Scale bar is 20μM for all images.



Supplemental figure S3. Comparison of original L255F mutant (*bam*^{bw}) and the transgenic L255F mutant with a Venus tag (*bam*^{L255F}*::Venus*) by the number of nurse-cell-positive egg chambers per ovary. ** = significant at p<0.01; *** = significant at p<0.001; NS = not significant.



Supplemental figure S4. GSC mitosis in the transgenic *bam::Venus* control and transgenic *bam^{L255F}::Venus* hypomorph. (a) No GSC mitosis and (b) active GSC mitosis in the *bam::Venus* control germarium. (c) No GSC mitosis and (d) active GSC mitosis in the *bam^{L255F}::Venus* hypomorphic mutant.



Supplemental figure S5. Ovaries of a *bam* wildtype and *bam* RNAi knockdown fly. (a) Ovaries of a *bam* wildtype fly. All egg chambers are results of successful Bam function. (b) Mutant ovaries of a nos-Gal4/+; UAS-*bam*^{HMS00029}/+ uninfected fly. Arrows indicate nurse-cell-positive egg chambers that represent successful Bam function. Scale bar for both images is 100μM.

Bam mutations	Mutagenesis forward primer	Mutagenesis reverse primer	Annealing temp. (°C)
D13A, D14A,	TGAGGGCAACgccgccCAGCAGTTGG	GGACACACGTCACGTGCATTAAG	70
L17A	CGACCAGCAGgctGACCACAATTTT AAGCAG	TCGTTGCCCTCAGGACAC	65
D18A, D19A, K22A,	ttttgccCAGATGGAGGAGCATTTGG	ttagcggcCAACTGCTGGTCGTCGTT	64
M24A	TTTTAAGCAGgctGAGGAGCATTTG GCC	TTGTGGTCCAACTGCTGG	62
E25A, E26A, H27A,	cgcTTTGGCCTTAATGGTGGAAG	gcggCCATCTGCTTAAAATTGTGG	60
L28A	GGAGGAGCATgctGCCTTAATGG	ATCTGCTTAAAATTGTGGTC	58
K108A, K109A, R111A,	tcggcaTCCGGAGGCGTGTTGGTC	ggcggcGGCCGGAGAACCGTCGAAT	71
K127A, E128A	GCAACTGCAGgccgctAATGTGTGGA ACCGGAAGAGTAAAG	TTCTGCTTTGGCCCGGTG	66
R133A, K134A, K136A,	agtgctGGCTCTGCGTCCGCGGAT	ggctgcGTTCCACACATTTTCCTTCTG CAGTTGC	71
E152A, K153A, H155A	ctggcaATGATTGGTCTGCACGGC	agcggcAATAGTTATGGGCAGTTTCT CAATATTATC	65
E165A, H166A,	CAATAGCTTAgccgctAACGCCGTGC	TTTAGATAAGGAGCTTTTTATTAT G	58
H180A, D181A, H182A,	cgctCTGACCGCCGATTTGGGC	gcagcCAGGGATCTGAACAGATTCA TCAAAC	66
K225A, E227A, R228A	gccgctTTCCTTGTCCAGCAGCGC	tacagcGGTGCACAGCACCTGCAG	69
R234A, R235A	TGTCCAGCAGgctgctACCTTGGAGG	AGGAAGCGCTCTACTTTG	61
E246A, K247A,	CTTCGATTTCgccgctTACGACGAGTG TGACAAGTTG	TGGCGATTCGCCTCCAAG	65
D249A, E250A	gccgctTTCCTTGTCCAGCAGCGC	tacagcGGTGCACAGCACCTGCAG	61
L255F	TGACAAGTTGtTTAAGGGTTTC	CACTCGTCGTATTTCTCG	58
D263A, K266A,	ttcgctCTGCTTTTAAAGCCCAAAATG	gttggcCAAATAGGATGCGAAACC	59
K270A, K272A,	cgctATGCGCAATCGAAACGGA	ggggcTAAAAGCAGTTTGAAGTTGT CC	62
R300A, R301A,	AATTGGTCTGgctgccTGGATCAAGG CTGCGCATC	AGCAATCTCTCCATGCGC	62
E320A, H321A, R322A	tgctTACTCCGGGGCCATGACC	gcggcCAGATCCATTTCCCAGTTAA ATACGTG	67
E336A, R337A	GTCGTTGAACgccgcaGCCATCCTTTT GTCC	TTGTGGCTTTCGGTCATG	59
E417A, E418A, E419A.	cgccTTTGAGGAGACCGAGGAAGTG C	gcggcCGACGCTGACGGCTGCTC	70

Supplemental table S1. Mutagenesis primers used to generate new *bam* mutants. Each row lists the corresponding primer sequences and annealing temperature used in the mutagenesis PCR step of the cloning procedure to generate the respective amino acid mutant.

Transgenic *bam* allele	Uninfected mean fertility	Infected mean fertility	Uninfected sample size	Infected sample size	Assay round
bam$^{D13A,\ D14A}$*::Venus*	72.2142857	90.3571429	28	28	two
bam^{L17A}*::Venus*	0.0000000	1.9600000	43	50	five
bam$^{D18A,\ H19A,\ K22A}$*::Venus*	71.9130435	90.8421053	23	19	one
bam^{M24A}*::Venus*	36.8928571	94.0909091	28	22	five
bam$^{E25A,\ E26A,\ H27A}$*::Venus*	100.2307692	102.0645161	26	31	one
bam^{L28A}*::Venus*	3.2000000	71.0000000	50	31	five
bam$^{K108A,\ K109A,\ R111A}$*::Venus*	59.9333333	98.6800000	30	25	five
bam$^{K127A,\ E128A}$*::Venus*	102.0000000	119.1111111	17	9	three
bam$^{R133A,\ K134A}$*::Venus*	63.8750000	107.5000000	16	10	three
bam$^{E152A,\ K153A,\ H155A}$*::Venus*	3.6666667	67.3421053	45	38	five
bam$^{E165A,\ H166A}$*::Venus*	81.3684211	100.8000000	19	10	four
bam$^{H180A,\ D181A,\ H182A}$*::Venus*	0.0000000	0.7714286	34	35	one
bam$^{K225A,\ E227A,\ R228A}$*::Venus*	0.0000000	0.9200000	29	25	one
bam$^{R234A,\ R235A}$*::Venus*	2.7058824	75.4814815	34	27	two
bam$^{E246A,\ K247A}$*::Venus*	73.2500000	104.8888889	20	18	four
bam$^{D249A,\ E250A}$*::Venus*	38.6896552	88.173913	29	23	two
bam$^{D263A,\ K266A}$*::Venus*	75.1320755	88.1111111	53	45	two
bam$^{K270A,\ K272A}$*::Venus*	85.4210526	99.5384615	19	13	four
bam$^{R300A,\ D301A}$*::Venus*	73.2727273	102.4583333	22	24	four
bam$^{E320A,\ H321A,\ R322A}$*::Venus*	0.0000000	0.0000000	41	39	three
bam$^{E336A,\ R337A}$*::Venus*	37.8800000	98.9375000	25	16	three
bam$^{E417A,\ E418A,\ E419A}$*::Venus*	87.9166667	111.4347826	36	23	one
bam::Venus	70.2702703	108.9200000	37	25	one
bam::Venus	66.8666667	92.5384615	30	26	two
bam::Venus	79.2666667	116.9000000	15	10	three
bam::Venus	81.0952381	101.1875000	21	16	four
bam::Venus	74.2800000	102.6086957	25	23	five

Supplemental table S2. Mean adult progeny per female seven days after first progeny eclosed, as uninfected and infected with *Wolbachia*. *Bam* alleles are listed in order of location of mutations from N- to C-terminal, with mean fertility of and sample size of surviving females at the end of the assay. The corresponding assay in which the transgenic flies were evaluated is indicated by the fertility assay round.