Wolbachia Endosymbionts Modify Drosophila Ovary Protein Levels in a Context-Dependent Manner

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ABSTRACT

Endosymbiosis is a unique form of interaction between organisms, with one organism dwelling inside the other. One of the most widespread endosymbionts is Wolbachia pipientis, a maternally transmitted bacterium carried by insects, crustaceans, mites, and filarial nematodes. Although candidate proteins that contribute to maternal transmission have been identified, the molecular basis for maternal Wolbachia transmission remains largely unknown. To investigate transmission-related processes in response to Wolbachia infection, ovarian proteomes were analyzed from Wolbachia-infected Drosophila melanogaster and D. simulans. Endogenous and variant host-strain combinations were investigated. Significant and differentially abundant ovarian proteins were detected, indicating substantial regulatory changes in response to Wolbachia. Variant Wolbachia strains were associated with a broader impact on the ovary proteome than endogenous Wolbachia strains. The D. melanogaster ovarian environment also exhibited a higher level of diversity of proteomic responses to Wolbachia than D. simulans. Overall, many Wolbachia-responsive ovarian proteins detected in this study were consistent with expectations from the experimental literature. This suggests that context-specific changes in protein abundance contribute to Wolbachia manipulation of transmission-related mechanisms in oogenesis.

IMPORTANCE

Millions of insect species naturally carry bacterial endosymbionts called Wolbachia. Wolbachia bacteria are transmitted by females to their offspring through a robust egg-loading mechanism. The molecular basis for Wolbachia transmission remains poorly understood at this time, however. This proteomic study identified specific fruit fly ovarian proteins as being upregulated or downregulated in response to Wolbachia infection. The majority of these protein responses correlated specifically with the type of host and Wolbachia strain involved. This work corroborates previously identified factors and mechanisms while also framing the broader context of ovarian manipulation by Wolbachia.

Symbiotic interactions between organisms, ranging from lethal parasitism to indispensable mutualism, frame the foundation of life. Endosymbionts face the same challenges as other microbes, which must replicate well and spread efficiently to be successful. However, the molecular mechanisms that contribute to endosymbiont transmission are not yet well understood. Endosymbiotic Wolbachia bacteria provide an excellent system to address this knowledge gap. These alphaproteobacteria of the Rickettsiales order are highly successful in nature, infecting filarial nematodes, crustaceans, mites, and over 40% of all insect species, including the well-established model organism Drosophila melanogaster (1–4). The presence of Wolbachia among this wide range of hosts is due to effective maternal transmission, analogous to mitochondrial (1, 5–7). The ovary produces egg chambers, composed of germ line and somatic cells, that mature over 3 to 4 days into completed eggs (8). Wolbachia bacteria are loaded into egg chambers through vertical and horizontal transmission (9–14), intracellular replication (15, 16), and achievement of transmission-enhancing localization patterns (17–20). The actin cytoskeleton also contributes to maternal Wolbachia transmission by facilitating germ line colonization through an unknown mechanism (21).

With the success of Wolbachia being reliant upon maternal germ line cells, it is in the interest of Wolbachia to enhance host fecundity (22–24). Wolbachia bacteria are thought to achieve this in part by increasing the frequency of germ line stem cell division (11). Other studies indicate that Wolbachia bacteria support ovary productivity by enabling proactive management of toxic iron (25–28), suppressing Sex- lethal (Sxl) mutations (29), and preventing generalized apoptosis in the germ line (11, 30). The specific factors involved in executing these Wolbachia impacts on the host germ line are not yet clear. Studies have used a variety of approaches to investigate expression-related host responses to Wolbachia (31–42). These analyses of Wolbachia-infected cultured cell lines, invertebrate body tissues, and...
intact host organisms to date have yielded a wealth of information. When considering how Wolbachia bacteria interact with and manipulate host germ line cells, the implications of this diverse set of findings are unclear, however. This study examines the hypothesis that consensus molecular interactions between Wolbachia and the host contribute to maternal Wolbachia transmission. The objective of this study was to assess the conservation of Wolbachia-host interaction mechanisms through analysis of the ovarian proteome.

MATERIALS AND METHODS

Wolbachia strain genotyping. Wolbachia genotyping was performed according to a diagnostic assay based on variable-number tandem-repeat (VNTR) and ISS markers (43). The profile of fragment sizes matched each sample with a known strain type. These same fragments were amplified from Drosophila simulans wRi as a negative control. To further distinguish wMelCS from the highly similar strain wMelPop, we used previously outlined diagnostic approaches (44). Sequencing was performed to identify a potential single nucleotide polymorphism (SNP) substitution at position 943,443 in the wMelCS genome. Samples were analyzed on an ABI 3100 genetic analyzer with sequencing analysis and GeneScan software (Applied Biosystems, CA). Octomom copy numbers were determined by quantitative real-time PCR (qRT-PCR) as previously reported (44). PCRs were carried out with Maxima SYBR green/ROX quantitative PCR (qPCR) master mix, using a CFX Connect real-time PCR detection system (Bio-Rad). Data were analyzed with CFX Manager V3.1. Relative Octomom copy numbers for each Wolbachia-infected host combination were calculated by methods reported previously (45).

Fly strains and rearing. The D. melanogaster genetic background used for all proteomic analyses was an uninfected wSp/CyoSh/TM6B strain. wMel and wMelCS strains described in previous studies were crossed into this line to ensure a uniform genetic background for all experiments (17, 44). The strain of D. simulans wSb/TM6B was cured of Wolbachia and the host contribute to maternal Wolbachia infection (Bindley Bioscience Center, Discovery Park, West Lafayette, IN) to be analyzed by a shotgun approach referred to as “discovery-based proteomics” (49–51).

Sample digestion. Gel bands were cut into 1-mm pieces and washed to remove the stain with 50:50 acetonitrile (ACN)–25 mM ammonium bicarbonate (ABC) (vol/vol). After washing, the samples were reduced and alkylated. Sequence-grade Lys-C–trypsin (Promega) was used to enzymatically digest the samples. All digestions were carried out with a Barocycler NEP2320 instrument (Pressure Biosciences) at 50°C under 20-kilopounds per square inch for 2 h. Peptides were recovered from gel samples by using 60% ACN–5% trifluoroacetic acid (TFA)–35% purified H2O with sonication in an ice bath. The supernatant was removed from the gels, and a vacuum centrifuge was used to dry samples. The resulting pellet was resuspended in 10 μL of 97% purified H2O–3% ACN–0.1% formic acid (FA). A 5-μL volume was used for nanoscale liquid chromatography–tandem mass spectrometry (NanoLC-MS/MS) analysis.

LC-MS/MS analysis. The samples were analyzed on a Nano Eksigent 425 high-performance liquid chromatography (HPLC) system coupled to a Triple TOF 5600 Plus instrument (ABSciex, Framingham, MA) (52). The gradient was 120 min at 300 nL/min over the cHiPLC-nanoflex system. The trap column was a Nano cHiPLC 200-μm by 0.5-mm ChromXP C18-CL 3-μm 120-Å column, followed by the analytical column, a Nano cHiPLC 75-μm by 15-cm ChromXP C18-CL 5-μm 120-Å column. The sample was injected into the Triple TOF 5600 Plus column through the Nanospray III source. Data acquisition was performed for 50 precursors at 50 ms/scan. Three technical replicates of this analysis were performed for each sample.

Proteomic data analysis. Initial data analysis was performed by using PeakView (ABSciex) and Mascot (Matrix Science) for database searches. D. melanogaster and D. simulans peptide information was compared to information in the respective databases for each host and assigned Uniprot identifiers accordingly. All isoform information corresponding to each protein was grouped together for classification as a single protein. To facilitate comparisons of D. melanogaster to D. simulans proteins, each D. simulans protein was assigned the name of its nearest D. melanogaster homolog. Intensity-based absolute quantification (iBAQ) of the protein amount (53) was used as a measure of initial protein detection for each sample type. Label-free quantification (LFQ) was performed by using MaxQuant (54) to identify proteins that satisfied a quality scoring function, enabling comparisons of protein quantity between infection conditions. Both iBAQ and LFQ data were recorded from 4 biological and 3 technical replicates for a combined total of 12 replicates per experimental condition. Proteins designated “reliable” were required to have been detected in 2 out of 3 technical replicates and 3 out of 4 biological replicates according to the LFQ data in order to be included in further data analyses. A coefficient of variation (CV) was also calculated for each significant protein by using the average of LFQ scores from all biological replicates. Only proteins exhibiting a CV below 50% were included in the final list of reliable hits. The reliable proteins were analyzed by using a one-way analysis of variance (ANOVA) approach to identify statistically significant proteins, based upon the LFQ scores of each biological replicate.

Differential protein abundance between sample types was determined by creating pairwise ratios of the average protein LFQ scores for each sample type. For D. melanogaster, differential abundance comparisons were made between the Dmel wMel/Dmel Uninf, Dmel wMelPop/Dmel Uninf, and Dmel wMel/Dmel wMelCS strains. For D. simulans, differential abundance comparisons were made between the Dsim wRi/Dsim Cured, Dsim wMel/Dsim Cured, and Dsim wMel/Dsim wRi strains. Proteins that showed an abundance change of >0.58 (log2-fold) (equivalent to a 1.5-fold change) were considered to represent differentially abundant proteins. In terms of regular numbers, these thresholds are indicated by a <0.67-fold change or a >1.5-fold change (38).

To assign the significant and differentially abundant proteins to functional.
Published variants were obtained by comparing threshold cycle (the amplified product. Data were analyzed by using Bio-Rad CFX man-

72°C for 30 s. Melting curves were examined to confirm the specificity of

for absolute quantification. The thermal cycling protocol for Wsp ampli-

tional classes, we first retrieved sequence information for each UniProt

for laboratory strains are listed. Distinguishing criteria for wMelCS and wMelPop, including the G-to-A transition at position 943443 and the Octomom copy number, are also shown (44).

a Diagnostic VNTR and insertion sequence element (ISS) regions were analyzed as described previously (43). The expected product size for a given variant as well as those

determined for laboratory strains are listed. Distinguishing criteria for

orthologous group assigned by eggNOG was selected as the initial

proteome, ovaries were dissected from all host-strain combina-

were analyzed per sample, for a total of 12 replicates per

the abundance of tandem repeats, the absence of additional

that

Wolbachia on

polymerase chain reaction (qPCR). Wolbachia titers were assessed by qPCR

sequence search was then performed

assign to each protein to 1 of 20 possible orthologous groups. The

first orthologous group assigned by eggNOG was selected as the initial functional classification for each of the proteins, followed by refinement of certain classifications in consultation with FlyBase and the scientific

literature.

DNA extraction for qPCR. Wolbachia titers were assessed by qPCR analysis of six biological replicates from each host-strain combination. All

flies were prepared as described above, and all sample types were run in parallel for each replicate. In running each replicate, ovary pairs were dissected from 5 females of each sample type. These pairs were homoge-
nized in 200 μl of 0.1 M Tris HCl, 0.1 M EDTA, and 1% SDS (pH 9.0) and

incubated for 30 min at 70°C. Twenty microliters of 3 M sodium acetate was added, and samples were mixed by shaking. After incubation for 30 min on ice, the samples were centrifuged at 14,000 rpm for 15 min at 4°C. Two hundred microliters of the supernatant containing DNA was col-

lected, and DNA was precipitated to a final volume of 50 μl by ethanol

precipitation. Briefly, 500 μl of absolute ethanol was added to 200 μl of

the supernatant. The sample was gently mixed and kept at −20°C for 1 h.

After centrifugation of the sample at 14,000 rpm for 15 min at 4°C, the

supernatant was removed carefully, and 1 ml of 70% ethanol was added to

the pellet. After 1 min, samples were centrifuged again at 14,000 rpm for

15 min at 4°C. After the supernatant was discarded, the DNA pellet was air
dried and resuspended in 50 μl of water. These DNA samples were diluted 1:10 for use in qPCR.

Real-time quantitative PCR analysis. Real-time PCRs were carried out with a CFX96 real-time PCR detection system (Bio-Rad). Each reaction was performed with a 20-μl final volume containing 10 μl of Maxima SYBR green-fluorescent qPCR master mix (Thermo Scientific), 0.5 μl of 5 mM each primer, and 2 μl of diluted DNA. Primers for the Wolbachia-
specific protein (Wsp) gene were used (44). Wsp plasmid standards rang-
ing from 10^5 to 10^8 copy numbers were used to generate a standard curve

for absolute quantification. The thermal cycling protocol for Wsp amplifi-

cation involved a 50°C incubation for 2 min and then denaturation for

10 min at 95°C, followed by 40 cycles of 95°C for 30s, 57°C for 1 min, and

72°C for 30 s. Melting curves were examined to confirm the specificity of

the amplified product. Data were analyzed by using Bio-Rad CFX man-
ger.3 with default threshold settings. Absolute Wolbachia copy numbers

were obtained by comparing threshold cycle (CT) values with a standard curve generated from the plasmid standard.

RESULTS

Each host-strain combination had ovarian proteins that were

reliable in abundance. To investigate the impact of Wolbachia on maternal transmission, this study focused on analyzing D. melanogaster and D. simulans ovaries of various infection statuses. D. melanogaster stocks that carried the native wMel strain (Dmel wMel) or the virulent wMelCS strain (Dmel wMelCS) (44) were derived from the same genetic background as uninfected control flies (Dmel Uninf). D. simulans stocks that carried the native wRi strain (Dsim wRi) or the artificially introduced wMel strain (Dsim wMel) were also generated (46) in the same genetic background as control flies cured with tetracycline (Dsim Cured). The identity of all Wolbachia strains was confirmed with diagnostic PCR assays, sequencing, and quantitative real-time PCR as described previously (43, 44, 57). The use of strain-specific markers confirmed that the D. melanogaster and D. simulans hosts infected with wMel carried the same wMel1 strain type (Tables 1 and 2). The other infected D. melanogaster line was verified to carry the wMelCS strain and not wMelSS or wMelPop variant types (Table 1), based upon the abundance of tandem repeats, the absence of additional Octomom repeats, and the absence of a specific G→A transition found in the wMelPop strain (44, 58). From this point forward, the confirmed Dmel wMel and Dsim wRi host-strain combinations are collectively referred to as “endogenous,” and the Dmel wMelSS and Dsim wMel combinations are referred to as “variant.”

To assess the impact of Wolbachia on the Drosophila ovary proteome, ovaries were dissected from all host-strain combina-

tions and analyzed by label-free LC-MS/MS. Four biological rep-

licates were collected for each sample type, and 3 technical rep-

licates were analyzed per sample, for a total of 12 replicates per sample type. This resulted in the initial identification of 927 pro-

teins from the D. melanogaster ovarian samples (see Table S1 in the supplemental material). A total of 853 of these proteins were

shared among all host-strain combinations (Fig. 1a). Further analysis determined that 549 of the shared proteins were based upon quality peptides in all D. melanogaster sample types (Fig. 1b; see also Table S1 in the supplemental material). In D. simulans,
TABLE 2 Quantitative PCR of host-specific Wolbachia variants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Slope</th>
<th>PCR efficiency</th>
<th>Dilution</th>
<th>Dmel wMel</th>
<th>Dmel wMelC5</th>
<th>Dsim wMel</th>
<th>Dsim wRi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3.108</td>
<td>2.0977</td>
<td>1:100</td>
<td>22.29</td>
<td>25.05</td>
<td>24.48</td>
<td>24.86</td>
</tr>
<tr>
<td>Target WD0513 gene</td>
<td>-3.085</td>
<td>2.1094</td>
<td>1:10</td>
<td>19.21</td>
<td>21.97</td>
<td>21.8</td>
<td>32.97</td>
</tr>
<tr>
<td></td>
<td>-3.085</td>
<td>2.1094</td>
<td>1:100</td>
<td>22.26</td>
<td>24.49</td>
<td>24.53</td>
<td>35.25</td>
</tr>
<tr>
<td>Fold change relative to control</td>
<td></td>
<td></td>
<td>1:10</td>
<td>1.00</td>
<td>0.85</td>
<td>0.80</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:100</td>
<td>1.00</td>
<td>1.46</td>
<td>0.93</td>
<td>0.00</td>
</tr>
<tr>
<td>Mean (SE)</td>
<td></td>
<td></td>
<td></td>
<td>1.00 (±0.0)</td>
<td>1.16 (±0.43)</td>
<td>0.87 (±0.09)</td>
<td>0.00 (±0.00)</td>
</tr>
</tbody>
</table>

834 total proteins were initially identified (see Table S2 in the supplemental material). A total of 762 of these proteins were shared among all D. simulans ovary proteomes analyzed (Fig. 1d). A total of 449 of these shared D. simulans protein identifications were based upon quality peptide information (Fig. 1c; see also Table S2 in the supplemental material). Taken together, these data indicate that 54 to 59% of the protein identifications initially associated with D. simulans and D. melanogaster ovarian proteomes were assigned with high confidence. This set of consensus quality proteins is pursued further in the analyses described below.

The next phase of analysis focused on identifying which consensus quality proteins were reliably detected during oogenesis for each host-strain combination. This required protein detection in at least 2 out of 3 technical replicates per biological sample and at least 3 out of 4 biological samples of each sample type. The coefficient of variation (Gini coefficient) was also calculated for each protein hit, and proteins with a CV below 50% were selected as described previously (59). These rigorous criteria defined 316 proteins as being "reliable" among all the D. melanogaster ovarian proteomes analyzed (Fig. 1c; see also Table S1 in the supplemental material). A total of 279 proteins were reliably detected within all ovarian proteomes of D. simulans (Fig. 1f; see also Table S2 in the supplemental material). All quantitative analyses of the ovarian proteomes described below focus on these reliable proteins.

Most Wolbachia-associated proteomic changes are restricted to a given host type. To identify ovarian proteins that exhibit significant abundance changes in Wolbachia-infected tissue, comparisons between sample types were performed by using ANOVA. For D. melanogaster, this analysis revealed 61 host proteins whose abundance changed significantly under one or more of the Wolbachia-infected conditions (see Table S3 in the supplemental material) (P < 0.05). The Wolbachia surface protein, Wsp, was also identified in Dmel wMel and Dmel wMelC5 samples only. For D. simulans, ANOVA identified 49 host proteins that exhibited significantly altered abundance in one or both Wolbachia-infected samples (see Table S4 in the supplemental material) (P < 0.05). These ovarian proteins are referred to here as "significant proteins."

To address the overall functional implications of the group of significant proteins, each protein was assigned to a functional class, based on information from the eggNOG v4.5 program and the Drosophila literature. This analysis grouped the significant proteins into 15 functional classes (Fig. 2a and c). Six functional classes were specific to either D. melanogaster or D. simulans and represented ≤10% of the total proteins. The remaining 9 functional classes were shared between host types. Translation-related proteins were highly represented, comprising up to half of the significant proteins overall. The other shared functional classes were carbohydrate transport and metabolism; chromatin structure and dynamics; cytoskeleton and cell motility; energy conversion; lipid transport and metabolism; protein modification, folding, and turnover; RNA binding, processing, and modification; and signal transduction (Fig. 2a and c). This implicates a diverse subset of ovarian cellular processes as being responsive to Wolbachia.

The similarity of D. melanogaster and D. simulans ovarian responses to Wolbachia was further assessed in terms of overlap between consensus significant proteins. As D. simulans annotation is less extensive than that of D. melanogaster, all D. simulans proteins were named as per the closest D. melanogaster homologs to facilitate this comparison. Out of 95 total significant proteins, this analysis identified 15 significant proteins as being shared between D. melanogaster and D. simulans ovarian proteomes (see Tables S3 and S4 in the supplemental material). These proteins were glycosen phosphorylase, the ATP synthase delta subunit, retinoid- and fatty acid-binding protein, heat shock proteins 26 and 27, the hnRNP protein Squid, and 9 different ribosomal proteins (see Tables S3 and S4 in the supplemental material). Thus, a...
limited redundancy of individual proteins was evident among the significant ovarian proteins of *D. melanogaster* and *D. simulans*. To assess the putative functional impact of *Wolbachia*-responsive significant proteins, the magnitude of protein abundance changes was examined. As in previous proteomics studies, differential abundance on the order of a $\geq 1.5$-fold change is predicted to indicate functional upregulation. Conversely, a $\leq 0.667$-fold change is predicted to indicate functional downregulation (38, 60–62). Comparisons of the significant protein data yielded 25 differentially abundant proteins in *D. melanogaster*, representing 11 functional classes (Fig. 2b and Table 3). Seventeen differentially abundant proteins were detected in *D. simulans*, comprised of 9 functional classes (Fig. 2d and Table 4). The 8 classes of differentially abundant proteins shared between host types were carbohydrate transport and metabolism; chromatin structure and dynamics; cytoskeleton and cell motility; energy production and conversion; lipid transport and metabolism; protein modification, folding, and turnover; RNA binding, processing, and modification; and translation, ribosomal structure, and biogenesis (Fig. 2b and d). This suggests that the differentially abundant proteins represent a distinct subset of significant proteins. The differential abundance data also indicated that the composition of each shared functional class is largely organism specific. The few differentially abundant proteins that were shared between hosts were glycogen phosphorylase, the ATP synthase delta subunit, and heat shock proteins 26 and 27.

**Differential protein abundance patterns associated with host and *Wolbachia* types.** To further define the impact of specific *Wolbachia* strains on the host ovary proteome, host-strain combinations were examined in terms of the commonalities that they share. One issue was to determine the extent of overlap between proteomic responses to endogenous and variant *Wolbachia* infections. In *D. melanogaster*, comparison of Dmel wMel to Dmel Uninf yielded 3 differentially abundant proteins, whereas comparison of Dmel wMelC3S to Dmel Uninf yielded 12 (Table 3). In *D. simulans*, comparison of Dsim wRi and Dsim Cured revealed 6 differentially abundant proteins, while comparison of Dsim wMel to Dmel Cured identified 13 (Table 4). This suggests that infections by variant *Wolbachia* strains had a more robust impact than infections by endogenous *Wolbachia* strains on ovarian proteomic responses at the level of differential abundance.

Another issue to address was the extent of bacterial versus host influence on the ovarian proteomic responses to *Wolbachia*. To assess the consistency of responses associated with a single *Wolbachia* strain, ovarian responses to wMel were tracked across host types. This analysis indicated that distinctive proteomic responses were evident in the natural *D. melanogaster* host compared to the ectopic *D. simulans* host (Tables 3 and 4). The similarity of host responses to multiple *Wolbachia* strains was also investigated. Direct comparison of Dmel wMelC3S to Dmel wMel identified 11 additional differentially abundant proteins, including the *Wolbachia* surface protein (Wsp) (Table 3). Most of these hits were due to oppositely directed protein abundance shifts under each *Wolbachia* infection condition. Direct comparison of Dsim wMel to Dmel wRi identified 8 differentially abundant proteins as well. However, nearly all these shifts were redundant with shifts already identified in comparisons between infected and uninfected *D. simulans* ovaries (Table 4). This suggests that ovarian proteomic responses to different *Wolbachia* strains were milder and more diversified in *D. melanogaster* than in *D. simulans*, where all-or-nothing responses were predominant.

Previous studies showed that high-titer *Wolbachia* infections exert the most extensive impact on host physiological processes (44, 63–65). This precedent raises questions about the role of *Wolbachia* titer in specifying *Wolbachia*-associated changes in the ovary proteome. Real-time quantitative PCR was performed to assess ovarian *Wolbachia* abundance. The data indicated that Dmel wMelC3S ovaries carried only 51% of the *Wolbachia* titer detected in Dmel wMel ovaries ($P = 0.047$) ($n = 60$ ovaries per condition) (see Fig. S1 in the supplemental material). Ovarian *Wolbachia* titers detected in Dsim wRi and Dsim wMel ovaries were not significantly different from each other or from those in Dmel wMel ovaries (see Fig. S1 in the supplemental material). This does not support a role for elevated *Wolbachia* titers as a
determinant of ovarian proteomic responses but alternatively favors consideration of molecular and cellular mechanisms intrinsic to each scenario.

**DISCUSSION**

In applying a proteomic approach to ovarian responses to *Wolbachia*, a central consideration is whether the data set substantiates current knowledge of infection. Based upon previous work, one expectation is that variant host-strain combinations should exhibit stress indicators (36, 40, 66, 67). Notably, the variant Dmel wMelCS and Dsim wMel combinations in this study exhibited depletion of dozens of ribosomal constituents, consistent with overall downregulation (68–70). Upregulation of heat shock and detoxification proteins was also seen, consistent with a stress response (71–73). Ovaries from the Dsim wMel combination have also been shown to exhibit extensive chromatin structuring defects in nurse cells, analogous to *squid* mutant organisms (16, 74). The downregulation of the Squid protein observed here informs the basis for this response.

Another expectation is that *Wolbachia* should strategically enhance ovarian survival and proliferation mechanisms to maximize transmission. The findings of this study corroborate the involvement of known factors while also identifying new candidate contributors. The upregulation of the iron-sequestering protein transferrin 1 is in agreement with previous reports that *Wolbachia* bacteria protect the germ line from iron-associated toxicity (25–28). An increased abundance of the retinoid- and fatty acid-binding protein, indicated to have heme-binding activity, may help to protect the germ line from oxidative stress as well (75). Upregulation of the Sxl effector protein, Female-specific independent of transformer, opens a speculative route for *Wolbachia* modulation of Sxl-induced germ line lethality (29, 76, 77). The downregulation of the cell division suppressor 14-3-3 zeta is also consistent with enhanced germ line stem cell division rates observed for *Wolbachia*-infected organisms (11, 78).

It is further expected that *Wolbachia* bacteria drive modifications of the ovarian environment that support *Wolbachia* persistence. Some evidence from this study supports that prediction. From a nutritional standpoint, an elevated abundance of proteases and proteasome subunits is consistent with the possibility of

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**TABLE 3 Differentially abundant proteins identified through comparison of *D. melanogaster* ovarian proteomes**

<table>
<thead>
<tr>
<th>Functional classification</th>
<th>Protein</th>
<th>Relative abundance</th>
<th>wMel/Uninf</th>
<th>wMel&lt;sup&gt;CS&lt;/sup&gt;/Uninf</th>
<th>wMel&lt;sup&gt;CS&lt;/sup&gt;/wMel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid transport and metabolism</td>
<td>Eip55E</td>
<td>1.271</td>
<td>1.587</td>
<td>1.248</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate transport and metabolism</td>
<td>Aldolase</td>
<td>0.919</td>
<td>0.630</td>
<td>0.685</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycogen phosphorylase</td>
<td>0.839</td>
<td>1.447</td>
<td>1.724</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Succinyl coenzyme A synthetase α subunit</td>
<td>NA</td>
<td>NA</td>
<td>0.591</td>
<td></td>
</tr>
<tr>
<td>Chromatin structure and dynamics</td>
<td>Vig2</td>
<td>0.868</td>
<td>1.330</td>
<td>1.533</td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton and cell motility</td>
<td>Ciboulot</td>
<td>0.858</td>
<td>1.380</td>
<td>1.608</td>
<td></td>
</tr>
<tr>
<td>Detoxification</td>
<td>Glutathione S-transferase D1</td>
<td>1.028</td>
<td>1.626</td>
<td>1.581</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peroxinectin-like</td>
<td>0.889</td>
<td>1.704</td>
<td>1.917</td>
<td></td>
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<tr>
<td></td>
<td>Transferrin 1</td>
<td>2.000</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Energy production and conversion</td>
<td>ATP synthase, δ subunit</td>
<td>0.888</td>
<td>1.367</td>
<td>1.540</td>
<td></td>
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<tr>
<td></td>
<td>Isocitrate dehydrogenase</td>
<td>0.776</td>
<td>1.314</td>
<td>1.693</td>
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<tr>
<td>Lipid transport and metabolism</td>
<td>Jabba</td>
<td>0.801</td>
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<td>1.603</td>
<td>1.539</td>
<td></td>
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<td></td>
<td>Heat shock protein 26</td>
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<td>1.639</td>
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<tr>
<td></td>
<td>Heat shock protein 27</td>
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<td>1.525</td>
<td>1.403</td>
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<tr>
<td></td>
<td>Hsc/Hsp70-interacting protein related</td>
<td>0.828</td>
<td>1.266</td>
<td>1.529</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regulatory particle non-ATPase 6</td>
<td>1.098</td>
<td>1.578</td>
<td>1.437</td>
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<tr>
<td>RNA binding, processing, and modification</td>
<td>Hoi-polloi</td>
<td>1.214</td>
<td>0.795</td>
<td>0.655</td>
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<tr>
<td></td>
<td>Modulo</td>
<td>1.035</td>
<td>0.687</td>
<td>0.663</td>
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</tr>
<tr>
<td></td>
<td>Rm62</td>
<td>0.999</td>
<td>0.644</td>
<td>0.644</td>
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<tr>
<td>Signal transduction</td>
<td>14-3-3-3ξ</td>
<td>0.611</td>
<td>0.535</td>
<td>0.909</td>
<td></td>
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<tr>
<td></td>
<td>Terribly reduced optic lobes</td>
<td>1.154</td>
<td>0.647</td>
<td>0.561</td>
<td></td>
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<tr>
<td>Translation, ribosomal structure, and biogenesis</td>
<td>Ribosomal protein S27</td>
<td>0.989</td>
<td>0.645</td>
<td>0.652</td>
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<tr>
<td></td>
<td>Seryl-tRNA synthetase</td>
<td>0.618</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Wolbachia protein</td>
<td><em>Wolbachia</em> surface protein</td>
<td>NA</td>
<td>NA</td>
<td>0.632</td>
<td></td>
</tr>
</tbody>
</table>

* Relative abundance represents a ratio of average LFQ scores for each sample type: Dmel wMel/Dmel Uninf, Dmel wMel<sup>CS</sup>/Dmel Uninf, and Dmel wMel<sup>CS</sup>/Dmel wMel. Ratios indicating protein up- or downregulation are shown in boldface type. NA, not applicable.
increased amino acid availability for Wolbachia (79). The upregulation of glycogen phosphorylase complements recent work using Brugia malayi nematodes, which indicated that Wolbachia bacteria induce the upregulation of glycolytic enzymes (80). An increased local availability of pyruvate is hypothesized to benefit Wolbachia (81). It was also recently shown that filamentous actin is important for stabilizing Wolbachia colonization of the host germ line (21). Wolbachia-associated upregulation of tropomyosin, a microfilament-stabilizing protein, is consistent with that model (82). Taken together, these data support the study outcomes as being representative while also associating the potential use of these transmission-enhancing mechanisms with new host-strain combinations.

It is notable that very few proteins were detected as being significant or differentially abundant across all sample types analyzed in this study. Data sets from previous Wolbachia-omics studies exhibit a wide range of Wolbachia-responsive host expression changes, indicating that contextual influences are substantial (31–42) (see Table S5 in the supplemental material). Analogous to those prior studies, our study provides substantial evidence of context-dependent responses to Wolbachia infection. Infections with endogenous Wolbachia strains had little effect on the host proteome compared to infections with variant Wolbachia strains, in agreement with data from previous work on heterologous symbiotic infections of cnidarians (83). Ovarian proteomic responses to Wolbachia also correlated poorly with Wolbachia titers, paralleling results from a previous fecundity study (84). This argues against the conservation of Wolbachia-ovary interactions in terms of specific protein abundance shifts. A combination of effects may contribute to this outcome, including technical limitations of the assay (51) as well as Wolbachia adaptation (85), modification of Wolbachia population structure (85), and/or selection (86). Regardless, the finite physical constraints of transmission inherently favor Wolbachia manipulation of the most functionally advantageous processes. Context-specific regulation of consensus ovarian mechanisms may contribute substantially to the achievement of this goal.

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