



SypC, a symbiont outer membrane vesicle protein, impacts the development of the squid–vibrio partnership

Jill T. Kuwabara^{ab}, Vera Beilinson^c, Alexis C. Hargadon^b, Grischa Y. Chen^{b,c}, Xiao-Meng Hu^{b,c}, Mark S. Ladinsky^c, Kathleen T. Hackett^d , Joseph P. Dillard^d , Karen L. Visick^e , Edward G. Ruby^{a,c} , and Margaret McFall-Ngai^{ab,c,1}

Affiliations are included on p. 9.

Contributed by Margaret McFall-Ngai; received September 3, 2025; accepted February 9, 2026; reviewed by Anne M. Krachler and George O'Toole

Bacterial outer membrane vesicles (OMVs) and the cargo they carry are increasingly recognized as a means of communication between microbial symbionts and the cells of their host. However, few studies have focused on the biochemical and molecular mechanisms underlying OMV signaling during symbiosis onset and development. We show here that SypC, an OMV protein of the bioluminescent symbiont *Vibrio fischeri*, is taken up by cells of the squid host *Euprymna scolopes* where it assumes a new function, i.e., the facilitation of symbiont-induced light-organ morphogenesis. SypC is a Wza-like outer membrane protein found in host-associated Vibrionaceae and is essential for *V. fischeri* biofilm formation. Colonization or direct treatment with *V. fischeri* OMVs triggers host development, which was reduced or delayed if the host is instead exposed to a Δ sypC mutant or Δ sypC OMVs. RNA-seq analyses comparing light organs colonized by either the mutant or its parent revealed differential expression of host genes associated with immune responses and tissue morphogenesis. In immunocytochemical imaging, SypC-bearing OMVs were taken up by the host's macrophage-like cells near the light-organ crypts, revealing the mechanism by which SypC travels through tissue to trigger morphogenesis. Taken together, the data provide evidence that in addition to its role in biofilm formation and colonization, SypC has a second function promoting the induction of symbiotic-tissue development. These findings provide a critical piece of a puzzle whereby a rich array of host and symbiont molecules work in concert to orchestrate normal symbiont colonization and host development within the first hours to days of symbiosis.

OMVs | transcriptomics | symbiosis | *Euprymna scolopes* | *Vibrio fischeri*

Most, if not all, Gram-negative bacteria produce outer membrane vesicles (OMVs), which have recently been recognized to be involved in communication with host cells (for review, see refs. 1–3). For many species, OMVs are released from the bacterial cell surface by a nonlytic budding mechanism of the outer membrane (2, 4) that can be recognized by the almost solely periplasmic and outer membrane derivation of their proteome (e.g., refs. 5, 6). The cargo present in OMVs also includes nucleic acids and components of the outer envelope, such as lipopolysaccharide (LPS) and peptidoglycan (PGN) derivatives. Following release from the cell surface of bacterial symbionts, these vesicles interact with surface receptors on host epithelial or blood cells, or can even enter the cytoplasm, where they are often recognized by intracellular receptors. Increasing evidence suggests that OMVs internalized in host cells can release their cargo into the host-cell cytoplasm and influence the function and viability of those cells (6–8). OMVs have been principally characterized in bacterial pathogenesis (9, 10), but recent studies have shown them to be critical elements of beneficial symbioses of both vertebrates (6) and invertebrates (see, e.g., refs. 11–13). For example, OMVs of the microbiota have been shown to enter the bloodstream of mammals, where they influence host immunity and disease response (12, 14), as well as to cross the blood–brain barrier, where they influence neurological health and disease (15).

Studies of the squid–vibrio model symbiosis have provided insights into the involvement of symbiont OMVs in modulating host–microbe interactions (16, 17). Host exposure to either ¹⁵N-labeled *V. fischeri* cells or OMVs derived from these cells results in indistinguishable patterns of heavy-isotope labeling of the host tissues (7). In this system, the luminous bacterium *Vibrio fischeri* resides in crypt spaces along the apical epithelium of the symbiotic light-emitting organ of the Hawaiian bobtail squid, *Euprymna scolopes* (18, 19). Each generation, the aposymbiotic (APO) (i.e., symbiont-free) light organ of the newly hatched juvenile host specifically recruits *V. fischeri* cells from the bacterioplankton within hours; these founders and their progeny remain the symbionts throughout the squid's lifespan. The host hatches with a light-organ morphology that potentiates symbiont

Significance

Bacterial symbionts can profoundly influence host development. Using the *Euprymna scolopes-Vibrio fischeri* model symbiosis, this study provides evidence for recruitment of the outer membrane protein SypC, which is necessary for *V. fischeri* biofilm formation, into the process of host morphogenesis that occurs early in the symbiosis. This divergent dual-function role that SypC plays requires it to be shed from the cell surface of *V. fischeri* in outer membrane vesicles (OMVs). Host macrophage-like cells phagocytose and traffic the OMVs to a distant site where they are essential for induction of light-organ development. Because many species of the Vibrionaceae share unusual structural features of SypC, this protein may be critical for host–vibrio interactions in both nonpathogenic and pathogenic associations.

Author contributions: J.T.K., V.B., G.Y.C., J.P.D., K.L.V., E.G.R., and M.M.-N. designed research; J.T.K., V.B., A.C.H., G.Y.C., X.-M.H., M.S.L., K.T.H., and K.L.V. performed research; J.P.D. and K.L.V. contributed new reagents/analytic tools; J.T.K., V.B., A.C.H., G.Y.C., X.-M.H., M.S.L., K.T.H., J.P.D., K.L.V., E.G.R., and M.M.-N. analyzed data; and J.T.K., V.B., A.C.H., G.Y.C., M.S.L., J.P.D., K.L.V., E.G.R., and M.M.-N. wrote the paper.

Reviewers: A.M.K., The University of Texas Health Science Center at Houston; and G.O., Dartmouth College Geisel School of Medicine.

The authors declare no competing interest.

Copyright © 2026 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

¹To whom correspondence may be addressed. Email: mcfallng@caltech.edu.

This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2524648123/-/DCSupplemental>.

Published March 11, 2026.

recruitment (Fig. 1A). Ciliated fields on the nascent symbiotic organ entrain environmental *V. fischeri* cells above pores on the organ surface (Fig. 1A', Left). These cells aggregate in host-derived mucus (Fig. 1A'). This behavior requires the production of an exopolysaccharide on the surface of *V. fischeri* cells, a function that is encoded by the symbiont's *syp* locus (20). Thus, the biofilm consists of two components: host mucus and the bacterial exopolysaccharide. After aggregating, the cells migrate through host tissues to colonize the crypt spaces (Fig. 1A', Right), where they induce the loss of the superficial ciliated fields over the first 4 to 5 d following colonization (18). This gradual process involves two conspicuous phenotypes: i) widespread cell death of the superficial ciliated cells (Fig. 1B); and ii) the migration of squid-host macrophage-like blood cells, or hemocytes, into the blood sinuses of the ciliated-appendage epithelia (Fig. 1C). Following an upregulation of matrix metalloprotease activity in the basement membrane (21), the dying cells are sloughed from the surface, i.e., they are not phagocytosed by the hemocytes in the blood sinus. The microbe-associated molecular patterns, or MAMPs, present in the OMVs of *V. fischeri* cells, specifically LPS and PGN derivatives, trigger most of this cell death and hemocyte migration, with symbiont light production being required for the full developmental program (22). While LPS and PGN are common to many bacterial species, this response is specific to *V. fischeri* because the signals originate in light-organ crypts, where only this species can persist (16, 23). Whereas a great deal of this process has been described, the mechanism by which the inductive message is transferred from the bacteria in the crypt spaces to the superficial ciliated fields has remained unresolved.

Investigations of *V. fischeri* OMVs have shown that LPS, as well as hundreds of proteins and several RNA types (tRNA, rRNA, and small noncoding RNAs or sRNAs), are trafficked into host cells. Thus far, three of these biomolecules in the OMVs have been deeply characterized, i.e., the LPS (24), the sRNA SsrA, also called tmRNA (17), and the outer membrane protein OmpU (5). The data showed that these molecules each impact a specific feature of early symbiosis development.

In this study, we present data from an examination of the importance of SypC [See SI Appendix, Fig. S1A; UniProt (25)], a *V. fischeri*

protein present in the OMVs (26) (SI Appendix, Fig. S1B), on host light-organ gene expression and development. SypC was selected for study because there is a deep understanding of components of the *V. fischeri* *syp* locus (20), which encodes proteins that produce an exopolysaccharide critical in symbiosis competency. SypC is an intrinsic outer membrane-spanning protein that is a paralog of the *V. fischeri* GfcE and its ortholog *Escherichia coli* Wza (SI Appendix, Fig. S1A) (27). It is essential for the export of the bacterium's exopolysaccharide (EPS) or Syp polysaccharide (28) and is the only protein encoded by the *syp* locus that is detected in OMVs (5). The data presented here provide evidence that SypC is critical for conveying the information that potentiates host light-organ development and promotes colonization of the crypts by *V. fischeri*.

Results

Δ sypC-Colonized Light Organs Have Altered Transcriptional Responses. To understand how colonization by a Δ sypC symbiont might differentially influence host gene expression, we performed an RNA-seq experiment at 24 h postinoculation (hpi). Relative to APO animals, *V. fischeri* colonization, regardless of the presence of *sypC* in the symbionts (i.e., WT or Δ sypC), modulated the expression of 396 genes, clearly distinguishing uncolonized squid from symbiotic ones. (Fig. 2A, Left, and B). This result is not unexpected, as multiple factors from *V. fischeri* (e.g., MAMPs and light production) are known to modulate host gene expression (22). Nevertheless, the presence of *sypC* did have a clear transcriptional impact, specifically affecting a distinct subset of host genes. Differential expression (DE) analysis (see PERMANOVA, in SI Appendix, Supplemental Results) comparing colonization by either the WT or the Δ sypC strain identified 138 *sypC*-dependent regulated genes (green and yellow, Fig. 2B). Hierarchical clustering showed that these genes had similar expression levels in APO and Δ sypC treatments, indicating that to be regulated, these genes require the presence of *sypC* in the symbionts (Fig. 2C, Left). Additionally, we identified 345 genes that were not significantly different after WT or Δ sypC colonization but instead exhibited an intermediate level of expression in Δ sypC relative to WT and APO (Fig. 2C, Right). These genes were statistically DE between

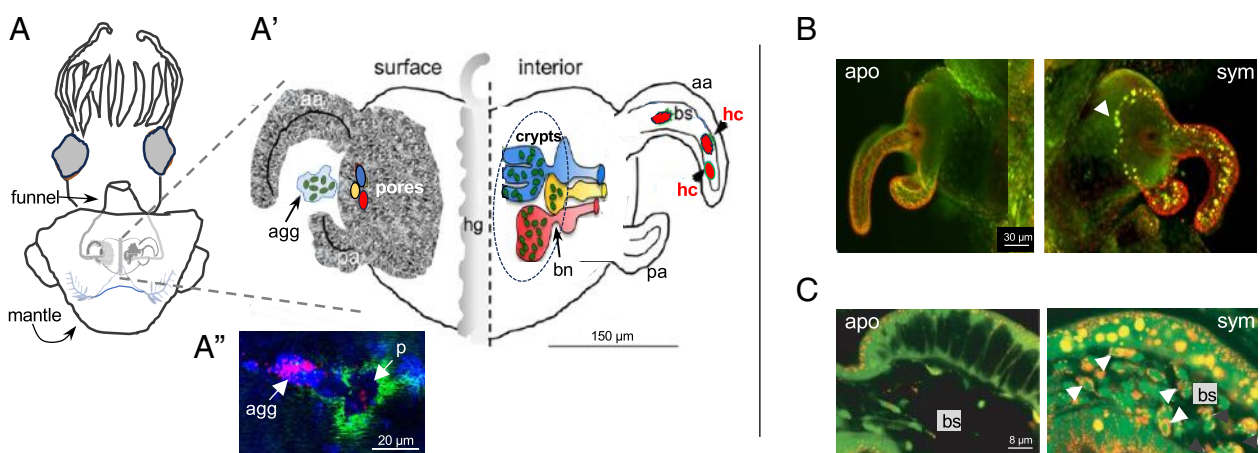


Fig. 1. Juvenile light organ features and colonization developmental events. (A) The morphology and anatomy of the symbiotic organ. Ventral view of an *E. scolopes* showing the location of the light organ beneath the mantle and funnel. (A') Expanded diagram of the light organ. *Left half*: surface features of the organ, showing the superficial ciliated fields (gray) and three pores, relative to the position of the hindgut (hg). *V. fischeri* cells (green) in the ambient seawater aggregate in mucus outside of the pores (agg). *Right half*: internal regions of the organ including 3 crypts and the migration-pathway bottlenecks at their entrances (one indicated, bn). *V. fischeri* cells (green) are located in the crypts; the blood sinuses (bs) of the superficial ciliated appendages (aa, anterior appendage; pa, posterior appendage) contain migrating hemocytes (hc, red). (A'') Confocal image of aggregated *V. fischeri* cells (magenta) in host mucus (blue) outside of a pore (p) surrounded by cilia (green). (B) Once the light organ is colonized, the superficial ciliated fields regress by programmed cell death; dying cells (yellow puncta) were visualized by acridine orange staining, characteristic of early stage cell death of symbiotic (sym) but not aposymbiotic (apo) light organs. (C) Close-up images of tissues of the blood sinuses (bs) of the ciliated appendages. After colonization by *V. fischeri* cells, the blood sinuses fill with hemocytes (white arrowheads).

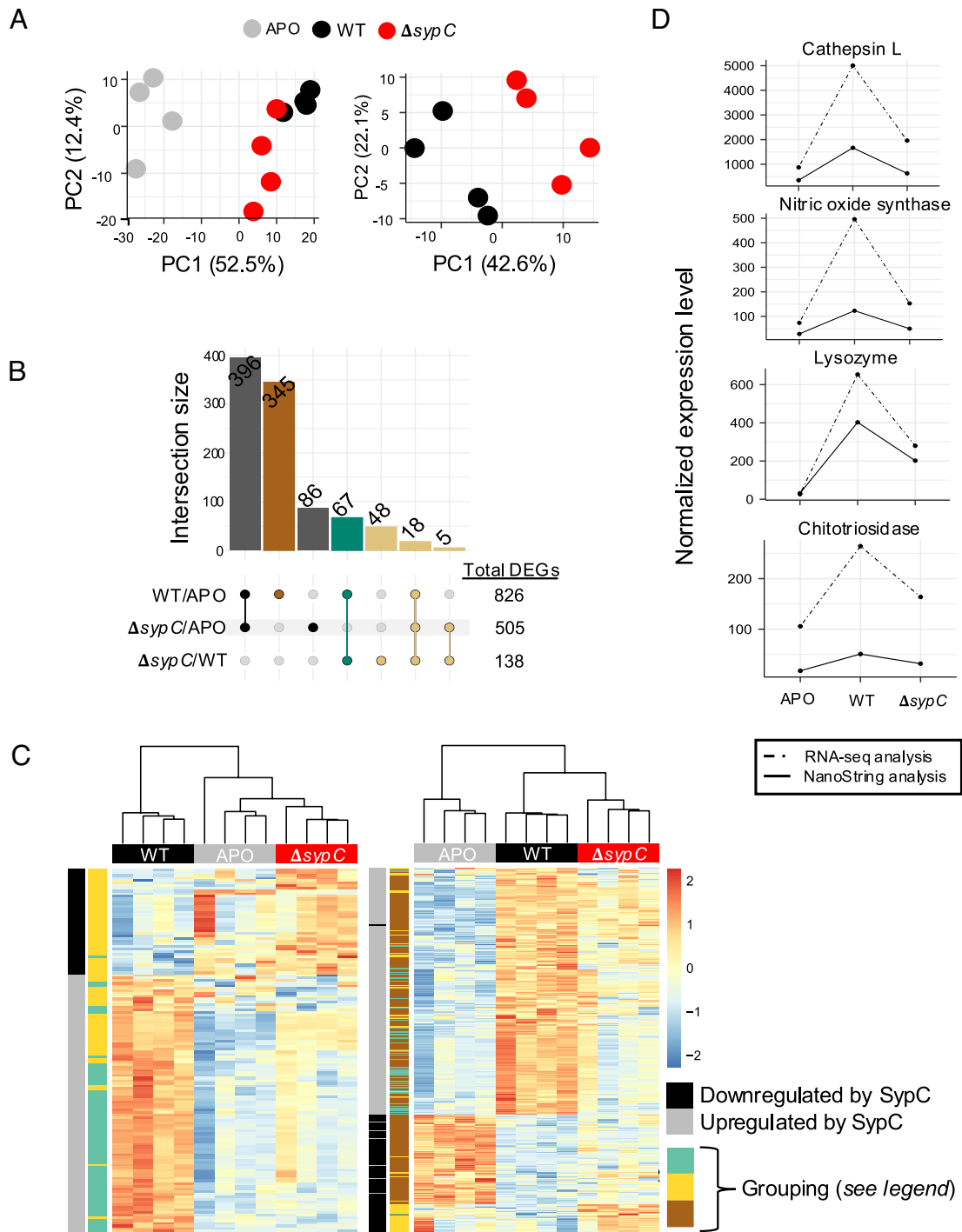


Fig. 2. Relative impact of the $\Delta sypC$ mutant on light-organ gene expression. (A) Principal component analysis (PCA) of host transcriptomes. *Left*: three conditions are shown: uncolonized (APO, gray), colonized with wild-type bacteria (WT, black), or colonized with the $\Delta sypC$ mutant ($\Delta sypC$, red). *Right*: Same data as *Left*, but with APO excluded to better highlight the separation between WT and $\Delta sypC$. Percent variance explained by each principal component is indicated on the axes. (B) UpSet plot of differentially expressed genes (DEGs). Comparisons include WT vs. APO and $\Delta sypC$ vs. APO ($\log_2 FC \geq |0.58|$, adjusted $P < 0.05$), and $\Delta sypC$ vs. WT ($\log_2 FC \geq |0.58|$, adjusted $P < 0.05$). (C) Expression patterns of selected DEG categories. *Left*: Green and yellow genes highlight that $\Delta sypC$ colonized animals resemble APO more closely for this subset. *Right*: Green, yellow, and brown gene groupings illustrate that $\Delta sypC$ represents an intermediate expression state between APO and WT. *Color coding*: Black: Downregulated by SypC ($\Delta sypC > WT$). Gray: Upregulated by SypC ($\Delta sypC < WT$). Green: DE in both WT vs. APO and $\Delta sypC$ vs. WT. Yellow: DE only in $\Delta sypC$ vs. WT. Brown: DE only in WT vs. APO. (D) Expression validation of selected genes comparing RNA-seq and NanoString analyses.

WT/APO, but not between either $\Delta sypC$ /APO or $\Delta sypC$ /WT (brown, Fig. 2B). Such a pattern suggests that *sypC* contributed to achieving full expression of these genes.

Examining all genes of interest (Fig. 2; brown, yellow, and green), GO-term enrichment analysis highlights the possibility that symbiont carriage of *sypC* suppresses pathways related to cytoskeletal organization and cellular motility, and upregulates

host responses involved in metabolism, immune modulation, cell adhesion, and extracellular remodeling (SI Appendix, Fig. S2), including production of a matrix metalloprotease by the ciliated epithelium, which is increased 24 h after colonization and is involved in light-organ morphogenesis (29). Twenty-five other upregulated host genes of interest were associated with cell death and proteolysis (SI Appendix, Fig. S3 A and B), supporting the

idea that the presence of the SypC protein promotes host-cell death in the ciliated fields (Fig. 3A).

To validate the RNA-seq results, NanoString analysis was performed on selected targets (Fig. 2D and SI Appendix, Fig. S3C). Previously studied symbiosis-associated genes such as cathepsin L, nitric oxide synthase, lysozyme, and chitotriosidase (reviewed

in ref. 18) were upregulated by symbionts encoding SypC, consistent with both cell-death enhancement and immune-response modulation. Most of these crucial developmental genes were downregulated in a colonization by a $\Delta sypC$ mutant compared to WT, further highlighting the importance of SypC during tissue morphogenesis.

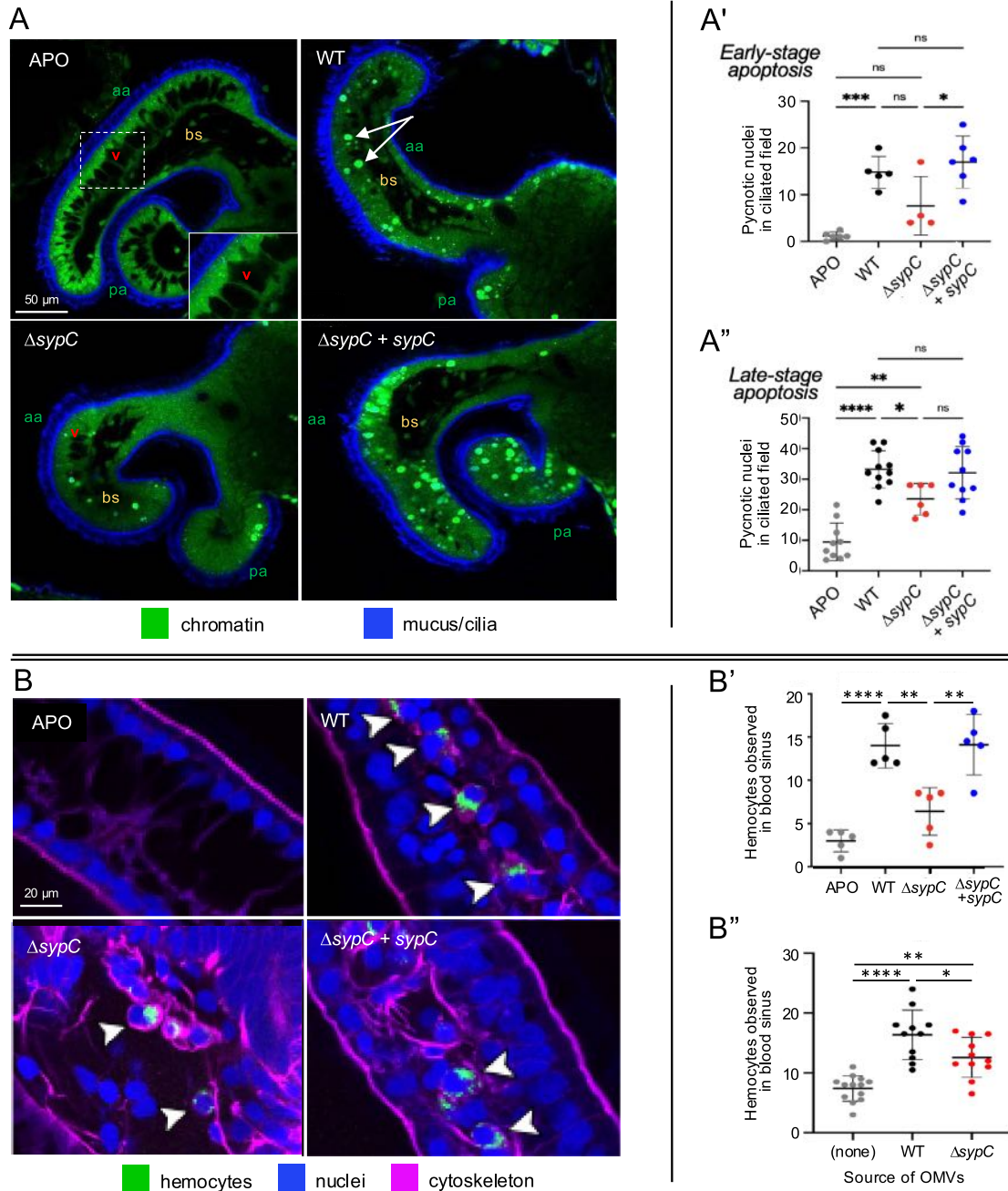


Fig. 3. Colonization by the *V. fischeri* $\Delta sypC$ mutant attenuates normal cellular phenotypes of the host. (A) Representative images of the superficial ciliated fields of light organs that were APO or colonized with wild-type *V. fischeri* (WT), $\Delta sypC$, or the complemented $\Delta sypC + sypC$ strains. At 24 h postcolonization, levels of symbiosis-induced early-stage apoptosis were determined in the ciliated field with each condition, as indicated by pycnotic nuclei (condensed chromatin, e.g., white arrows) in these regions. Fluorochromes used: cell death was indicated by acridine-orange stained condensed chromatin (green); mucus was stained with wheat-germ agglutinin (blue). aa, anterior appendage; bs, blood sinus; pa, posterior appendage; v, vacuoles within the lateral epithelial cells of the ciliated field. (A') Quantification of nuclei indicating early-stage cell death. (A'') Quantification of late-stage apoptosis was determined using the fluorochrome TUNEL (terminal deoxynucleotidyl transferase dUTP nicked-end labeling). (B) Representative images of hemocytes (blood cells, white arrowheads) trafficking into the blood sinus of the anterior appendage under the four colonization conditions. Fluorochromes used: hemocytes were identified by DNase-1 staining (green); F-actin cytoskeleton was stained with phalloidin (magenta); nuclei, TO-PRO-3 (blue). (B') The number of hemocytes present in the blood sinus of the anterior appendage was quantified at 24 h after colonization by different *V. fischeri* strains. (B'') Hemocyte trafficking was also determined in animals exposed only to OMVs that had been isolated from either WT or $\Delta sypC$ strains. For all conditions in A' and A'', and B' and B'' each data point represents a biological replicate. Each experiment was repeated 3 times with the same outcome. Results are presented as mean \pm 1 SD. Comparisons between multiple groups were analyzed using one-way ANOVA with Tukey test. ns: not significant, $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

Colonization by the Δ sypC Mutant Affects Certain Features of Host Development. In response to colonization by the Δ sypC mutant, several typical wild-type-induced developmental phenotypes were significantly reduced (Fig. 3). Both early-stage (characterized by condensed chromatin; Fig. 3 A and A') and late-stage (characterized by fragmented chromatin; Fig. 3A'') cell death were often statistically different; however, due to natural genetic variation of juveniles during development, some clutches only showed significant differences during late-stage cell death. Similarly, the number of hemocytes migrating into the blood sinuses of the anterior appendages of the ciliated fields was consistently statistically reduced in Δ sypC-colonized animals (Fig. 3 B and B'). Treatment of juveniles with *V. fischeri* OMVs was sufficient to induce the migration of hemocytes to the blood sinus (23), even in the absence of the bacteria themselves (Fig. 3B''), indicating that OMVs alone were adequate to trigger this key developmental event. Further, the reduced level of hemocyte migration that resulted from a Δ sypC colonization (Fig. 3B') was phenocopied when animals were exposed to OMVs produced by the mutant strain relative to wild-type OMVs (Fig. 3B'').

SypK, another protein encoded by the *syp* locus and essential for biofilm formation (28), contains the conserved RfbX flippase domain found in Wzx (30). It likely transports subunits of the Syp polysaccharide from the cytoplasm into the periplasm where they are assembled. SypK is predicted to be associated with the cytoplasmic membrane and thus, unlike the outer membrane SypC, was not detected in OMVs (5). In contrast to the Δ sypC mutant, colonization by the Δ sypK mutant did not significantly affect the level of hemocyte migration (SI Appendix, Fig. S4). These results indicate that it is the absence of the SypC protein itself, not a disruption of exopolysaccharide production, that is responsible for the inability of Δ sypC to induce normal hemocyte migration.

Certain other morphological events in the developmental program are also absent or delayed in Δ sypC colonized animals, such as the loss of the ciliated ridge on the light organ's surface (31) (SI Appendix, Fig. S5A) at 24 hpi. However, squids colonized by Δ sypC did retain several other normal developmental phenotypes, such as the typical regression of the superficial ciliated fields, which did not differ significantly between WT- and Δ sypC-colonized animals at 72 hpi (SI Appendix, Fig. S5 B–D). In addition, a phenotype occurring more deeply in the symbiont's migration path, i.e., the symbiont-induced closure of the migration path "bottleneck" (32, 33), was similar in animals colonized by either the WT or Δ sypC strain (SI Appendix, Fig. S5E).

Given that the peptidoglycan monomer TCT (tracheal cytoxin) induces hemocyte migration into the blood sinuses of the appendages (26), we hypothesized that the Δ sypC would be deficient in TCT production. To test this notion, we measured the amount of TCT produced by WT and Δ sypC cells in culture and determined that the mutant produced roughly the same level of TCT as the WT strain (See SI Appendix, Fig. S6 A and B). This result suggests that the developmental defect caused by the Δ sypC symbiont is not in the production of TCT but instead in the ability of this TCT to induce hemocyte migration normally. Interestingly, the experimental addition of high levels of TCT directly to seawater can itself induce hemocyte migration in both uncolonized and Δ sypC-colonized animals (SI Appendix, Fig. S6C), indicating that such a pharmacological treatment with TCT alone can be sufficient to overcome the typical requirement that SypC be present to enable induction of hemocyte migration.

Symbiont SypC Protein Is Found within Host Hemocytes. Because the SypC protein itself is required for normal host responses to either colonization by symbionts or treatment with OMVs, we

investigated whether this protein's effect was due to accumulation within host cells. Prior studies have shown that components of symbiont-produced OMVs can enter host cells, including LPS, which induces early-stage cell death (24), and small RNAs (17). These findings suggest a mechanism by which OMV-associated SypC (5) could also be delivered into host cells. To determine whether SypC is, in fact, translocated within the light organ, we introduced into wild-type *V. fischeri* a gene encoding an mCherry-SypC fusion protein that was constitutively expressed from its plasmid (SI Appendix, Table S1). Thus, this SypC fusion protein was well expressed, functional, and could easily be detected in bacterial cell extracts using a fluorescent antibody made against mCherry (SI Appendix, Fig. S7). When juvenile animals were examined at 24 hpi, the SypC fusion protein was observed within the colonized light organ by immunocytochemistry using an anti-mCherry antibody (Fig. 4). As expected, the symbionts themselves were strongly labeled. The SypC fusion protein was also observed in the cytoplasm of the epithelial cells lining the crypts (Fig. 4A); however, when colonized by wild-type symbionts carrying mCherry alone, no antibody reaction was observed in hemocytes (SI Appendix, Fig. S8), indicating that these cells internalize OMVs, but not entire *V. fischeri* symbionts. Further, the SypC-fusion labeling was in proximity to the nucleus, but excluded from the nuclear region (Fig. 4 B and C). Examination of tissues distant from the crypts revealed labeled SypC (Fig. 4 D–G') appearing even as remotely as the blood sinus of the external ciliated appendages (Fig. 5A). In colonized animals, hemocytes migrate to the appendages prior to the onset of cell death (34); thus, we asked whether SypC might be associated with these tissue-targeting cells and, if so, whether the presence of SypC might be connected to cell-death-driven morphogenesis.

Colocalization of the SypC label with a hemocyte-specific marker confirmed that this symbiont protein was internalized and carried into the sinuses of the appendages by these host cells (Fig. 4 D and E–E'). We next hypothesized that the SypC protein entered the phagocytic hemocytes through the uptake of symbiont OMVs they encountered while traversing the crypts (18). We tested this by asking whether OmpU, another symbiont protein present in OMVs (5), also colocalized with the hemocytes in the appendages. As predicted, an antibody to an mCherry-OmpU fusion protein labeled these host cells (Fig. 4 F and G–G'), indicating that the uptake of SypC was not specific to that protein, and likely occurred during the phagocytosis of OMVs.

The Progression of SypC Transported to the Blood Sinuses by Migrating Hemocytes Is Associated with the Induction of a Proteolytic Gene.

The path of SypC-containing hemocytes was tracked from the light-organ crypts to the blood sinuses of the superficial ciliated appendages. At 9 hpi, SypC was first observed in the hemocytes near the crypt spaces, presumably after they had phagocytosed symbiont OMVs (Fig. 5 A–D). By 12 hpi, labeled hemocytes were most easily detected distally near the region of the pores (Fig. 5E). Finally, by 17 hpi, hemocytes containing SypC were primarily in the blood sinuses of the appendages (Fig. 5F). Hemocytes collected from symbiotic animals at 24 hpi had increased levels of the C8 subunit of the proteasome, a proteolytic complex that is induced in hemocytes of the wild-type colonized light organ during cell death of the appendages (35). In contrast, no such induction was seen in hemocytes collected from Δ sypC colonized squid (Fig. 5G).

Discussion

SypC is a putative outer membrane transport protein involved in Syp-polysaccharide secretion and biofilm formation, activities that are critical for the initiation of symbiosis by planktonic *V. fischeri*

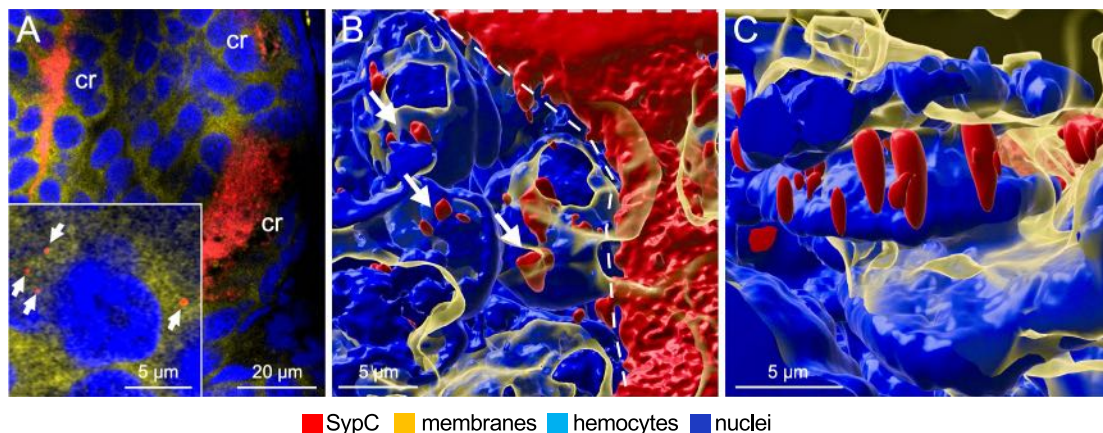


Fig. 4. Components and activities of migrating hemocytes. Localization of SypC within and near the symbionts at 24 hpi. (A) Abundance of antibody labeling of *V. fischeri* SypC in the light-organ crypt spaces (cr). *Insert*, mCherry-SypC fusion protein detected by fluorescent mCherry antibody (white arrows) within the cytoplasm of host cells lining the crypts. (B) Imaris rendering of higher-magnification confocal images showing SypC (white arrows) within host cells in regions near nuclei. The dashed line indicates the boundary between the host cells (*Left*) and the symbiont-containing crypts. CellMask Orange-stained membranes in and surrounding the cytoplasm. (C) Imaris rendering demonstrating that SypC is extranuclear. (D) Labeling of the *V. fischeri* SypC-fusion protein within the blood sinus (bs) of an anterior appendage (aa) of the superficial ciliated field of the organ; low magnification image showing the general location of higher magnification area. (E–E'') Higher magnification images showing the association of SypC with hemocytes in the blood sinus. DAPI-stained nuclei, (blue); fluorescent antibody to mCherry portion of mCherry-SypC fusion protein (red); CellMask Orange-stained hemocytes (turquoise). (F) Labeling of the *V. fischeri* OmpU protein within the blood sinus; low magnification image as in D. (G–G'') Higher magnification images showing the association of OmpU with hemocytes in the blood sinus. DAPI-stained nuclei, (blue); fluorescent antibody to OmpU (yellow); CellMask Orange-stained hemocytes (turquoise). Images of experimental controls can be found in *SI Appendix, Fig. S8*.

cells (for review, see ref. 20), as well as for their colonization of the light organ (36). Recent studies have shown that, of the 18 proteins encoded by the well-studied *syd* locus, SypC is the only one that is present in *V. fischeri* OMVs (5), likely due to its being embedded within the outer membrane. Here, we present data on host phenotypes that rely on the presence of SypC for normal symbiosis onset. Expression of hundreds of juvenile-host genes is dependent on colonization by symbionts that encode SypC (Fig. 2). Further, in the early hours of the host–symbiont partnership, SypC-bearing OMVs are taken up by phagocytic blood cells, or hemocytes, of the juvenile host and trafficked through its tissues to the outer surface of the light organ. The SypC delivered to the surface is essential for inducing, either directly or indirectly, normal development of the symbiosis.

Prior to the work described here, the presence of symbiont OMVs in the bloodstream of the squid host (17) had suggested that bacterial proteins like SypC, present in phagocytosed OMVs, might impact not only the light organ, but other tissues as well (37). Our findings add to the growing recognition that OMV trafficking through the body of a host is an evolutionarily conserved mechanism of host–symbiont communication (38, 39). Such long-range trafficking of OMVs has been documented in a

variety of eukaryotic hosts spanning from plants, choanoflagellates, and sponges to squids and vertebrates (5, 6, 40, 41). In mammals, not only do OMVs of the gut microbiome have either beneficial or pathogenic influences locally on the gastrointestinal epithelium, they also cross into the bloodstream where they are transported to distant tissues with wide-ranging effects (for reviews, see refs. (42, 43)). Recent studies of pregnant women have provided evidence that this phenomenon even occurs with the delivery of OMVs from the mother’s microbiota to the developing fetus (44). Once the OMVs are delivered, they can have an array of effects (38). For example, notable here is the role of the *V. fischeri* OMVs in inducing normal cell death in the light organ’s superficial ciliated epithelium (45); such OMV-induced cell death has also been reported in a diversity of other host animals, from sponges to mammals (40, 46).

The activity of SypC in bacterial-induced animal development is only one of a variety of bacterial and host features that work in concert to promote a normal and productive symbiosis. SypC is unusual in that its role in inducing host development is so distinct from its role in biofilm secretion. Proteins that have taken on a divergent role have been termed as “dual-function” (see, e.g., ref. 47). These proteins are not uncommon within a bacterial species, or between bacterial species,

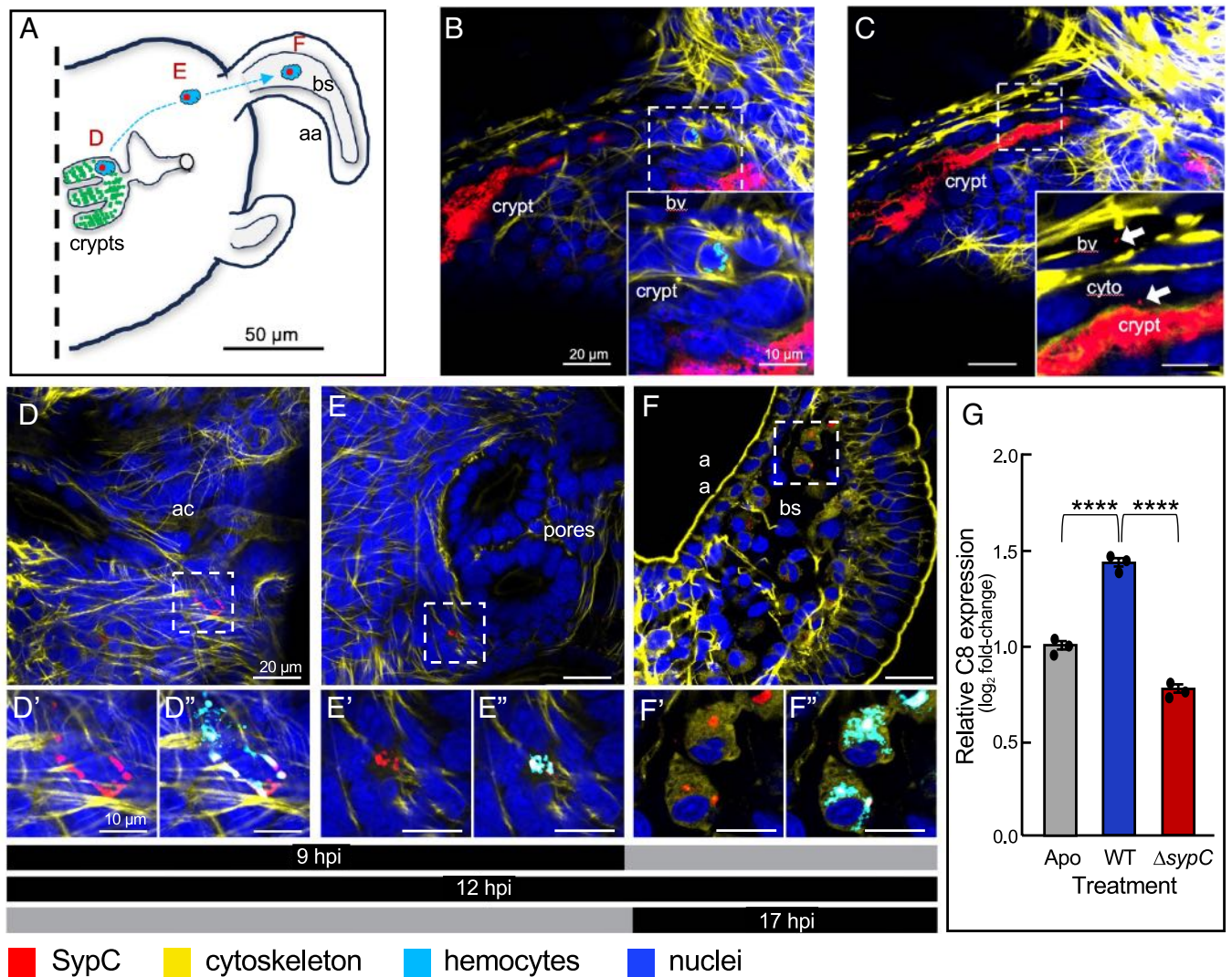


Fig. 5. Migration of SypC-bearing hemocytes to the blood sinus. (A) Tracking of host hemocytes during the first 17 h postinfection (hpi) as they migrate from the crypts to the blood sinus (bs) of the anterior appendage (aa). The presence of SypC-bearing hemocytes was indicated by the colocalization of a mCherry-SypC fusion protein (red) detected by a fluorescent mCherry antibody, with the CellMask Orange-stained hemocytes (turquoise). The hemocytes were observed near the medial crypt tissues at 9 hpi (B–D), progressed through the lateral tissues of the light organ by 12 hpi (E), and became situated in the blood sinus of the anterior appendage as soon as 17 hpi (F). The areas indicated by the dashed white boxes are enlarged in the insets and lower images. SypC could also occasionally be seen free in the blood vessels (bv) and in the cytoplasm of the crypt epithelium (white arrows) (C). The black timeline indicates at which image locations (D, E, and/or F) the SypC-carrying hemocytes could be observed when viewed at 9, 12, or 17 hpi. Nuclei were stained with DAPI (blue) and cytoskeleton was labeled with rhodamine phalloidin (yellow). ac, antechamber; cyto, host cytoplasm. (G) qRT-PCR determination of the expression level of the C8 subunit of the proteasome. Relative expression of the C8 gene in hemocytes in uncolonized juveniles (Apo) or 24 h postcolonization by either wild-type (WT) or Δ sypC symbionts. Differences are indicated by a one-way ANOVA with Tukey's multiple comparison test ($n = 3$). Data are presented as the mean \pm 1 SEM. **** $p < 0.0001$.

but it is rare to have trans-Domain functions. While this study of the Syp protein may be the first to describe a bacterial-animal dual-function protein, such proteins have previously been reported between plants and their rhizobial symbionts (48, 49).

Studies of the OMVs have demonstrated that they contain cargo other than SypC that impacts light-organ morphogenesis, such as another outer membrane protein, OmpU (5). In addition, nonprotein cargo of the OMVs influences organ development, including i) MAMPs such as LPS (24) and peptidoglycan derivatives (23, 26) that directly induce the developmental program; and, ii) the small RNA, SsrA (also called tmRNA), which influences host development and symbiont persistence by modulating the host's immune system (17). Several other non-OMV elements are critical for morphogenesis including the symbiont's production of light (22) and modulation of crypt pH (5). The role of the host in this dialogue is similarly complex, coordinating hemocyte migration from the light-organ crypts where

OMVs are engulfed, to the blood sinuses of the superficial epithelium where their contents are delivered (Fig. 5). The host responds to this delivery by upregulating expression of genes, such as those encoding the proteasome in the hemocytes as well as metalloproteinases at the base of the developing epithelium. These events enable the normal cell-death process that underlies the morphogenetic program (for review, see ref. 18). Overall, the level of complexity characterizing host-symbiont codevelopment is reminiscent of that evident during embryogenesis: the migration of cells and diffusion of morphogens from one tissue to another, delivering signals and triggering developmental programs. In both progressions, the specification of cell fates is required for a normal, healthy outcome, demanding coordination of several factors both across time and space, and in balanced proportions. The principal difference is that, with a few exceptions (50, 51), embryonic development typically requires coordination only within a single genome; as described here, in a beneficial symbiosis the two

evolutionarily distinct genomes of the bacterium and its host must communicate.

A common feature of animal associations with *Vibrio* species, whether pathogenic or beneficial, may be the functional diversification of SypC, reflected in both the protein's structure and its role in symbiosis. A simulation of SypC using AlphaFold2 (52) suggests the protein may have evolved from another outer membrane transporter, Wza (53), with an increase in domain type (54) and number (SI Appendix, Fig. S1). The UniProt database (25) currently lists 14 SypC homologs in conspecifics or congeners of *V. fischeri*. These proteins all have subunits consisting of ~703 amino acids, have similar secondary/tertiary structures, and most have been reported as proteins of mutualistic or pathogenic bacteria of aquatic animals. At the 50+% level of amino acid identity, dozens of proteins are listed, ~2/3 of which are in the Vibrionaceae and have a similar structure, with 700 (\pm 5) amino acids. For example, a SypC homolog has been found in the hydrothermal-vent bacterium, *Vibrio diabolicus* (55). In addition, and relevant here, a *sypC* gene encoding a protein of 699 aa, with 68% sequence identity to *V. fischeri* SypC has been reported as cargo in the OMVs of *Vibrio tasmaniensis*, a pathogen of oysters (56). These phylogenetic patterns of SypC-like proteins suggest a widespread, essential function in host–symbiont interactions of the Vibrionaceae.

The results presented here provide another piece of the puzzle of how a host and its species-specific bacterial symbiont communicate at each new interaction during their 5 My-old partnership. In particular, they address the challenge of how symbiont signals produced deep in the light-organ crypts are transmitted to, and drive host development in, the surface epithelium. The data provide evidence that the transmission is not indirect, i.e., broadcast from the crypt spaces where the symbionts reside by signal transduction cascades passing through the tissues to the surface. Instead, signal delivery appears to be direct, i.e., by the hemocyte-trafficking of OMVs and their cargo to distant tissues that undergo morphogenesis. Many more questions concerning the coordination of this process remain to be addressed. For example: What are the molecular interactions of the SypC protein with the host macrophages that trigger host morphogenesis? Are these interactions direct, or are they indirect, perhaps by affecting the composition or activity of other outer membrane proteins in the OMVs? Do the mechanisms underlying the described behaviors involve the host's immune system or other symbiont MAMPs? Finally, is the structure of the SypC molecule, which appears to be unique to *Vibrio* spp., critical for interactions with the host?

Materials and Methods

Animal Collection and Care. All animal protocols and experiments were approved by the California Institute of Technology Institutional Animal Care and Use Committee. Adult *Euprymna scolopes* Hawaiian bobtail squid were collected from Maunaloa Bay, Oahu, HI, and either maintained in flow-through seawater tanks at Kewalo Marine Laboratory (Honolulu, HI) or transferred to recirculating tanks at Caltech (Pasadena, CA) and maintained as previously described (57). Juveniles bred from wild-caught adults were used for all experiments. After embryonic day 10, egg clutches were moved to a natural 12:12-hour light–dark cycle. Upon hatching, juvenile squid were collected, washed, and placed in i) 0.2 μ m-pore size filter-sterilized artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, CA) (FSIO = filter-sterilized Instant Ocean), ii) natural ocean seawater collected from the pier pump of Scripps Institution of Oceanography, La Jolla, CA (SIO seawater), or iii) filter-sterilized ocean seawater (FSOW), as indicated in the figure legends of a given experiment.

Bacterial Strains and Host Colonization Assay. *V. fischeri* strains and plasmids used in this study are listed in SI Appendix, Table S1. The ability of different strains to colonize newly hatched juvenile *E. scolopes* was determined as described elsewhere (58, 59) (SI Appendix, Supplemental Methods).

Imaging of Cell Death in the Appendages. Juvenile squid were collected at 24 h postinoculation, placed in 0.0001% acridine orange (Thermo Fisher Scientific, Irwindale, CA) in F50W for 1 min, and washed with F50W. Animals were anesthetized in 2% ethanol and their mantles and funnels were removed to expose the light organ. Light organ appendages were visualized to determine the number of acridine orange positive (early-stage cell death) nuclei using fluorescence confocal microscopy (60) (See SI Appendix, Table S2 for details). Late-stage cell death, characterized by DNA fragmentation, was detected by TUNEL staining, according to the manufacturer's instructions (Promega, Madison, WI).

Construction of Protein Fusions in *V. fischeri*. Because an antibody raised to the SypC protein itself was not easily detected by ICC, N-terminal mCherry translational fusions were constructed by amplification of *mCherry* from pSMV3-*mCherry-zapA* (61) and either *sypC* or *ompU* from the *V. fischeri* ES114 genome by standard Gibson assembly into pVSV105 (62). All fusions include an internal linker sequence (5'-CTCGAGGGATCCGGA-3') between *mCherry* and the gene of interest (See SI Appendix, Table S1). pCMC37 was cloned with the native signal peptide sequence of *ompU* on the 5' end of the fusion sequence. Transfer of plasmids into *V. fischeri* strains was performed by standard biparental conjugation protocol (63) using the *E. coli* donor strain MFDpir (64).

Immunocytochemistry of SypC and OmpU. Juvenile squid were colonized within 3 h of hatching, at 1 h postdusk with 10,000 CFU of bacteria per ml (either WT + mCherry, WT + mCherry-SypC conjugate, or WT + mCherry-OmpU conjugate). Colonization was confirmed via luminometer prior to fixation. Squid were fixed at 4 and 10 h postdawn (15 and 21 h postcolonization) in 4% PFA in marine PBS (mPBS) for 12 h at 4 °C with agitation, then rinsed four times for 15 min in mPBS. Light organs were dissected out of the squid and permeabilized for two days in mPBS/1% Triton X-100 (mPBST) at 4 °C with agitation. Blocking solution (0.5% BSA, 1% goat serum in mPBST) was added and samples were incubated in the dark overnight at 4 °C with agitation. Either an anti-mCherry monoclonal antibody (Thermo Fisher Scientific) or the corresponding rat IgG isotype control (Thermo Fisher Scientific) was added at a concentration of 1:100 in blocking solution and incubated in the dark for 14 d at 4 °C with agitation; fresh antibody was replaced at 7 d. Samples were rinsed 4 \times 1 h with mPBST and goat anti-rat IgG secondary antibody (Alexa Fluor 647 conjugated, Thermo Fisher Scientific) was added at 1:1000 concentration in blocking solution overnight. Samples were rinsed 5 \times 5 min in mPBST, and 1 \times 5 min in 5 \times SSC, then counterstained with 1:1,000 CellMask Orange Plasma Membrane Stain, and 1:50 DAPI (Thermo Fisher Scientific), in mPBST for two days at 4 °C with agitation.

Light organs were mounted in Vectashield (Fisher Scientific, Hampton, NH) on slides for confocal microscopy imaging. Lasers were set to the negative control to eliminate background autofluorescence and identical imaging parameters were used across all samples. Specific confocal microscope and imaging acquisition parameters are listed in SI Appendix, Table S2. Three-dimensional reconstructions of 0.3 μ m thick Z-stacks were generated using Imaris 10.1 (Bitplane, Zurich, Switzerland).

Electron Tomography. Samples of the light organ were prepared for electron microscopy. For sample preparation and details of the electron tomography and scanning electron microscopy methods, see SI Appendix, Table S2 and Supplemental Methods.

TCT Production by *V. fischeri*. Analyses of the production of this MAMP by cultures of *V. fischeri* have been reported elsewhere and can be found in SI Appendix, Supplemental Methods.

OMV Isolation. OMVs were isolated from *V. fischeri* cultures after 24 h of growth in LBS at 28 °C as previously described (5) (see SI Appendix, Supplemental Methods for details).

RNA Extraction for RNAseq and NanoString Experiments. Juvenile squid were hatched into *V. fischeri*-free natural ocean water. Animals hatched on the same day and from the same clutch (i.e., with a related genetic background) were separated into 3 treatments groups: APO (no exposure to *V. fischeri*), WT (exposed to wild-type *V. fischeri* strain ES114 at ~5,000 CFU/mL), and Δ *sypC* (exposed to a *V. fischeri* Δ *sypC* derivative at ~5,000 CFU/mL); n = 20 animals per sample, with 4 biological replicates. After 24 h of exposure, total light-organ RNA was isolated using an RNeasy Mini Kit (Qiagen, Germantown, MD) and Qiagen QIAshredder according to the manufacturer's instructions. Samples were treated with TURBO

DNase (Thermo Fisher Scientific) to remove DNA contamination. RNA quality and concentration were determined by spectrophotometry using a NanoPhotometer (Implen, Westlake City, CA), and the concentration was confirmed using Qubit RNA BR assay kit (Thermo Fisher Scientific). For RNAseq analysis, the Illumina TruSeq Stranded mRNA Sample Prep with polyA selection v4.0 protocol was used for library preparation. Sequencing of light-organ tissue samples was performed at the NYU Genome Center on an Illumina HiSeq 4000 using a paired-end, 100-nucleotide-length run mode that produced ~35 to 85 million reads/sample.

For NanoString codeset analysis, RNA was prepared from APO, WT, and *ΔsypC* animals as described above. Raw nCounter data were passed into Rs NanoStringDiff (v1), which computed \log_2FC and q value to identify differentially expressed genes (DEGs); two host genes, serine hydroxymethyltransferase and REL_NF- κ B, were chosen for normalization. Clutch number (as a proxy for genetic background) was included as a covariate in the model. nSolver (v4.0) was used to normalize raw NanoString data for visualization.

Determination of Differential Gene Expression. Raw PE reads were quality- and adapter-trimmed using fastp (v0.24.0) with default parameters (65). Cleaned reads where then pseudoaligned, to the most recently published *E. scolopes* reference genome and annotations (v2.2) (66), using kb-python with bulk RNA-seq and reverse stranded tags (kb-python: v0.28.2, kallisto v0.50.0, bustools: v0.43.2) (67). On average, 64.4% of reads successfully pseudoaligned. Gene-level counts were then passed into R's DESeq2 (v1.44.0) for differential expression analysis (68). Genetic background, or clutch, was included as a covariate in the model. DEG were identified for each pairwise comparison, due to high within sample variation, with P -value adjusted globally (DE: global padj < 0.05, $\log_2FC \pm 0.5$). Plots were made with ggplot2 (v3.5.1), ComplexUpsetR (v1.3.3), and variancePartition (v1.34.0).

- V. Tiku, M. W. Tan, Host immunity and cellular responses to bacterial outer membrane vesicles. *Trends Immunol.* **42**, 1024–1036 (2021).
- M. Toyofuku, S. Schild, M. Kaparakis-Liaskos, L. Eberl, Composition and functions of bacterial membrane vesicles. *Nat. Rev. Microbiol.* **21**, 415–430 (2023).
- L. Turnbull *et al.*, Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **7**, 11220 (2016).
- L. Zavan *et al.*, The mechanism of *Pseudomonas aeruginosa* outer membrane vesicle biogenesis determines their protein composition. *Proteomics* **23**, e2200464 (2023).
- J. B. Lynch *et al.*, Ambient pH alters the protein content of outer membrane vesicles, driving host development in a beneficial symbiosis. *J. Bacteriol.* **201**, e00319-19 (2019).
- M. G. Sartorio, E. J. Pardue, N. E. Scott, M. F. Feldman, Human gut bacteria tailor extracellular vesicle cargo for the breakdown of diet- and host-derived glycans. *Proc. Natl. Acad. Sci. U.S.A.* **120**, e2306314120 (2023).
- S. K. Cohen *et al.*, Tracking the cargo of extracellular symbionts into host tissues with correlated electron microscopy and nanoscale secondary ion mass spectrometry imaging. *Cell Microbiol.* **22**, e13177 (2020).
- D. N. Villagelii, D. R. Samuelson, The role of bacterial membrane vesicles in human health and disease. *Front. Microbiol.* **13**, 828704 (2022).
- F. S. Abolhasani *et al.*, Outer membrane vesicles in gram-negative bacteria and its correlation with pathogenesis. *Front. Immunol.* **16**, 1541636 (2025).
- J. Li *et al.*, Outer membrane vesicles produced by coral-associated *Vibrio coralliilyticus* inhibit bacteriophage infection and its ecological implications. *Microbiol. Res.* **281**, 127607 (2024).
- H. Gao *et al.*, Outer membrane vesicles from a mosquito commensal mediate targeted killing of *Plasmodium* parasites via the phosphatidylcholine scavenging pathway. *Nat. Commun.* **14**, 5157 (2023).
- J. B. Lynch, R. A. Alegado, Spheres of hope, packets of doom: The good and bad of outer membrane vesicles in interspecies and ecological dynamics. *J. Bacteriol.* **199**, e00012-17 (2017).
- G. Wu *et al.*, Outer membrane vesicles induce the mussel plantigrade settlement via regulation of c-di-GMP. *Biofouling* **39**, 359–370 (2023).
- Y. Shen *et al.*, Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. *Cell Host Microbe* **12**, 509–520 (2012).
- A. Kaisanlahti *et al.*, Bacterial extracellular vesicles – brain invaders? A systematic review. *Front. Mol. Neurosci.* **16**, 1227655 (2023).
- M. S. Aschtgen *et al.*, Rotation of *Vibrio fischeri* flagella produces outer membrane vesicles that induce host development. *J. Bacteriol.* **198**, 2156–2165 (2016).
- S. Moriano-Gutierrez *et al.*, The noncoding small RNA SsrA is released by *Vibrio fischeri* and modulates critical host responses. *PLoS Biol.* **18**, e3000934 (2020).
- S. V. Nyholm, M. J. McFall-Ngai, A lasting symbiosis: How the Hawaiian bobtail squid finds and keeps its bioluminescent bacterial partner. *Nat. Rev. Microbiol.* **19**, 666–679 (2021).
- K. L. Visick, E. V. Stabb, E. G. Ruby, A lasting symbiosis: How *Vibrio fischeri* finds a squid partner and persists within its natural host. *Nat. Rev. Microbiol.* **19**, 654–665 (2021).
- B. L. Fung, J. J. Esin, K. L. Visick, *Vibrio fischeri*: A model for host-associated biofilm formation. *J. Bacteriol.* **206**, e0037023 (2024).
- T. Koropatnick, M. S. Goodson, E. A. Heath-Heckman, M. McFall-Ngai, Identifying the cellular mechanisms of symbiont-induced epithelial morphogenesis in the squid–association. *Biol. Bull.* **226**, 56–68 (2014).
- M. McFall-Ngai, E. A. Heath-Heckman, A. A. Gillette, S. M. Peyer, E. A. Harvie, The secret languages of coevolved symbioses: Insights from the *Euprymna scolopes-Vibrio fischeri* symbiosis. *Semin. Immunol.* **24**, 3–8 (2012).

Statistical Analyses. All statistical analyses were conducted using Prism 8 (Graphpad Software, La Jolla, CA). Statistical analyses between two groups were analyzed using unpaired Student's t test. Comparisons between multiple groups were analyzed using two-way ANOVA with Tukey test. Statistical variability is expressed as mean \pm 1 SD; ns: not significant, $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. N values for experiments are indicated in figure legends as appropriate.

Data, Materials, and Software Availability. The data supporting the findings of this study are available within the article and its *SI Appendix*. The transcriptomic raw sequencing data generated in this study have been deposited in Gene Expression Omnibus under accession numbers GSE307160 (69) and GPL36208 (70). Relevant code for this manuscript is available at https://github.com/verabeil/escolopes_vfischeri_sypc.git (71).

ACKNOWLEDGMENTS. We thank Zachary A. Yahiku and Hannah Osland for their technical help. Dr. William Goldman (University of North Carolina at Chapel Hill) provided purified TCT. Dr. Joram Piatigorsky provided valuable advice. This work was supported by funding from the NIH Grants R37-AI50661 to M.M.-N. and E.G.R. and R01-GM135254 to E.G.R. and M.M.-N.

Author affiliations: ^aPacific Biosciences Research Center, Kewalo Marine Laboratory, University of Hawaii at Manoa, Honolulu, HI 96813; ^bDivision of Biosphere Sciences and Engineering, Carnegie Science, Pasadena, CA 91125; ^cDivision of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125; ^dDepartment of Medical Microbiology and Immunology, University of Wisconsin-Madison, School of Medicine and Public Health, Madison, WI 53705; and ^eDepartment of Microbiology and Immunology, Loyola University Medical Center, Maywood, IL 60153

- M. S. Aschtgen, K. Wetzel, W. Goldman, M. McFall-Ngai, E. Ruby, *Vibrio fischeri*-derived outer membrane vesicles trigger host development. *Cell Microbiol.* **18**, 488–499 (2016).
- J. S. Foster, M. A. Apicella, M. J. McFall-Ngai, *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. *Dev. Biol.* **226**, 242–254 (2000).
- C. UniProt, UniProt: The universal protein knowledgebase in 2025. *Nucleic Acids Res.* **53**, D609–D617 (2025).
- T. A. Koropatnick *et al.*, Microbial factor-mediated development in a host-bacterial mutualism. *Science* **306**, 1186–1188 (2004).
- E. S. Yip, B. T. Grublesky, E. A. Husa, K. L. Visick, A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. *Mol. Microbiol.* **57**, 1485–1498 (2005).
- S. Shibata, E. S. Yip, K. P. Quirke, J. M. Ondrey, K. L. Visick, Roles of the structural symbiosis polysaccharide (*syp*) genes in host colonization, biofilm formation, and polysaccharide biosynthesis in *Vibrio fischeri*. *J. Bacteriol.* **194**, 6736–6747 (2012).
- T. A. Koropatnick, M. S. Goodson, E. A. Heath-Heckman, M. McFall-Ngai, Identifying the cellular mechanisms of symbiont-induced epithelial morphogenesis in the squid-vibrio association. *Biol. Bull.* **226**, 56–68 (2014).
- Y. Hong, M. A. Liu, P. R. Reeves, Progress in our understanding of Wzx flippase for translocation of bacterial membrane lipid-linked oligosaccharide. *J. Bacteriol.* **200**, e00154-17 (2018).
- J. A. Doi, M. J. McFall-Ngai, A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. *Biol. Bull.* **189**, 347–355 (1995).
- T. Essock-Burns, C. Bongrand, W. E. Goldman, E. G. Ruby, M. J. McFall-Ngai, Interactions of symbiotic partners drive the development of a complex biogeography in the squid-vibrio symbiosis. *mBio* **11**, e00853-20 (2020).
- L. K. Sycuro, E. G. Ruby, M. McFall-Ngai, Confocal microscopy of the light organ crypts in juvenile *Euprymna scolopes* reveals their morphological complexity and dynamic function in symbiosis. *J. Morphol.* **267**, 555–568 (2006).
- T. A. Koropatnick, J. R. Kimbell, M. J. McFall-Ngai, Responses of host hemocytes during the initiation of the squid-vibrio symbiosis. *Biol. Bull.* **212**, 29–39 (2007).
- J. R. Kimbell, T. A. Koropatnick, M. J. McFall-Ngai, Evidence for the participation of the proteasome in symbiont-induced tissue morphogenesis. *Biol. Bull.* **211**, 1–6 (2006).
- K. E. Zink *et al.*, A small molecule coordinates symbiotic behaviors in a host organ. *mBio* **12**, e03637-20 (2021).
- S. Moriano-Gutierrez *et al.*, Critical symbiont signals drive both local and systemic changes in diel and developmental host gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 7990–7999 (2019).
- J. D. Cecil, N. Sirisaengtaksin, N. M. O'Brien-Simpson, A. M. Krachler, Outer membrane vesicle-host cell interactions. *Microbiol. Spectr.* **7**, 7.1.06 (2019).
- J. M. Bomberger *et al.*, Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog.* **5**, e1000382 (2009).
- K. Wang, C. Jia, B. Zhang, J. Chen, J. Zhao, Outer membrane vesicles from commensal microbes contribute to the sponge *Tedania* sp. development by regulating the expression level of apoptosis-inducing factor (AIF). *Commun. Biol.* **7**, 952 (2024).
- A. Woznica *et al.*, Bacterial lipids activate, synergize, and inhibit a developmental switch in choanoflagellates. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 7894–7899 (2016).
- S. Chen, Q. Lei, X. Zou, D. Ma, The role and mechanisms of gram-negative bacterial outer membrane vesicles in inflammatory diseases. *Front. Immunol.* **14**, 1157813 (2023).
- R. Stentz, A. L. Carvalho, E. J. Jones, S. R. Carding, Fantastic voyage: The journey of intestinal microbiota-derived microvesicles through the body. *Biochem. Soc. Trans.* **46**, 1021–1027 (2018).

44. A. Kaisanlahti *et al.*, Maternal microbiota communicates with the fetus through microbiota-derived extracellular vesicles. *Microbiome* **11**, 249 (2023).
45. M. S. Aschtgen *et al.*, Insights into flagellar function and mechanism from the squid–*Vibrio* symbiosis. *NPJ Biofilms Microbiomes* **5**, 32 (2019).
46. S. Dhital, P. Deo, I. Stuart, T. Naderer, Bacterial outer membrane vesicles and host cell death signaling. *Trends Microbiol.* **29**, 1106–1116 (2021).
47. B. M. Coffey, S. S. Akhand, G. G. Anderson, MgtE is a dual-function protein in *Pseudomonas aeruginosa*. *Microbiology (Reading, Engl.)*. **160**, 1200–1213 (2014).
48. D. Liu, A. K. Bhunia, Anchorless bacterial moonlighting metabolic enzymes modulate the immune system and contribute to pathogenesis. *ACS Infect. Dis.* **10**, 2551–2566 (2024).
49. F. Sorroche *et al.*, The *ex planta* signal activity of a *Medicago* ribosomal uL2 protein suggests a moonlighting role in controlling secondary rhizobial infection. *PLoS One* **15**, e0235446 (2020).
50. K. E. Malter *et al.*, A bacterial membrane-disrupting protein stimulates animal metamorphosis. *mBio* **16**, e0357324 (2025).
51. B. Qi, M. Han, Microbial siderophore enterobactin promotes mitochondrial ion uptake and development of the host via interaction with ATP synthase. *Cell* **175**, 571–582.e511 (2018).
52. J. Jumper *et al.*, Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–589 (2021).
53. R. F. Collins, J. P. Derrick, Wza: A new structural paradigm for outer membrane secretory proteins?. *Trends Microbiol.* **15**, 96–100 (2007).
54. A. M. Burroughs, S. Balaji, L. M. Iyer, L. Aravind, A novel superfamily containing the beta-grasp fold involved in binding diverse soluble ligands. *Biol. Direct* **2**, 4 (2007).
55. D. Goudenege *et al.*, Genome sequence of *Vibrio diabolus* and identification of the exopolysaccharide HE800 biosynthesis locus. *Appl. Microbiol. Biotechnol.* **98**, 10165–10176 (2014).
56. A. S. Vanhove *et al.*, Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. *Environ. Microbiol.* **17**, 1152–1165 (2015).
57. A. C. Hargadon *et al.*, An acidic microenvironment produced by the V-type ATPase of *Euprymna scolopes* promotes specificity during *Vibrio fischeri* recruitment. *Commun. Biol.* **7**, 1642 (2024).
58. E. J. Koch, T. Miyashiro, M. J. McFall-Ngai, E. G. Ruby, Features governing symbiont persistence in the squid–vibrio association. *Mol. Ecol.* **23**, 1624–1634 (2014).
59. L. M. Naughton, M. J. Mandel, Colonization of *Euprymna scolopes* squid by *Vibrio fischeri*. *J. Vis. Exp.* **10**, e3758 (2012).
60. M. K. Montgomery, M. McFall-Ngai, Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development* **120**, 1719–1729 (1994).
61. L. Yang *et al.*, Bacterial growth dynamics in a rhythmic symbiosis. *Mol. Biol. Cell* **35**, ar79 (2024).
62. A. K. Dunn, D. S. Millikan, D. M. Adin, J. L. Bose, E. V. Stabb, New rfp- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression in situ. *Appl. Environ. Microbiol.* **72**, 802–810 (2006).
63. K. L. Visick, K. M. Hodge-Hanson, A. H. Tischler, A. K. Bennett, V. Mastrodomenico, Tools for rapid genetic engineering of *Vibrio fischeri*. *Appl. Environ. Microbiol.* **84**, e00850-18 (2018).
64. L. Ferrieres *et al.*, Silent mischief: Bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *J. Bacteriol.* **192**, 6418–6427 (2010).
65. S. Chen, Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *Imeta* **2**, e107 (2023).
66. T. F. Rogers *et al.*, Gene modelling and annotation for the Hawaiian bobtail squid. *Euprymna scolopes*. *Sci. Data* **11**, 40 (2024).
67. P. Melsted *et al.*, Modular, efficient and constant-memory single-cell RNA-seq preprocessing. *Nat. Biotechnol.* **39**, 813–818 (2021).
68. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
69. J. T. Kuwabara, V. Beilinson, Data from "SypC, a symbiont outer membrane vesicle protein, impacts the development of the squid-vibrio partnership". Gene Expression Omnibus. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE307160>. Deposited 3 September 2025.
70. J. T. Kuwabara, V. Beilinson, E. G. Ruby, Data from "SypC, a symbiont outer membrane vesicle protein, impacts the development of the squid-vibrio partnership". Gene Expression Omnibus. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL36208>. Deposited 3 September 2025.
71. V. Beilinson, Data from "SypC, a symbiont outer membrane vesicle protein, impacts the development of the squid-vibrio partnership". GitHub. https://github.com/verabeil/escolopes_vfischeri_sypc. Deposited 25 February 2026.

Supporting Information for

SypC, a Symbiont Outer-Membrane-Vesicle Protein, Impacts the Development and Persistence
of the Squid-Vibrio Partnership

Jill T. Kuwabara, Vera Beilinson, Alexis C. Hargadon, Grischa Y. Chen, Xiao-Meng Hu, Mark
S. Ladinsky, Kathleen T. Hackett, Joseph R. Dillard, Karen L. Visick, Edward G. Ruby,
Margaret McFall-Ngai

Margaret McFall-Ngai
Email: mcfallng@caltech.edu

This PDF file includes:

- Supporting text
- Figures S1 to S8
- Tables S1 to S2
- SI References

37 **Supporting Information Text**

38

39 **SUPPLEMENTAL METHODS**

40 **Bacterial strains and media**

41 *V. fischeri* strain ES114 was used as the wild-type strain, and as the parent strain for a number of
42 mutants (See SI Appendix, table S1). To prepare an inoculum for light-organ colonization
43 experiments (1), strains were cultured overnight in Luria-Bertani salt medium (LBS) (2). To
44 maintain plasmid constructs, chloramphenicol (Cm) was added to LBS at a concentration of 2.5
45 mg/ml. The strains were then subcultured (1:100) into seawater tryptone medium (SWT) (3) and
46 grown to mid-log phase at 28 °C with shaking. The SWT culture was diluted into seawater to a
47 final concentration of between 0.5 and 1 x 10⁴ CFU/ml to serve as an inoculum for the newly
48 hatched juvenile squid.

49 **Construction of protein fusions in *V. fischeri***

50 Briefly, N-terminal *mCherry* translational fusions were constructed by amplification of *mCherry*
51 from pSMV3-*mCherry-zapA* (4) and either the *sypC* or *ompU* gene from the *V. fischeri* ES114
52 genome by standard Gibson assembly into pVSV105 (5). All fusions include an internal linker
53 sequence (5'-CTCGAGGGATCCGGA-3') between *mCherry* and the gene of interest. pCMC37
54 (See SI Appendix, table S1) were cloned with the native signal peptide sequence of *ompU* and
55 *sypC*, respectively, on the 5' end of the fusion sequence. Transfer of plasmids into *V. fischeri*
56 strains was performed by a standard biparental conjugation protocol (6) using the *E. coli* donor
57 strain MFDpir (7).

58 **Host colonization assay**

59 Within 2 h of hatching, juvenile squid were colonized by overnight exposure to *V. fischeri* cells
60 or kept aposymbiotic (APO, *i.e.*, exposed only to the ~10⁶ non-specific bacteria per ml in the

61 bacterioplankton), or in filter-sterilized Instant Ocean (Instant Ocean, Spectrum Brands,
62 Blacksburg, CA) (FSIO). Colonization was monitored by checking luminescence of the animal
63 with a TD-20/20 luminometer (Turner Designs, San Jose, CA). The number of colony-forming
64 units (CFU) in the light organ of squid was determined by plating dilutions of homogenized
65 light-organ contents on LBS plates as previously described (4). Relative light units (RLU) were
66 determined by normalizing the luminescence reading by the number of CFU in each individual
67 light organ.

68 **TCT production by *V. fischeri***

69 To determine the amount of peptidoglycan monomer (tracheal cytotoxin; TCT) exported from
70 wild-type and $\Delta sypC$ mutant cells, peptidoglycan (PG) fragments were purified from (90 mL) of
71 *V. fischeri* supernatant. For binding the PG from the supernatant, 6-mL C18 SepPak cartridges
72 (Waters Corp., Milford, MA) were prepared by washing the cartridges with 6 mL methanol,
73 followed by two washes with 6 mL 0.1% trifluoroacetic acid (TFA). TFA was added to the
74 culture supernatant sample to a final concentration of 0.1%. The sample was loaded on the
75 cartridge, and the volume of liquid was slowly pulled through the cartridge using a syringe on a
76 short length of tubing attached to the bottom of the cartridge. The cartridge was washed twice
77 with 6 mL 0.1% TFA. PG fragments were eluted from the cartridge with 3 mL methanol. The
78 samples were evaporated under vacuum, and each was suspended in 50 μ L of LC/MS grade
79 H₂O.

80 The samples were analyzed by UPLC/MS (ultra-performance liquid chromatography/mass
81 spectrometry) using a Waters Acquity UPLC CSH C18 column, 1.7 μ m particle size, 2.1 x 150
82 mm using a flow rate of 0.5 mL/min. A volume of 10 μ L of the sample was injected, and the
83 column temperature was maintained at 40 °C. Separation was achieved using a linear gradient

84 spread over 5 min of 2-15% acetonitrile in 2% formic acid. Absorbance was measured at 206 nm
85 and 254 nm. Mass spectrometry was performed in the positive electrospray ionization mode
86 (ESI⁺) with the capillary voltage at 0.8 kV and the cone voltage at 15.

87 **Imaging of squid light organs**

88 Juvenile squid were fixed in 4% paraformaldehyde (PFA) in marine phosphate-buffered saline
89 (mPBS; 50 mM sodium phosphate buffer, 450 mM NaCl, pH 7.4) overnight at 4 °C, then washed
90 three times for 30 min in mPBS. Staining was performed on dissected light organ tissue;
91 CellMask Orange Plasma Membrane Stain (Thermo Fischer Scientific, Irwindale, CA) highlights
92 the lipid-rich vesicles within cells. To visualize hemocytes, light organs were stained with
93 deoxyribonuclease 1/Alexa Fluor 488 conjugate (DNase-1) (Thermo Fisher Scientific) in 1%
94 Triton X-100 in mPBS (mPBST) for 24 h, at 4 °C. To visualize late-stage cell death
95 (characterized by DNA fragmentation), light organs were stained with TUNEL according to the
96 manufacturer's protocol (Promega, Madison, WI). Light organs were counterstained overnight
97 with either DAPI or TO-PRO-3 (Thermo Fisher Scientific) to visualize host nuclei, and
98 phalloidin (Invitrogen, Carlsbad, CA) to visualize host actin cytoskeleton and the host cell
99 boundary. Samples were mounted on slides with Vectashield (Vector Laboratories, Newark, CA)
100 and imaging was performed on an upright Zeiss LSM 980 laser-scanning confocal microscope
101 (LSCM) at Caltech, a Zeiss LSM 710 at Kewalo Marine Laboratory, or an inverted Zeiss LSM
102 800 LSCM at the Caltech Biological Imaging Facility (See SI Appendix, table S2). Images and
103 figures were edited in either Photoshop CS6 (Adobe) or Fiji (ImageJ).

104 **Sample preparation for electron microscopy**

105 Squid light organs were dissected from the animal body cavity and immediately pre-fixed with
106 3% glutaraldehyde, 1% paraformaldehyde, 5% sucrose in marine PBS. Whole light organs were

107 rinsed with fresh mPBS containing 10% Ficoll (Sigma-Aldrich, St. Louis, MO), placed into brass
108 planchettes (Ted Pella, Inc., Redding, CA) and rapidly frozen with a Wohlwend Compact-03
109 high pressure freezer (Technotrade International Inc., Manchester, NH). The vitrified samples
110 were transferred under liquid nitrogen to cryotubes (Nunc) containing a frozen solution of 2.5%
111 osmium tetroxide and 0.05% uranyl acetate in acetone. Tubes were loaded into an AFS-2 freeze-
112 substitution machine (Leica Microsystems, Wetzlar, Germany) and processed at -90 °C for 72 h,
113 warmed over 12 h to -20 °C, held at that temperature for 6 h, then warmed to 4 °C for 2 h.
114 Fixative was removed and samples were rinsed 4-times with cold acetone, then infiltrated with
115 Epon-Araldite resin (Electron Microscopy Sciences, Hatfield, PA) over 48 h. Samples were flat-
116 embedded between two Teflon-coated glass microscope slides and resin polymerized at 60 °C
117 for 48 h.

118 Embedded light organs were viewed by phase-contrast microscopy to identify optimal
119 specimens. These were excised with a #11 scalpel and glued to the tips of plastic sectioning
120 stubs. Semi-thin (170 nm) serial sections were cut with an EM UC6 ultramicrotome (Leica
121 Microsystems) using a diamond knife (Diatome, Ltd., Bern, Switzerland). Sections were
122 transferred to Formvar-coated copper-rhodium 1-mm slot grids (Electron Microscopy Sciences,
123 Port Washington, PA) and stained with uranyl acetate and lead citrate. Gold beads (10 nm) were
124 placed on both surfaces of the grids to serve as fiducial markers for tomographic image
125 alignment.

126 **Electron tomography**

127 Grids were placed in a Model 2040 dual-axis tomography holder (E.A. Fischione Instruments,
128 Export, PA) and imaged with a Tecnai T12-G2 transmission electron microscope (120 KeV,
129 Thermo Fisher Scientific) equipped with a XP1000 2k x 2k CCD camera (Gatan, Inc.

130 Pleasanton, CA). Tomographic tilt-series and large-area montaged overviews were acquired
131 automatically using the SerialEM software package (8). For tomography, samples were tilted
132 $\pm 62^\circ$ and images collected at 1° intervals. The grid was then rotated 90° and a similar series
133 taken about the orthogonal axis. Tomographic data was calculated, analyzed, and modeled using
134 the IMOD software package (8-10) on iMac Pro and Mac Studio M1 computers (Apple, Inc.).

135 **Scanning electron microscopy**

136 Tissue was fixed with 4% PFA in mPBS for 24 h, post-fixed with 1% osmium tetroxide in 0.1 M
137 sodium cacodylate, dehydrated through an ethanol series, and dried in a Tousimis Samdri-795
138 critical point dryer. Samples were mounted on aluminum stubs with double-stick carbon tape and
139 coated with gold/palladium in a Hummer 6.2 sputter coater. Samples were viewed and digital
140 images were acquired with a Hitachi S-4800 field emission scanning electron microscope at an
141 accelerating voltage of 5 kV.

142 **Outer membrane vesicle (OMV) purification**

143 Single-membrane OMVs were isolated from *V. fischeri* cultures after 24 h of growth in LBS at
144 28°C as previously described, and contained almost entirely outer-membrane embedded (*e.g.*,
145 SypC) and periplasmic proteins (11). Briefly, the cells were pelleted at $8,000 \times g$ at 4°C for 10
146 min. The culture supernatant was filtered through a $0.22\text{-}\mu\text{m}$ polyethersulfone membrane filter to
147 remove any contaminating cells. OMVs were then collected from the filtered supernatant by
148 centrifugation at $173,000 \times g$ for 2 h at 4°C using a Type 45 Ti rotor in an Optima XE-90
149 ultracentrifuge (Beckman Coulter, Brea, CA). Pellets containing OMVs were resuspended in
150 Dulbecco's phosphate-buffered saline supplemented with NaCl (dPBS-NaCl: 422 mM NaCl, 2.7
151 mM KCl, 1.5 mM KH_2PO_4 , 7.7 mM Na_2HPO_4 , 900 μM CaCl_2 , 500 μM MgCl_2). OMVs were
152 further purified with a discontinuous density gradient (sucrose layers of 25, 35, 40, 45, 50, and

153 55%, wt/vol) and centrifugation at 90,000 x g for 15 h at 4 °C using an MLA-50 rotor in an
154 Optima MAX-XP ultracentrifuge (Beckman Coulter). The OMVs were pelleted at 173,000 g
155 and 4°C for 4 h and resuspended in dPBS-NaCl. The total OMV protein concentration was
156 measured using a Qubit 2.0 Fluorometer (Thermo Fisher). Samples were diluted to equal
157 concentrations with dPBS-NaCl before addition to FSIO for squid exposure experiments. Squid
158 were treated with 100 mg of OMV proteins per ml for 18 h.

159 **RNA extraction and qRT-PCR analysis**

160 Hemocytes were isolated from both aposymbiotic and colonized juvenile squids as previously
161 described (12). The isolated hemocyte samples were treated with RNAlater (Invitrogen) for 1 h,
162 and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD). Genomic
163 DNA contamination was removed by treatment with TURBO DNase (Thermo Fisher Scientific).
164 The integrity and quality of the RNA were assessed by agarose gel electrophoresis and
165 NanoDrop spectrophotometry (Thermo Fisher Scientific). RNA concentration was quantified
166 using the high-sensitivity Qubit RNA HS Assay Kit (Invitrogen). For each sample, 50 ng of
167 RNA was reverse-transcribed into cDNA using the LunaScript RT SuperMix Kit (New England
168 Biolabs, Ipswich, MA) in a 20 µL reaction.

169 Primers for the C8 subunit of proteasome gene were designed using Primer 5.00 (PREMIER
170 Biosoft International, San Francisco, CA), and primers for the 40S gene, serving as the
171 endogenous reference were adopted from (13). Selected primer pairs showed single melting
172 peaks and acceptable amplification performance. The expression of 40S gene was stable across
173 all samples in this experiment. Quantitative real-time PCR was performed using SsoAdvanced
174 Universal SYBR Green Supermix (Bio-Rad, Hercules) on a QuantStudio 5 system (Applied

175 Biosystems, Foster City, CA). Relative gene expression levels of C8 subunit of proteasome gene
176 were calculated using the $2^{-\Delta\Delta C_t}$ method (14).

177

178 **SUPPLEMENTAL RESULTS**

179 **PERMANOVA analyses of RNAseq data**

180 PERMANOVA indicated significant global differences among conditions ($R^2 = 0.31$, $p = 0.012$).

181 Pairwise PERMANOVA showed that APO differed from both WT ($R^2 = 0.35$, adj. $p = 0.0495$)

182 and $\Delta sypC$ ($R^2 = 0.26$, adj. $p = 0.0495$), whereas WT and $\Delta sypC$ were not significantly different

183 ($R^2 = 0.11$, $p = 0.72$). Pairwise tests for homogeneity of multivariate dispersion were non-

184 significant (all $p > 0.10$), confirming that these results are not driven by unequal dispersion.

185 Importantly, the lack of global separation between WT and $\Delta sypC$ did not invalidate the

186 differential expression results. Instead, it indicated that $\Delta sypC$ colonization does not broadly

187 reprogram host transcription, but produces focused, pathway-specific changes—consistent with

188 the PCA, where WT and $\Delta sypC$ clustered tightly together and apart from APO. The lack

189 of PERMANOVA significance between WT and $\Delta sypC$ likely reflected high biological

190 variability relative to the scale of transcriptional differences between these groups. This pattern

191 underscored that the presence of bacteria was the dominant driver of host transcriptional state,

192 whereas the loss of a single bacterial gene ($\Delta sypC$) led to more targeted effects. To resolve these

193 subtler differences, we implemented a tiered DEG framework (brown, yellow, and green

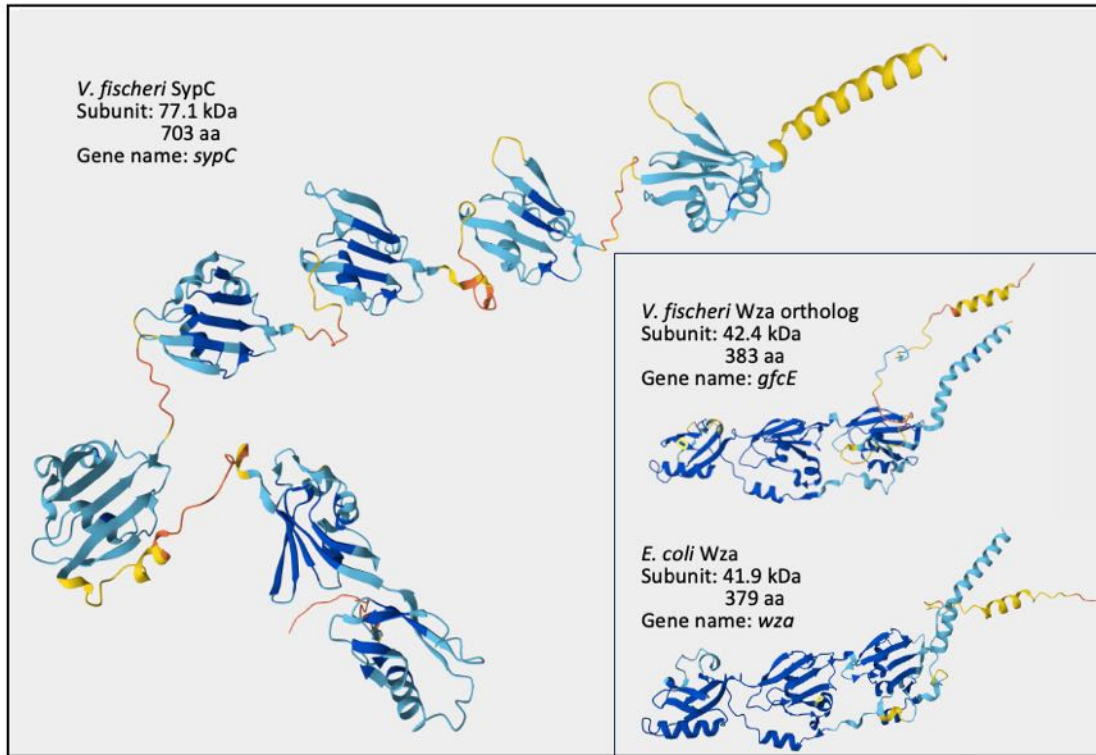
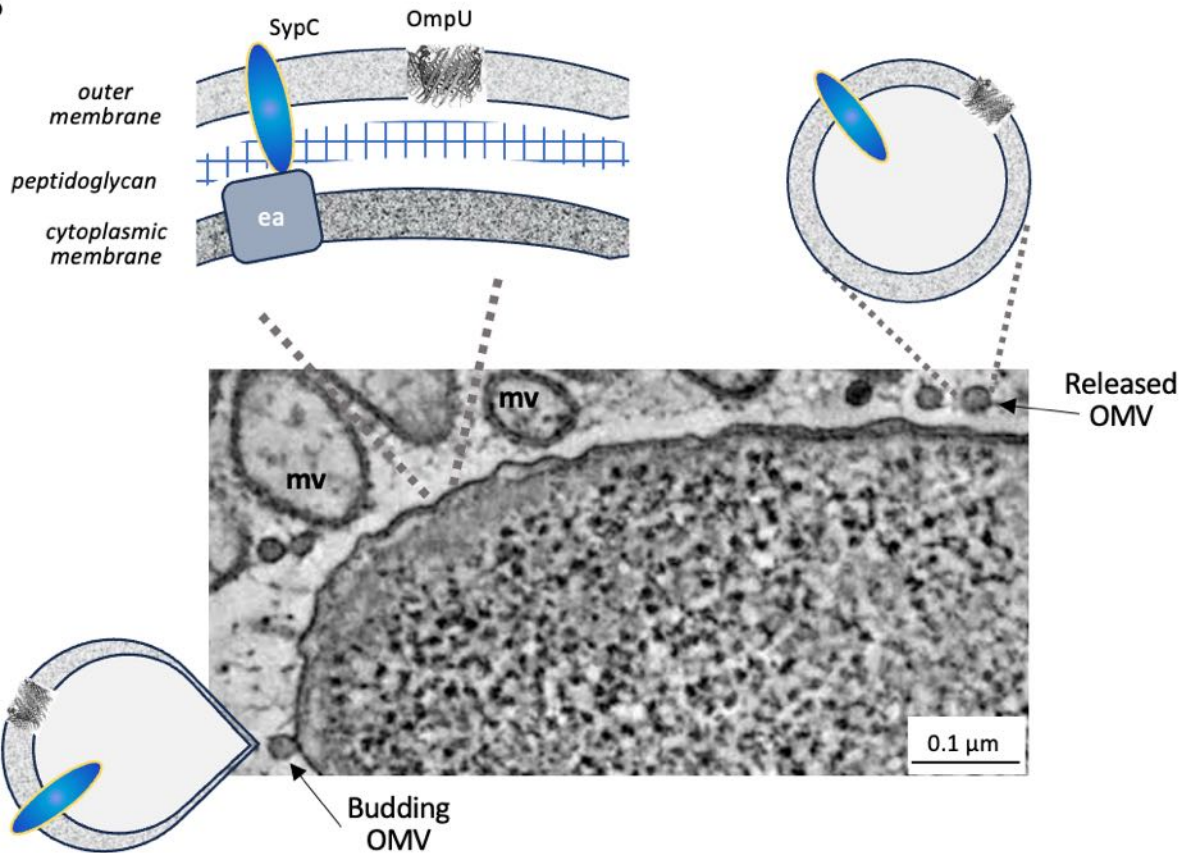
194 categories). Within this system, the “green” DEGs represent the strongest candidates (Fig. 2B

195 and C), as they were not differentially expressed between APO and $\Delta sypC$, but were distinct in

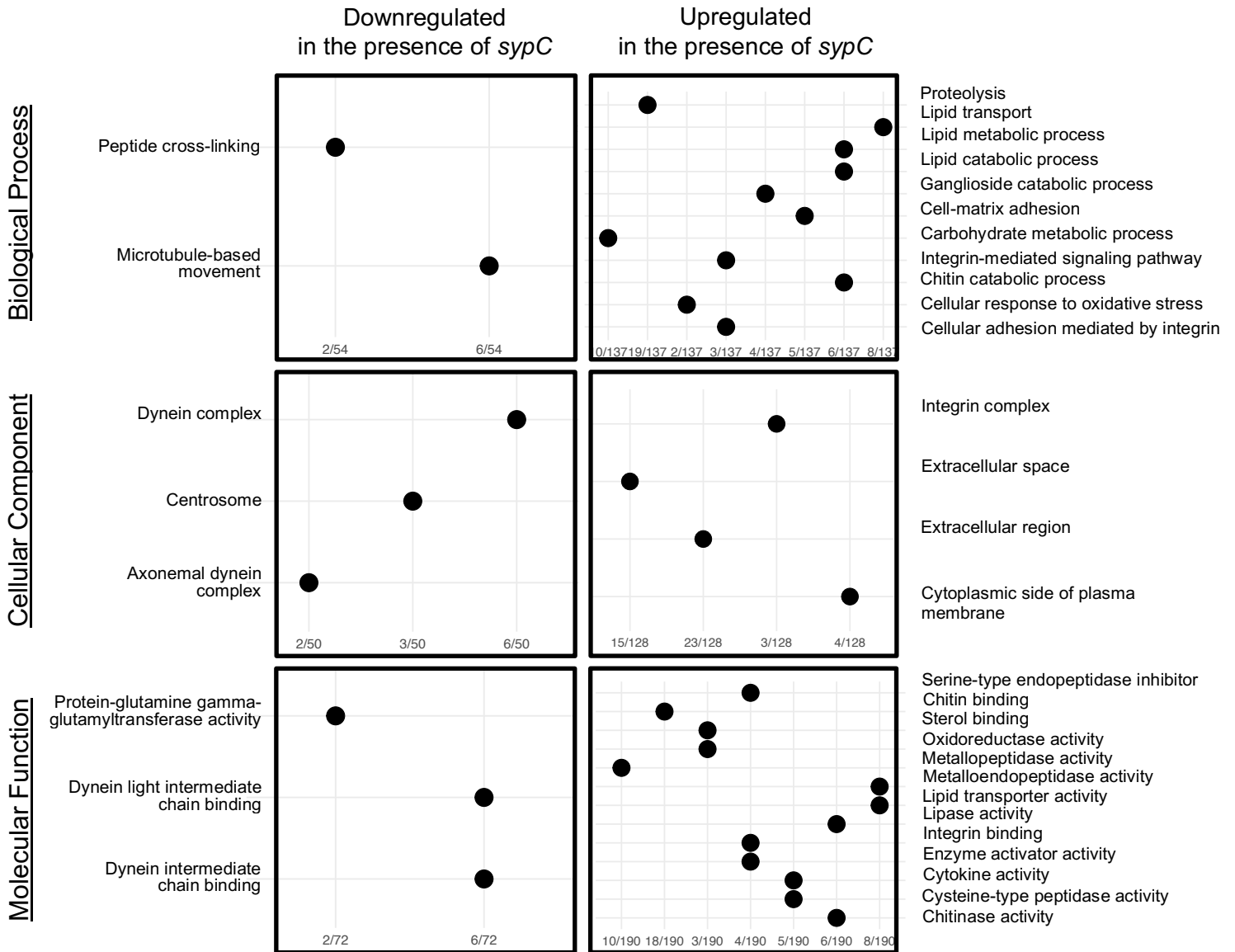
196 WT, suggesting that these genes are specifically dependent on SypC activity rather than general

197 colonization status.

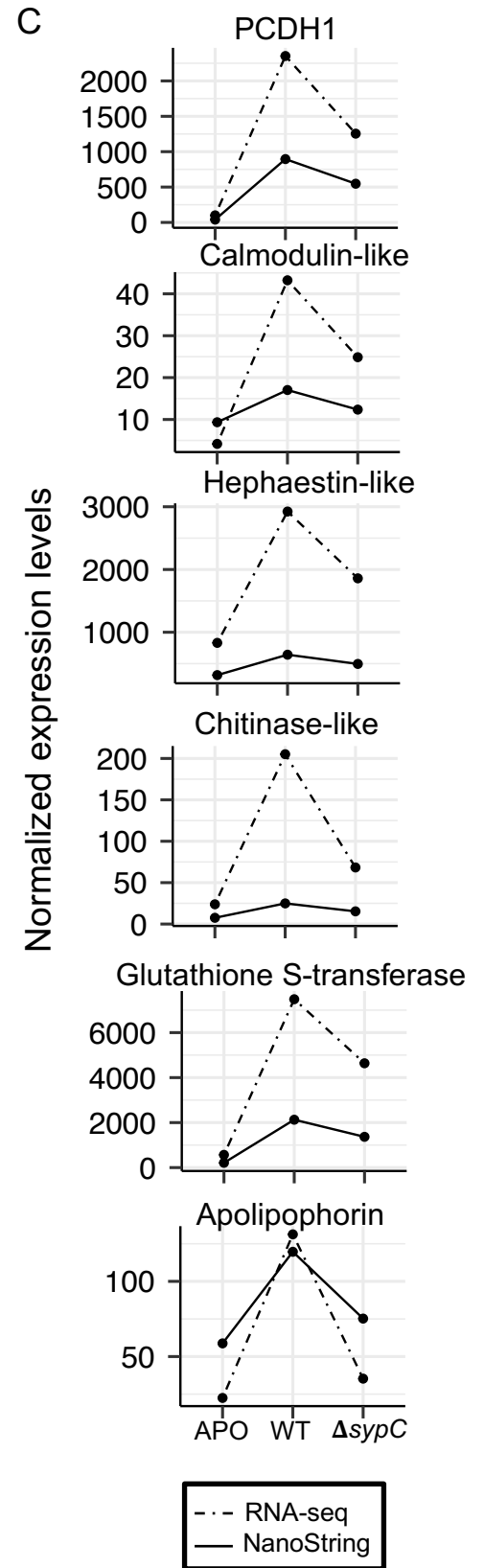
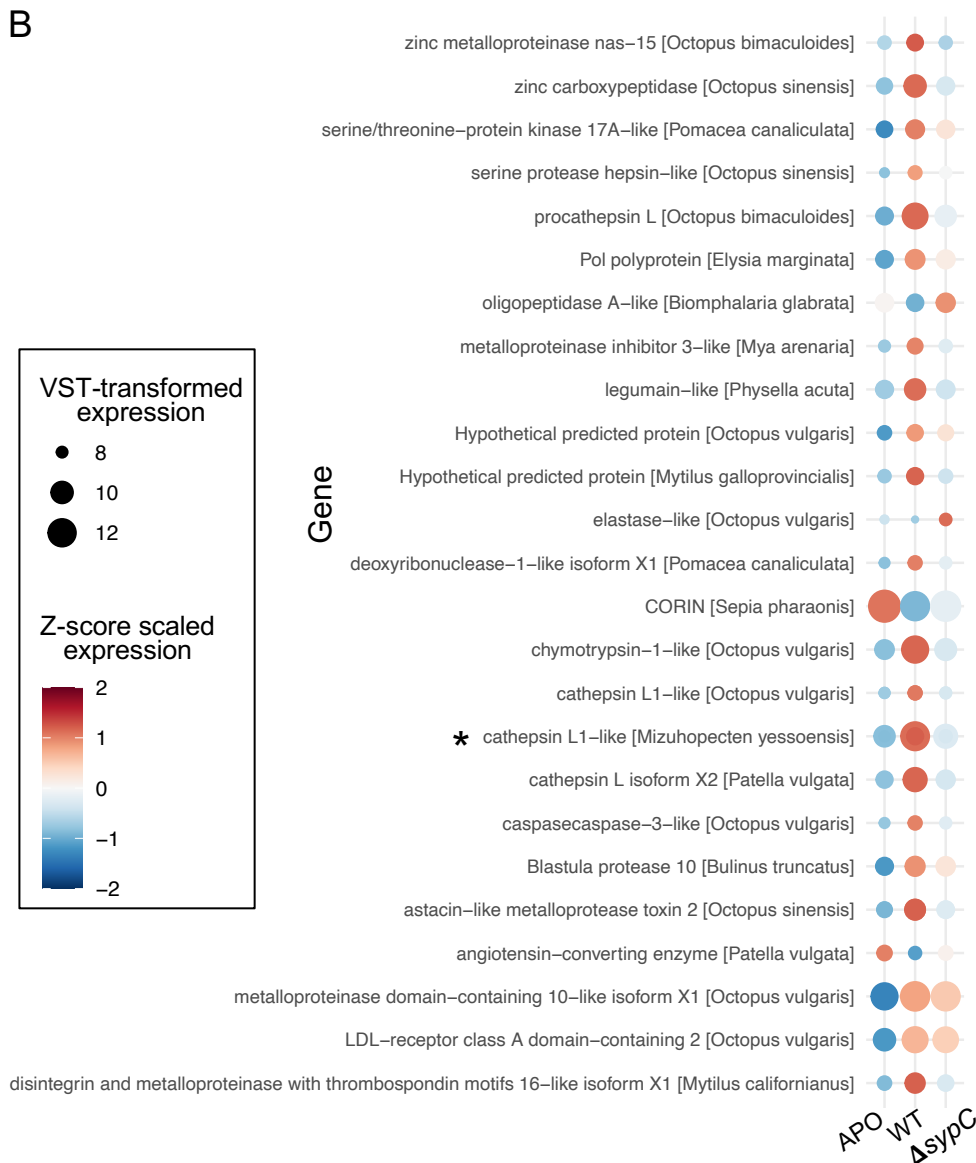
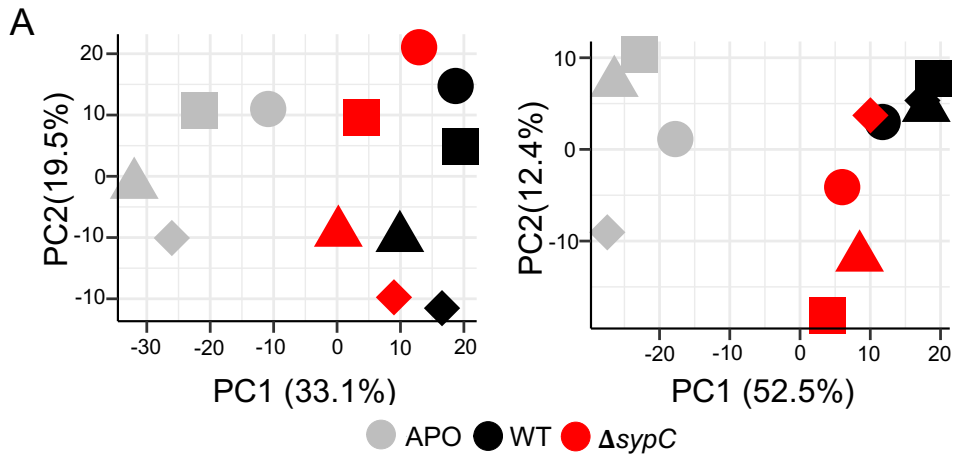
198

A**B**

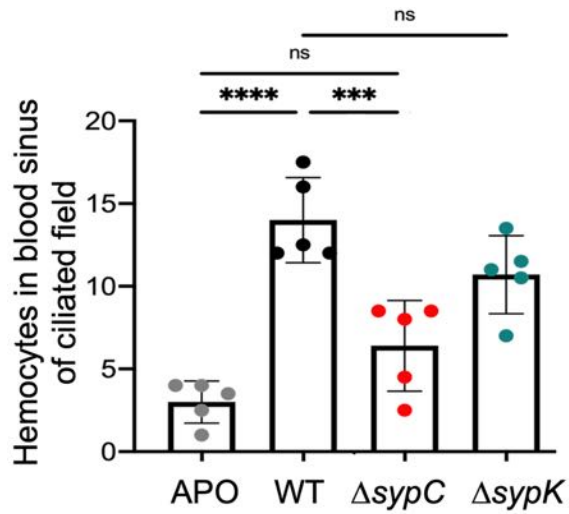
200 **Fig. S1.** Characteristics of *V. fischeri* SypC. (A) Features of the *V. fischeri* SypC protein
201 (adapted from UniProt [Universal Protein Resource database]; see Results). SypC-like proteins
202 only occur in closely related *Vibrio* (*Aliivibrio*) spp. *Inset:* Wza, a protein widely occurring in
203 Gram-negative bacteria, is involved in export of exopolysaccharides; *sypC* is likely a paralog of
204 *V. fischeri wza* (aka, *gfcE*). (B) *V. fischeri* OMVs. A transmission electron micrograph of a
205 portion of a *V. fischeri* symbiont surrounded by the microvilli (mv) of the host's crypt epithelial
206 cells. Features the size of OMVs occur associated with (*i.e.*, budding) or close to (*i.e.*, released)
207 the *V. fischeri* surface. *ea*, other essential Syp proteins making up the molecular export apparatus
208 for *V. fischeri* Syp exopolysaccharide. OmpU is the major outer membrane porin of *V. fischeri*.



211 **Fig. S2.** GO-term enrichment of DEGs. Over-representation analysis (ORA) was performed
 212 using Fisher’s Exact Test. GO terms shown are significantly enriched among genes that are
 213 either downregulated by the presence of the symbiont SypC ($\Delta sypC > WT$) or upregulated by
 214 SypC ($\Delta sypC < WT$). The gene ratio number at the bottom of the data=point columns indicates
 215 the proportion of DEGs associated with a given GO term relative to the total number of DEGs
 216 tested (*i.e.*, overlapping DEGs / total DEGs in the query set).



218 **Fig. S3.** Additional analyses supporting transcriptomic differences. (A) PCA plots illustrating the
219 impact of egg clutch (*i.e.*, genetic background) on host-gene expression. *Left*: PCA without
220 clutch modeled as a covariant reveals clustering by genetic background; *right*: PCA with clutch
221 included as a covariant reduces background-driven clustering, supporting the inclusion of clutch
222 in the differential expression model. Four replicates performed, each indicated by a different
223 symbol. (B) Differentially expressed genes, most associated with cell death and proteolysis.
224 Asterisk (*) indicates a cathepsin L gene, whose expression pattern was also examined by
225 NanoString (Fig. 2D). (C) Comparison of RNA-seq and NanoString expression levels for several
226 additional genes not shown in Fig. 5D.

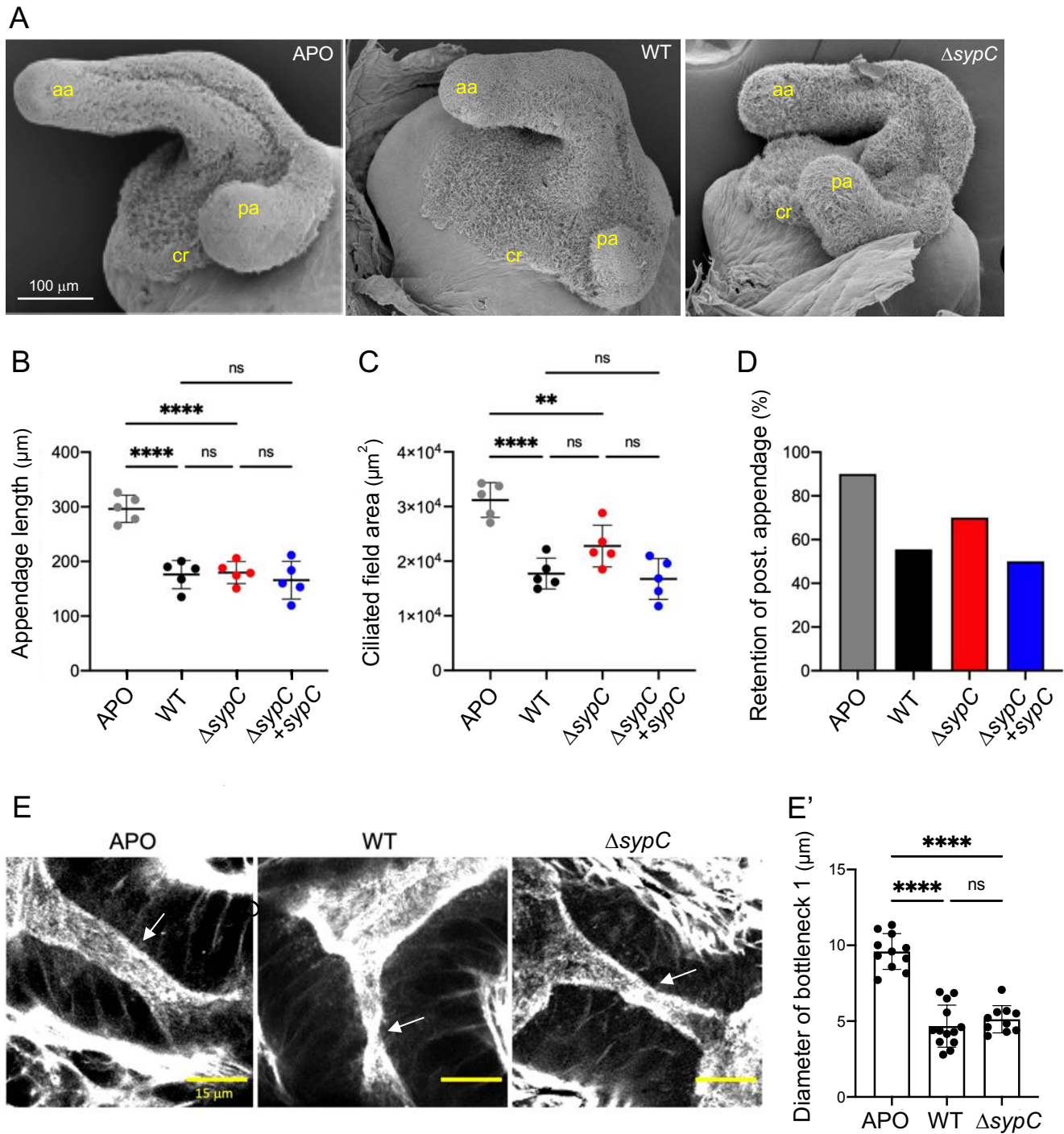


227

228

229

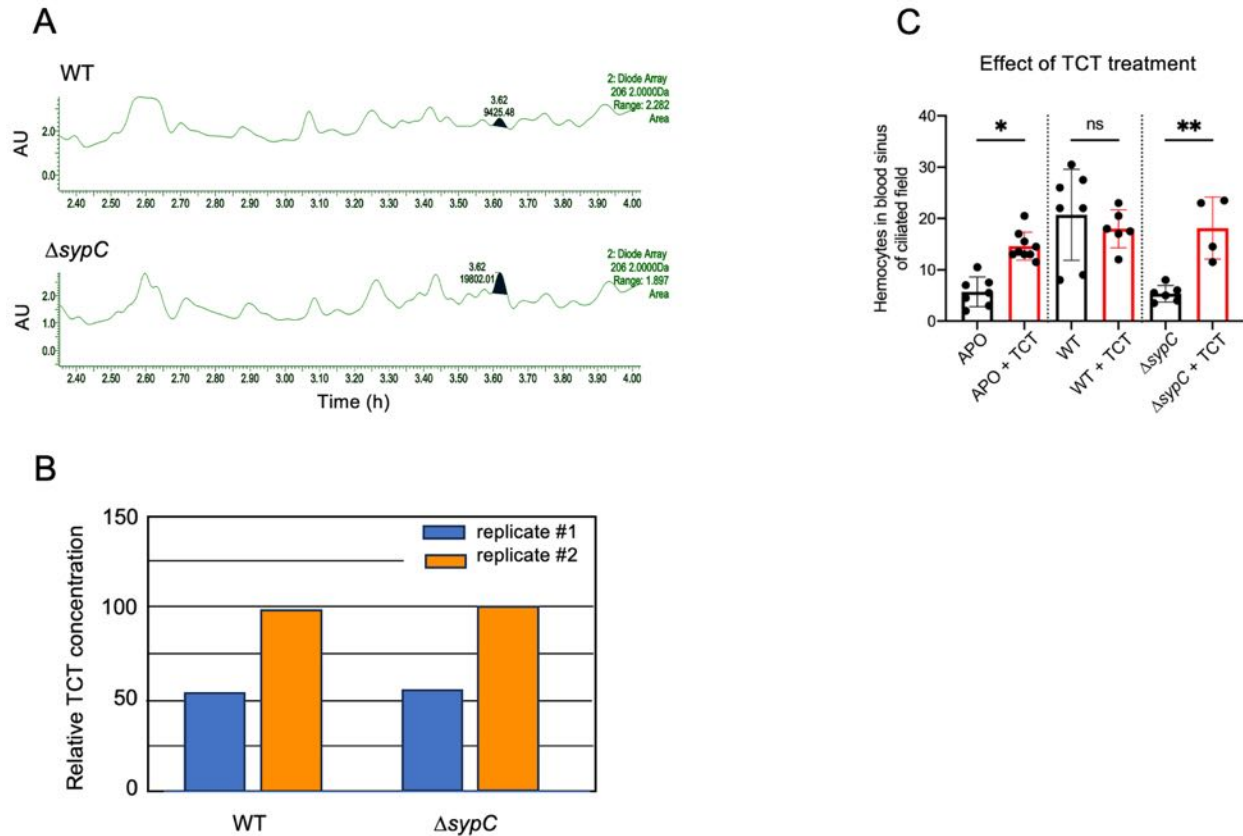
230 **Fig. S4.** Normal hemocyte trafficking requires a SypC-specific function other than Syp
 231 polysaccharide formation. Quantification of hemocytes in the blood sinus of the anterior
 232 appendage at 24 h post-inoculation. Animals were colonized with the WT, $\Delta sypC$ or $\Delta sypK$
 233 strain; n=5 per condition. The experiment was repeated 3 times with the same results.



234

235 **Fig. S5.** Patterns of morphogenesis. (A) SEM images of the ciliated fields of the light organ at 24
 236 hpi, showing the loss of the ciliated ridge (cr) in the wild-type (WT) colonized animal, but its
 237 retention in both the aposymbiotic (APO) and $\Delta sypC$ -colonized animals; aa, anterior appendage;
 238 pa, posterior appendage. Measurements of anterior appendage length (B) and epithelial-field area

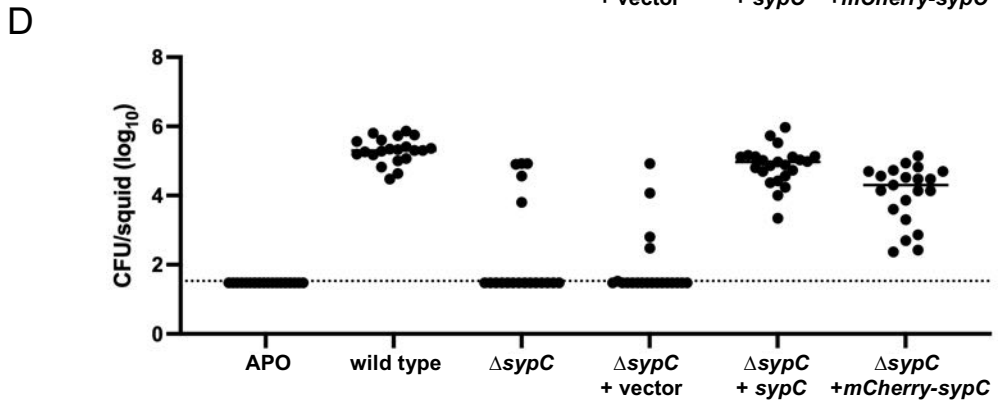
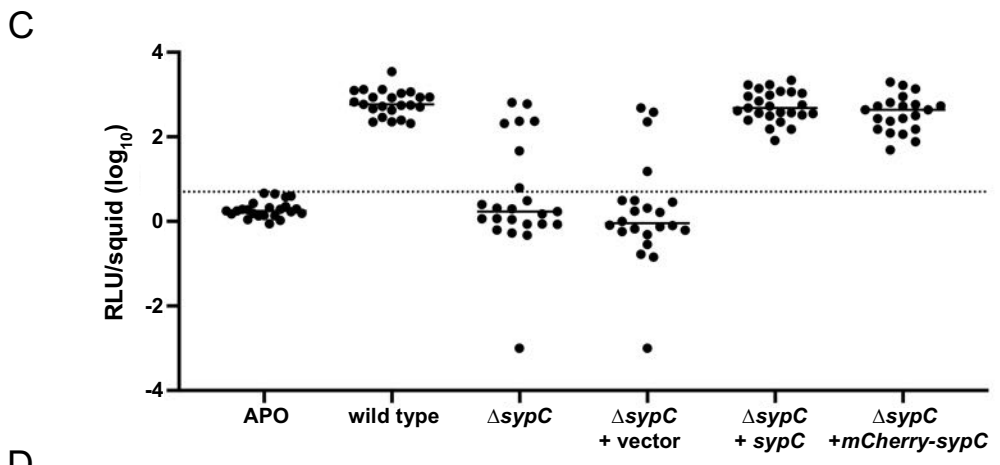
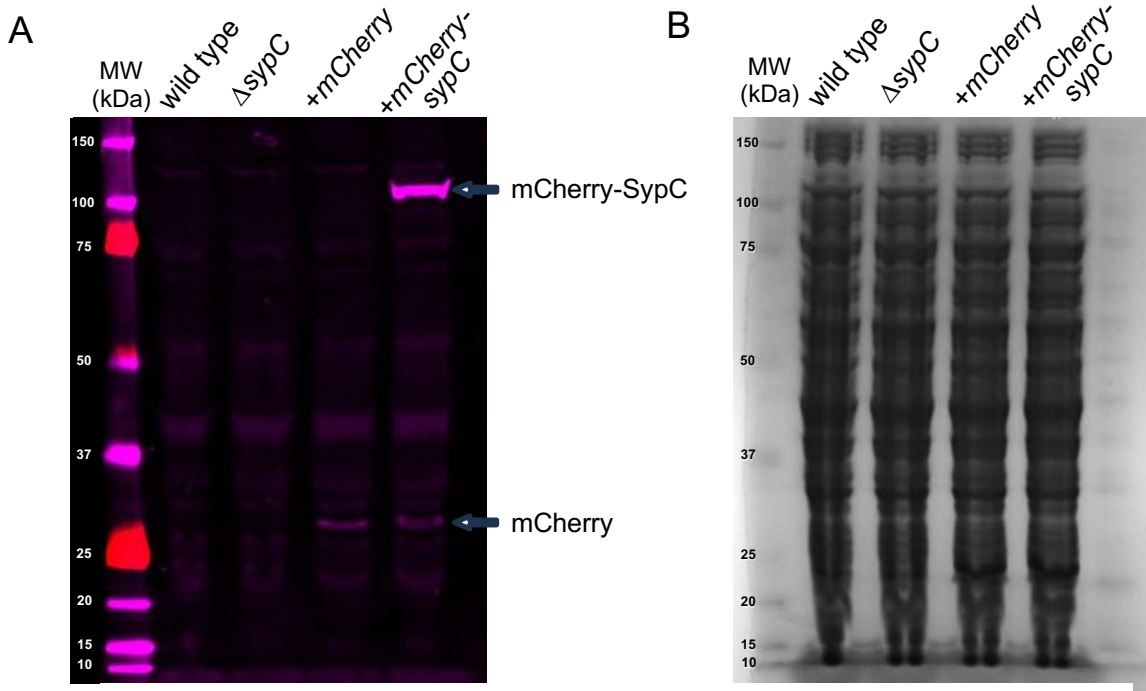
239 (C) at 72 hpi are not significantly different between any of the colonized animal (WT, $\Delta sypC$, or
240 the $\Delta sypC + sypC$ complemented strain). Results are the mean \pm 1 SD. Comparisons between
241 multiple groups were analyzed using one-way ANOVA with Tukey test. ns: not significant, $P >$
242 0.05; ** $P \leq 0.01$; **** $P \leq 0.0001$. (D) Percentage of animals retaining a posterior appendage 72
243 hpi; n=5 per condition. (E) Confocal images of the bottleneck leading into crypt 1 (white arrows)
244 in the migration path between the antechamber and the crypt (see Fig. 1A'); the bottleneck
245 constricts to a similar extent in response to colonization by either the WT or $\Delta sypC$ strain. (E')
246 Quantification of bottleneck diameter at 24 hpi. Each data point represents a biological replicate.
247 Results are the mean \pm 1 SD. Comparisons between multiple groups were analyzed using one-
248 way ANOVA with Tukey test. ns: not significant, $P > 0.05$; **** $P \leq 0.0001$.



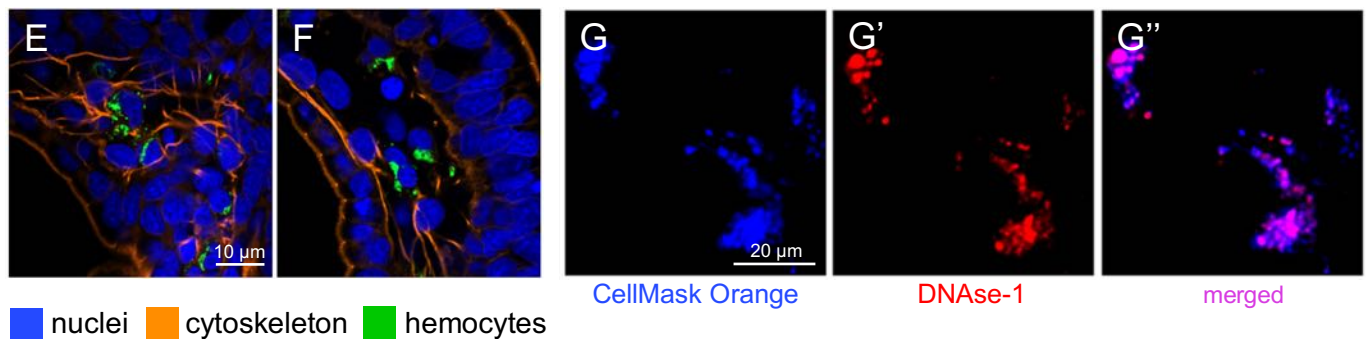
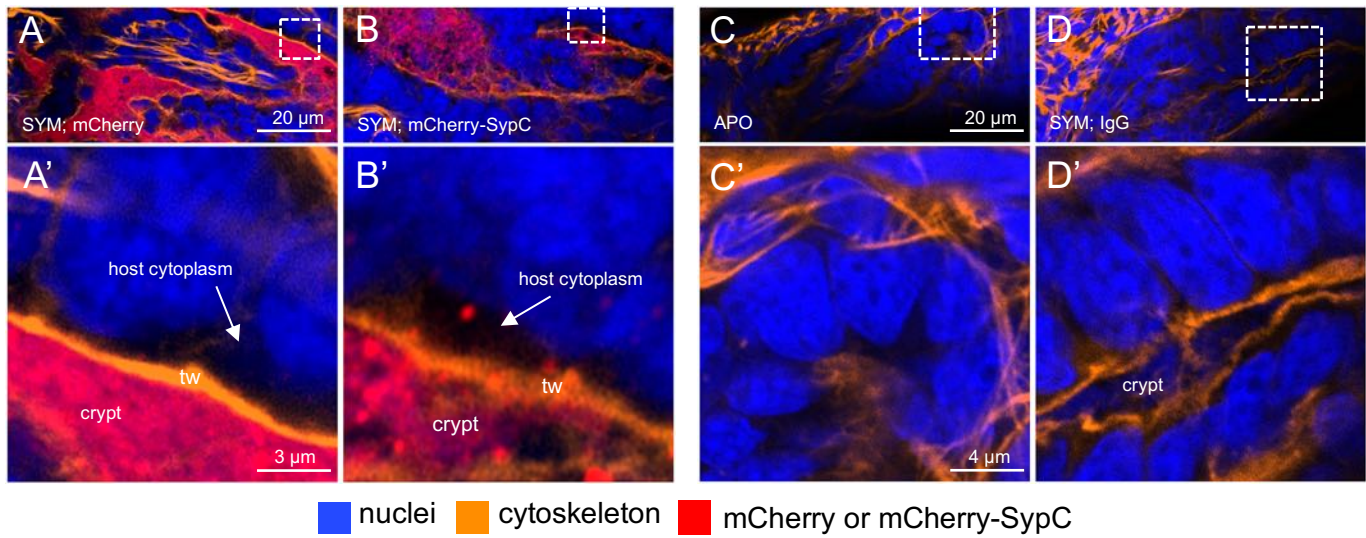
249

250 **Fig. S6.** Effect of a *sypC* deletion on the release of, and host response to, peptidoglycan
 251 monomer (TCT). (A) Example of HPLC chromatograms comparing the level of TCT released in
 252 culture by WT and $\Delta sypC$ *V. fischeri* cells. Blackened sections indicate TCT peak areas. AU =
 253 relative absorbance units. (B) Normalized quantification of TCT exported by cultures of WT or
 254 $\Delta sypC$ in two replicate experiments. (C) Quantification of hemocyte number in the blood sinus
 255 of the anterior appendage at 24 hpi. Animals were either uncolonized, or colonized with either
 256 the WT parent or $\Delta sypC$ strain, with or without the addition of 1 mM TCT to the surrounding
 257 seawater. Each data point represents a biological replicate. Results are the mean \pm 1 SD.
 258 Comparisons between multiple groups were analyzed using one-way ANOVA with Tukey test.
 259 ns: not significant, $P > 0.05$; $*P \leq 0.05$; $**P \leq 0.01$.

260
261
262



263 **Fig. S7.** Detection of the mCherry-SypC fusion protein. (A) A fluorescent antibody made to the
264 mCherry protein identified a strongly expressed protein corresponding to the molecular weight of
265 a mCherry-SypC fusion protein. Less strongly expressed was the mCherry protein itself, when
266 encoded on a plasmid used to fluorescently label *V. fischeri* cells. (B) Extracts of wild-type or
267 $\Delta sypC$ strains of *V. fischeri*, as well as strains carrying an mCherry-expressing plasmid or the
268 fusion-protein expressing plasmid, were separated on an SDS-PAGE gel and probed by western
269 blot with the mCherry antibody (A). Arrows indicate the expected molecular weights of the
270 fusion protein and the mCherry protein itself. (C and D) The ability of the *mCherry-sypC*
271 construct to complement a $\Delta sypC$ mutation in symbiosis. Juvenile squid were either uncolonized
272 (APO) or colonized by wild type *V. fischeri* or one of 4 mutant derivatives. Colonization was a
273 3-h exposure to 2000-5000 CFU/ml for all conditions except $\Delta sypC$, which was an inoculum of
274 10,000 CFU/ml for 24 h. Phenotypes were scored at 24 h. Colonization success was determined
275 by either (C) bioluminescence (relative light units, RLU), or (D) CFU per squid. Carriage of an
276 *mCherry-sypC* construct complemented the symbiosis-defective $\Delta sypC$ mutant essentially as
277 well as a wild-type copy of *sypC*. The dotted lines indicate the limits of detection of each
278 measurement.



279

280 **Fig. S8.** mCherry-SypC localization. Control images labelled with mCherry antibody supporting
 281 the conclusions drawn from Fig. 4. (A) Constitutively expressed mCherry protein is confined
 282 within the epithelium-lined crypts of a *V. fischeri*-colonized (SYM) host. (A') Enlargement of
 283 area in white dashed box. As expected, the bacterium's cytoplasmic mCherry protein remains
 284 confined within the symbiont cells and does not appear in the epithelial cell cytoplasm. In
 285 contrast, labeling of the mCherry-SypC fusion protein can be seen not only within the symbionts
 286 in the crypts (B), but also as focused loci located within the host-cell cytoplasm (B'). No sign of
 287 labeling is seen in either aposymbiotic (APO) tissues treated with fluorescent mCherry antibody
 288 (C), or in symbiotic tissues treated with control IgG (D). Enlargements of areas within the white
 289 dashed boxes are below the parent image. Close-up of the blood sinus region of the ciliated
 290 anterior appendages of SYM animals (Fig. 1A') treated with either fluorescent mCherry antibody

291 (E) or control IgG (F). All tissues were imaged at 22 hpi. nuclei, DAPI (blue); cytoskeleton,
292 rhodamine phalloidin (orange); hemocytes, DNase-1 (green); antibody to mCherry (red)
293 identifies either the free protein, or the SypC fusion protein; tw, terminal web of the actin
294 cytoskeleton at the outer edge of the host epithelial cells. Colocalization of cell membranes
295 (CellMask Orange; blue) and hemocytes (DNase-1; red) in the blood sinus (G-G'').

Table S1. Strains, plasmids and primers**BACTERIAL STRAINS**

Common name	Species/strain	Chromosomal fe	Plasmid	Selection	Description	Reference
ES114	<i>V. fischeri</i> ES114	wild type			wild-type squid symbiont	Boettcher & Ruby 1990
ES114mCherry-sypC	<i>V. fischeri</i> ES114	wild type	pCMC24	Cm 2.5	pCMC24 (<i>mCherry-sypC</i> fusion)	this work
ES114 mCherry	<i>V. fischeri</i> ES114	wild type	pCMC35	Cm 2.5	pCMC35 (<i>mCherry</i> -expressing control for <i>mCherry</i> fusions)	this work
ES114 mCherry-ompU	<i>V. fischeri</i> ES114	wild type	pCMC37	Cm 2.5	pCMC37 (signal peptide- <i>mCherry-ompU</i> into ES114)	this work
Δ sypC	<i>V. fischeri</i> ES114	Δ sypC			<i>sypC</i> mutant	Shibata <i>et al.</i> 2012
Δ sypC complemented	<i>V. fischeri</i> ES114	Δ sypC	pSS25	Cm 2.5	<i>sypC</i> mutant with native <i>sypC</i> complement	Shibata <i>et al.</i> 2012
Δ sypC vector control	<i>V. fischeri</i> ES114	Δ sypC	pVSV105	Cm 2.5	<i>sypC</i> mutant carrying plasmid vector	this work
Δ sypC mCherry-sypC	<i>V. fischeri</i> ES114	Δ sypC	pCMC24	Cm 2.5	<i>sypC</i> mutant carrying <i>mCherry-sypC</i>	this work
Δ sypC mCherry	<i>V. fischeri</i> ES114	Δ sypC	pCMC35	Cm 2.5	<i>sypC</i> mutant carrying <i>mCherry</i> label	this work
Δ ompU mCherry-ompU	<i>V. fischeri</i> ES114	Δ ompU	pCMC37	Cm 2.5	<i>ompU</i> mutant carrying signal peptide- <i>mCherry-ompU</i>	this work
DH5 α λ pir	<i>E. coli</i> DH5 α λ pir	<i>uidA</i> ::pir+			<i>E. coli</i> conjugation strain	Dunn <i>et al.</i> 2006
MFDpir	<i>E. coli</i> MFDpir	Δ dapA::(erm-pir)			DAP deficient <i>E. coli</i> for biparental conjugation	Ferrieres <i>et al.</i> 2010

PLASMIDS

Name	Selection	Description	Reference
pCMC24	Cm 2.5	pCMC7 with internal ATG deleted in the <i>mCherry-sypC</i> fusion	this work
pCMC35	Cm 2.5	pCMC7 with excised <i>sypC</i> from <i>mCherry-sypC</i> fusion	this work
pCMC37	Cm 2.5	pVSV105 vector with fused signal peptide- <i>mCherry-ompU</i>	this work
pSS25	Cm 2.5	native <i>sypC</i> complementation plasmid	Shibata <i>et al.</i> 2012
pVSV105	Cm 2.5	<i>V. fischeri</i> complementation vector	Dunn <i>et al.</i> 2006

PRIMERS

Oligonucleotide ID	Sequence 5' to 3'	Description	Reference
40S-qF3	AAGGCTTTGTCCACCTTCCT	Reference gene S19 ribosomal protein amplification	Moriano- Gutierrez <i>et al.</i> , 2019
40S-qR3	TAAATGCTCCAACACCAGCA	Reference gene S19 ribosomal protein amplification	Moriano- Gutierrez <i>et al.</i> , 2019
C8-qF2	ACGCTCAAAAGGCAGTGG	Target gene C8 subunit of proteasome amplification	this work
C8-qR2	GCCGAACCTATATGCGTATC	Target gene C8 subunit of proteasome amplification	this work

Table S2. Imaging characteristics

Figure	Instrument, upright/inverted	Location	Objective	Detector type	Dyes, Excitation/Emission, laser used
3A	Zeiss LSM 710 upright	Kewalo Marine Laboratory	40x/1.2 water	GaAs-PMT	Acridine orange (502/525 nm), WGA (633 nm)
3B	Zeiss LSM 710 upright	Kewalo Marine Laboratory	40x/1.2 water	GaAs-PMT	DNase-1 (AF488, 490/525 nm), Rhodamine phalloidin (540/565 nm), TO-PRO-3 (642/661 nm)
4A	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 63x/1.40 Oil DIC M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
4B (A processed)	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 63x/1.40 Oil DIC M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
4C (A processed)	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 63x/1.40 Oil DIC M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
4D	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 20x/0.8 Air M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
4E-E"	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 63x/1.40 Oil DIC M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
4F	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 20x/0.8 Air M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
4G-G"	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 63x/1.40 Oil DIC M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)
5A	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
5A	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
5A	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
5A	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
5A	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
S6A	Hitachi S-4800	University of Hawaii-Manoa	400x	none	none
S6E	Zeiss LSM 710 upright	Kewalo Marine Laboratory	40x/1.2 water	GaAs-PMT	DNase-1 (AF488, 490/525 nm), Rhodamine phalloidin (540/565 nm), TO-PRO-3 (642/661 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DAPI (350/470 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DAPI (350/470 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DAPI (350/470 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DAPI (350/470 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	GAR-AF647 (650/671 nm), Rhodamine phalloidin (540/565 nm), DNase-1 (AF488, 490/525 nm), DAPI (350/470 nm)
S9A-A'	Zeiss LSM 900 upright	Caltech, Pasadena, CA	Plan-Apochromatic 40x/1.30 Oil DIC (UV) VIS-IR M27	GaAsP-Pmt	DNase-1 (AF488, 490/525 nm), CellMask Orange Plasma Membrane (554/567 nm), DAPI (350/470 nm)

Table S2. Imaging characteristics (page 2)

Figure	Track: laser wavelength, gain, detection wavelength	Averaging, bit depth	Snap or Z-stack
3A		4, 16	Z-stack
3B	Track 1: (AF647) 640 nm, 1.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.04%, 500V, 410-647, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.1%, 500V, 400-490	4, 16	Z-stack
4A	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
4B (A processed)	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
4C (A processed)	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
4D	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
4E-E"	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
4F	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
4G-G"	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (CMO) 561 nm, 0.5%, 500V, 410-647, Track 3: (DAPI) 405 nm, 0.3%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
5A	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
5A	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
5A	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
5A	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
5A	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
S6A	none	none	Snap
S6E	Track 1: (AF647) 640 nm, 1.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.04%, 500V, 410-647, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.1%, 500V, 400-490	4, 16	Z-stack
S9A-A'	Track 1: (AF647) 640 nm, 1.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.04%, 500V, 410-647, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.1%, 500V, 400-490	4, 16	Snap
S9A-A'	Track 1: (AF647) 640 nm, 0.5%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Z-stack, 0.5um steps
S9A-A'	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Snap
S9A-A'	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Snap
S9A-A'	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Snap
S9A-A'	Track 1: (AF647) 640 nm, 0.2%, 591V, 645-700, Track 2: (Rhodamine) 561 nm, 0.03%, 500V, 410-617, Track 3: (FITC) 488 nm, 0.10%, 500V, 410-546, Track 4: (DAPI) 405 nm, 0.2%, 500V, 400-490	4, 16	Snap
S9A-A'	Track 1: (CMO) 561 nm, 2%, 500V, 535-617, Track 2: (AF488) 488 nm, 0.5%, 500V, 410-546, Track 3: (DAPI) 405 nm, 0.6%, 500V, 400-495	4, 16	Snap

296 **SI References**

297

298

- 299 1. B. D. Bennett, T. Essock-Burns, E. G. Ruby, HbtR, a heterofunctional homolog of the
300 virulence regulator TcpP, facilitates the transition between symbiotic and planktonic
301 lifestyles in *Vibrio fischeri*. *mBio* **11** (2020).
- 302 2. J. Graf, P. V. Dunlap, E. G. Ruby, Effect of transposon-induced motility mutations on
303 colonization of the host light organ by *Vibrio fischeri*. *J Bacteriol* **176**, 6986-6991 (1994).
- 304 3. K. J. Boettcher, E. G. Ruby, Detection and quantification of *Vibrio fischeri* autoinducer
305 from symbiotic squid light organs. *J Bacteriol* **177**, 1053-1058 (1995).
- 306 4. L. Yang *et al.*, Bacterial growth dynamics in a rhythmic symbiosis. *Mol Biol Cell* **35**,
307 ar79 (2024).
- 308 5. A. K. Dunn, D. S. Millikan, D. M. Adin, J. L. Bose, E. V. Stabb, New rfp- and pES213-
309 derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux*
310 expression *in situ*. *Appl Environ Microbiol* **72**, 802-810 (2006).
- 311 6. K. L. Visick, K. M. Hodge-Hanson, A. H. Tischler, A. K. Bennett, V. Mastrodomenico,
312 Tools for rapid genetic engineering of *Vibrio fischeri*. *Appl Environ Microbiol* **84** (2018).
- 313 7. L. Ferrieres *et al.*, Silent mischief: bacteriophage Mu insertions contaminate products of
314 *Escherichia coli* random mutagenesis performed using suicidal transposon delivery
315 plasmids mobilized by broad-host-range RP4 conjugative machinery. *J Bacteriol* **192**,
316 6418-6427 (2010).
- 317 8. D. N. Mastronarde, S. R. Held, Automated tilt series alignment and tomographic
318 reconstruction in IMOD. *J Struct Biol* **197**, 102-113 (2017).
- 319 9. J. R. Kremer, D. N. Mastronarde, J. R. McIntosh, Computer visualization of three-
320 dimensional image data using IMOD. *J Struct Biol* **116**, 71-76 (1996).
- 321 10. D. N. Mastronarde, Correction for non-perpendicularity of beam and tilt axis in
322 tomographic reconstructions with the IMOD package. *J Microsc* **230**, 212-217 (2008).
- 323 11. J. B. Lynch *et al.*, Ambient pH alters the protein content of outer membrane vesicles,
324 driving host development in a beneficial symbiosis. *J Bacteriol* **201** (2019).
- 325 12. S. Moriano-Gutierrez *et al.*, The noncoding small RNA SsrA is released by *Vibrio*
326 *fischeri* and modulates critical host responses. *PLoS Biol* **18**, e3000934 (2020).

- 327 13. S. Moriano-Gutierrez *et al.*, Critical symbiont signals drive both local and systemic
328 changes in diel and developmental host gene expression. *Proc Natl Acad Sci U S A* **116**,
329 7990-7999 (2019).
- 330 14. K. J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time
331 quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25**, 402-408 (2001).
332
333



SypC: A symbiont morphogen packaged in bacterial vesicles

Anne Marie Krachler^{a,1}

Animals develop in constant association with microorganisms. Across diverse host–microbe partnerships, microbial signals shape tissues, tune immune responses, and even influence the formation of organs specialized to house symbionts. What remains less clear is how microbial molecules reach the host cells that execute these developmental programs. Many of these processes occur deep within host tissues, physically separated from the microbes themselves.

Outer-membrane vesicles (OMVs)—nanometer-scale spheres shed from the outer membranes of Gram-negative bacteria—have emerged as one potential solution to this delivery problem. In pathogenic systems, OMVs have long been studied as vehicles for virulence factors. Vesicles from pathogens carry toxins, effectors, and immunomodulatory molecules that can damage host tissues or manipulate immune signaling (1–3). These vesicles can fuse with host membranes, enter cells by endocytosis, and in some cases even traverse epithelial barriers, effectively transporting bacterial cargo across host tissues (4). A recurring feature across these interactions is the intimate engagement between OMVs and host cells, which readily internalize vesicles and respond to their contents. Yet vesicle-mediated communication is not limited to pathogenic encounters. In beneficial host–microbe relationships, similar vesicle exchange may help coordinate developmental processes between partners. In this issue, Kuwabara et al. show that OMVs produced by the luminous symbiont *Vibrio fischeri* deliver a signal that promotes organ morphogenesis in its squid host (5). Their work identifies a specific vesicle-associated protein—SypC—that functions as a morphogen-like factor, providing an unusually clear example of how a bacterial protein has a dual function and is interpreted by the host to drive its development. OMVs are well suited for such roles. They package diverse cargo—including proteins, lipids, polysaccharides, and nucleic acids—while protecting these molecules from degradation in extracellular environments. Vesicles also allow bacteria to influence host cells without direct contact, extending the reach of microbial signals beyond the immediate site of colonization (6). Beyond host interactions, OMVs participate in biofilm development, nutrient scavenging, horizontal gene transfer, and microbial competition, highlighting their versatility in bacterial ecology (7). Despite this functional diversity, most studies of OMVs have focused on their roles in infection. Their functions in beneficial symbioses have only begun to receive comparable attention. Conceptually, vesicle-mediated transport offers an attractive mechanism for delivering concentrated microbial signals across host

tissues to regulate processes such as apoptosis, proliferation, or differentiation. However, direct mechanistic examples of defined vesicle cargo driving host development have been scarce. In many cases, host responses have been attributed to broad classes of microbial-associated molecular patterns—lipopolysaccharide, or peptidoglycan fragments—rather than to specific vesicle proteins with dedicated developmental roles (8).

Due to its unique binary nature, the partnership between the Hawaiian bobtail squid *Euprymna scolopes* and its bacterial symbiont *V. fischeri* provides an ideal system for dissecting such interactions. Within hours after hatching, newly hatched juvenile squid recruit *V. fischeri* from the surrounding seawater to a specialized light organ. Once established within crypts of this organ, the bacteria produce bioluminescence that helps camouflage the squid during nighttime hunting. Over the past thirty years, we have come to understand that colonization does more than provide light: It initiates a cascade of host developmental changes that transform the immature organ into a mature symbiotic structure (9). During this process, ciliated epithelial fields that initially help capture bacteria (10) regress, immune cells migrate into the tissue, and the organ remodels to form crypt structures that house the symbionts. Previous work in this system revealed that bacterial molecules such as lipopolysaccharide and tracheal cytotoxin—a peptidoglycan fragment—trigger apoptosis of the ciliated epithelium, a key step in the maturation of the light organ (8). These discoveries helped establish the squid–*Vibrio* association as a classic example of microbes directing host development. Over the past decade, attention has increasingly turned to OMVs as additional mediators of this dialog. Previous studies demonstrated that *V. fischeri* releases vesicles capable of inducing host responses associated with the onset of symbiosis (11, 12). These vesicles stimulate the recruitment of hemocytes—the squid’s macrophage-like immune cells—to the developing light organ and

Author affiliations: ^aDepartment of Microbiology and Molecular Genetics, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX 77030

Author contributions: A.M.K. wrote the paper.

The author declares no competing interest.

Copyright © 2026 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

See companion article, “SypC, a symbiont outer membrane vesicle protein, impacts the development of the squid–vibrio partnership,” 10.1073/pnas.2524648123.

¹Email: anne.marie.krachler@uth.tmc.edu.

Published April 20, 2026.

contribute to epithelial apoptosis and tissue remodeling. Such observations suggested that OMVs might serve as delivery vehicles for microbial signals during the earliest stages of colonization.

In this issue, Kuwabara et al., show that OMVs produced by the luminous symbiont *Vibrio fischeri* deliver a signal that promotes organ morphogenesis in its squid host (5).

Kuwabara et al. now provide a molecular handle on this process by identifying SypC as a key vesicle cargo that influences host morphogenesis (5). SypC is a Wza-like outer-membrane protein encoded within the symbiosis polysaccharide (Syp) locus, a regulatory and biosynthetic pathway that controls biofilm formation during squid colonization (13). Biofilms formed by Syp polysaccharides allow *V. fischeri* cells to aggregate within host mucus before entering the crypts of the light organ. This aggregation step is critical for successful colonization (14). Until now, Syp proteins were viewed primarily through the lens of bacterial physiology. The new study reveals an additional dimension. OMVs containing SypC stimulate host tissue remodeling, whereas vesicles lacking SypC produce a weaker or delayed developmental response. Similarly, squid colonized with a Δ sypC mutant exhibit impaired maturation of the light organ. Transcriptomic analyses further show that the presence of SypC influences host gene-expression programs associated with immune signaling and tissue morphogenesis (5). These findings suggest that SypC performs a kind of double duty. Within the bacterium, it contributes to the formation of biofilms that enable colonization. Packaged into OMVs, the same protein helps initiate developmental changes in the host. Such “moonlighting” functions are not unprecedented in host-microbe biology. In the human gut, the polysaccharide A produced by *Bacteroides fragilis* shapes immune development while also contributing to bacterial surface architecture (15). In plant symbioses, lipochitooligosaccharide Nod factors produced by *Rhizobium* bacteria induce root nodule formation while coordinating bacterial infection processes (16). These examples illustrate a recurring evolutionary theme: Microbial molecules that facilitate colonization often also participate in shaping host responses. Another intriguing aspect of the study concerns the route by which vesicles access host tissues. Imaging experiments show that OMVs containing SypC are internalized by hemocytes located near the crypts of the light

organ. These immune cells appear to take up vesicles and transport them within host tissue, potentially distributing microbial signals beyond the immediate site of bacterial colonization. In this scenario, immune cells function not only as sentinels guarding against infection but also as intermediaries that relay symbiont-derived signals to developmental pathways. Of course, the squid host contributes its own regulatory machinery to this process. Remodeling of the light organ involves host apoptosis pathways, extracellular-matrix remodeling enzymes, and reactive oxygen species that help regulate symbiont populations (17). SypC therefore operates within a broader dialog between host and microbe, where bacterial signals trigger responses that the host interprets and executes using its own developmental toolkit.

The idea that microbes can produce morphogen-like signals is gaining traction across biological systems: Bacterial metabolites influence intestinal stem cell proliferation in vertebrates (18). Symbiont-derived molecules regulate larval settlement in marine invertebrates (19). And in plants, microbial signals control the formation of entire organs dedicated to symbiotic nitrogen fixation (20). In many of these cases, extracellular vesicles have emerged as potential vehicles for signal delivery. Seen in this broader context, SypC represents one of the clearest demonstrations that a specific bacterial protein packaged into vesicles can function as a developmental signal within an animal host. Rather than acting merely as a generic immune stimulant, SypC appears to engage host pathways that guide tissue remodeling during the establishment of symbiosis.

Many questions remain. How exactly does SypC influence host signaling once delivered to host cells? Does the protein interact directly with host receptors, or does it modify vesicle structure in ways that alter host responses? Are additional vesicle cargo molecules cooperating with SypC to orchestrate the developmental program of the light organ? Whatever the answers, the work by Kuwabara et al. underscores an emerging view of symbiosis: Microbes are not simply passengers in animal bodies, but active participants in development. By packaging molecules like SypC into extracellular vesicles, bacteria gain a means of reaching across tissue boundaries to influence host biology. The squid-*Vibrio* partnership thus offers a vivid example of how microbial physiology and host development can become intertwined, each shaping the other through a steady exchange of molecular signals.

1. N. Sirisaengtaksin, E. J. O'Donoghue, S. Jabbari, A. J. Roe, A. M. Krachler, Bacterial outer membrane vesicles provide an alternative pathway for trafficking of *Escherichia coli* O157 type III secreted effectors to epithelial cells. *mSphere* **8**, e0052023 (2023).
2. F. G. Zingl et al., Outer membrane vesicles of *Vibrio cholerae* protect and deliver active cholera toxin to host cells via porin-dependent uptake. *mBio* **12**, e0053421 (2021).
3. P. Deo et al., Outer membrane vesicles from *Neisseria gonorrhoeae* target PorB to mitochondria and induce apoptosis. *PLoS Pathog.* **14**, e1006945 (2018).
4. E. J. O'Donoghue, A. M. Krachler, Mechanisms of outer membrane vesicle entry into host cells. *Cell. Microbiol.* **18**, 1508–1517 (2016).
5. J. T. Kuwabara et al., SypC, a symbiont outer membrane vesicle protein, impacts the development of the squid-vibrio partnership. *Proc. Natl. Acad. Sci. U.S.A.* **123**, e2524648123 (2026).
6. S. K. Cohen et al., Tracking the cargo of extracellular symbionts into host tissues with correlated electron microscopy and nanoscale secondary ion mass spectrometry imaging. *Cell. Microbiol.* **22**, e13177 (2020).
7. J. Berleman, M. Auer, The role of bacterial outer membrane vesicles for intra- and interspecies delivery. *Environ. Microbiol.* **15**, 347–354 (2013).
8. T. A. Koropatnick et al., Microbial factor-mediated development in a host-bacterial mutualism. *Science* **306**, 1186–1188 (2004).
9. J. A. Doyno, M. J. McFall-Ngai, A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. *Biol. Bull.* **189**, 347–355 (1995).
10. K. A. Gundlach et al., Ciliated epithelia are key elements in the recruitment of bacterial partners in the squid-vibrio symbiosis. *Front. Cell Dev. Biol.* **10**, 974213 (2022).
11. M. S. Aschtgen et al., Rotation of *Vibrio fischeri* flagella produces outer membrane vesicles that induce host development. *J. Bacteriol.* **198**, 2156–2165 (2016).
12. M. S. Aschtgen, K. Wetzel, W. Goldman, M. McFall-Ngai, E. Ruby, *Vibrio fischeri*-derived outer membrane vesicles trigger host development. *Cell. Microbiol.* **18**, 488–499 (2016).
13. E. S. Yip, B. T. Grublesky, E. A. Husa, K. L. Visick, A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. *Mol. Microbiol.* **57**, 1485–1498 (2005).
14. J. F. Brooks II et al., Global discovery of colonization determinants in the squid symbiont *Vibrio fischeri*. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 17284–17289 (2014).
15. S. K. Mazmanian, C. H. Liu, A. O. Tzianabos, D. L. Kasper, An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **122**, 107–118 (2005).

16. P. Lerouge *et al.*, Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* **344**, 781–784 (1990).
17. S. K. Davidson, T.A. Koropatnick, R. Kossmehl, L. Sycuro, M. J. McFall-Ngai, NO means "yes" in the squid-vibrio symbiosis: Nitric oxide (NO) during the initial stages of a beneficial association. *Cell. Microbiol.* **6**, 1139–1151 (2004).
18. J. E. Kim *et al.*, Gut microbiota promotes stem cell differentiation through macrophage and mesenchymal niches in early postnatal development. *Immunity* **55**, 2300–2317.e6 (2022).
19. N. J. Shikuma *et al.*, Marine tubeworm metamorphosis induced by arrays of bacterial phage tail-like structures. *Science* **343**, 529–533 (2014).
20. G. E. Oldroyd, Speak, friend, and enter: Signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol.* **11**, 252–263 (2013).