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Supplemental Information

A *Drosophila* Model for *Clostridium difficile* Toxin CDT Reveals Interactions with Multiple Effector Pathways

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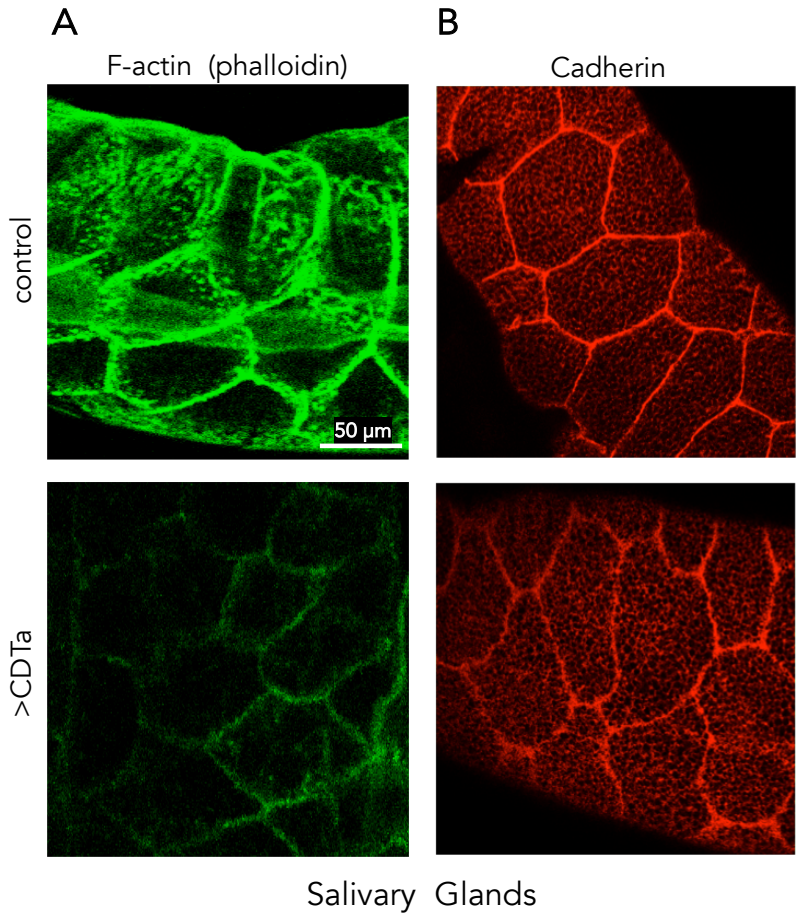

Figure S1. CDTa expression disrupts F-actin network and tissue morphology in salivary glands, related to Fig 3.

Salivary glands from wt (control) or CDTa-expressing third instar larvae under the control of the MS1096-GAL4 (>CDTa) were dissected and stained with fluorescent Phalloidin, which binds actin filaments (F-actin) (left panels) or with an anti-Cadherin antibody, which stains adherens junctions (right panels). Scale bar represents 50 μm.

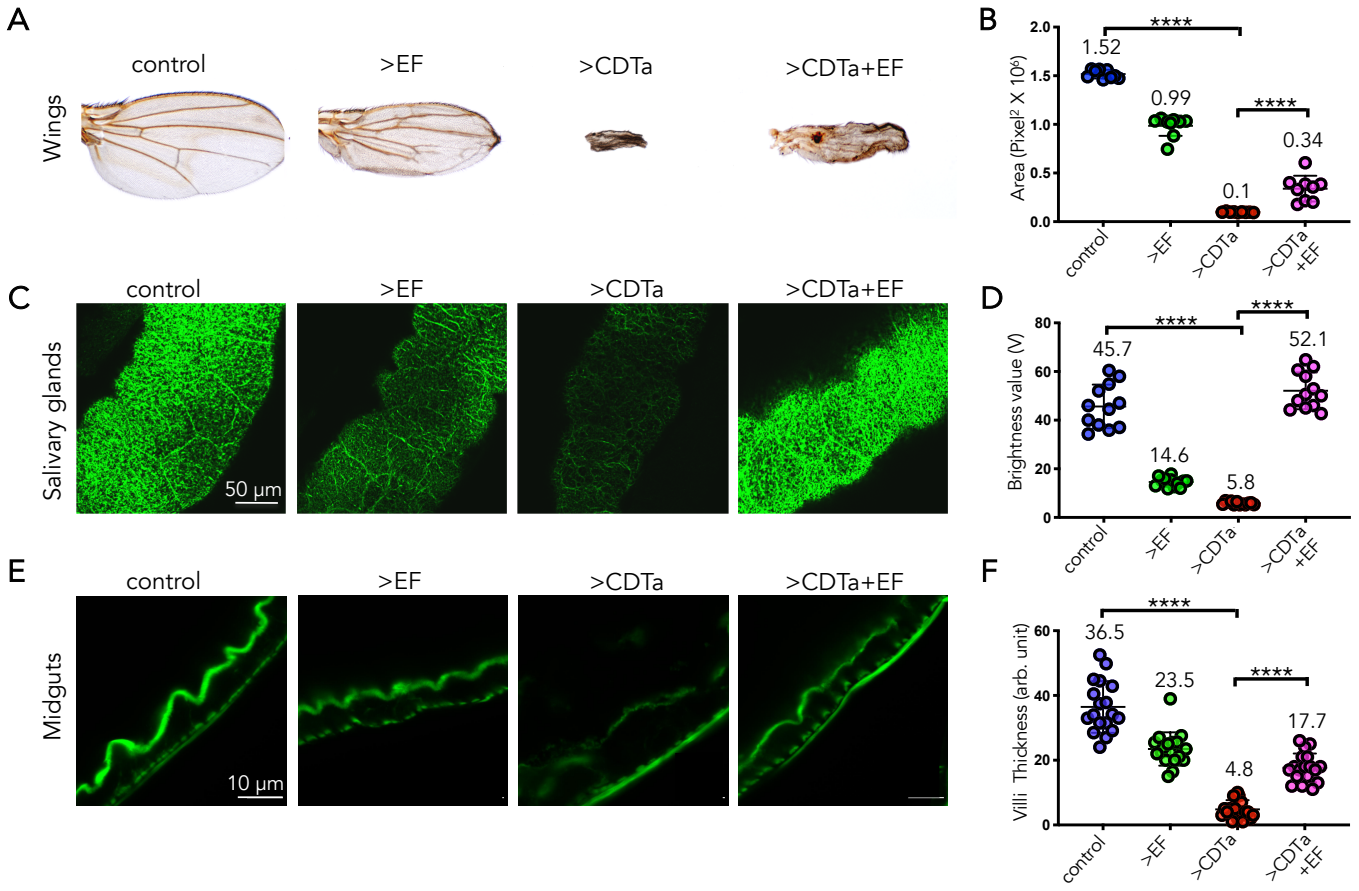

Figure S2. Anthrax EF toxin partially rescues CDTa phenotypes in wings, salivary glands and guts. Related to Fig 4.

(A) Wings from male flies of indicated genotypes grown at 25ºC. Male flies expressing CDTa under the control of wing-specific tubGAL80ts MS1096 (>CDTa), tubGAL80ts MS1096 EF (>EF) or in combination (>CDTa+EF) are shown. Co-expression of anthrax EF toxin partially rescues the strong CDTa wing phenotype. (B) Mounted wings shown in A were photographed and surface area was quantified with Photoshop® ($F=490.6$, $df=3$, $p<0.0001$). (C) Salivary glands from third instar larvae grown at 25ºC and stained with fluorescent Phalloidin. Genotypes are the same as in panel A. EF expression fully

rescues the loss of F-actin seen in CDTa-expressing glands. (D) Fluorescence levels were measured with ImageJ ($F=175.3$, $df=3$, $p<0.0001$). (E) One week-old adult control males, or expressing EF, CDTa or both under tubGAL80ts NP1 were grown at RT, further incubated for 24hrs at 31^{o}C and dissected. F-actin in gut cells was stained with fluorescent Phalloidin. EF partially rescues villi thickness when co-expressed with CDTa. Midgut images are luminal views. Scale bar represents 50 μm in upper panels and 10 μm in lower panels. (F) Comparative measurements of villi thickness were quantified with Photoshop® and analyzed using Prism8 ($F=105.5$, $df=3$, $p<0.0001$).

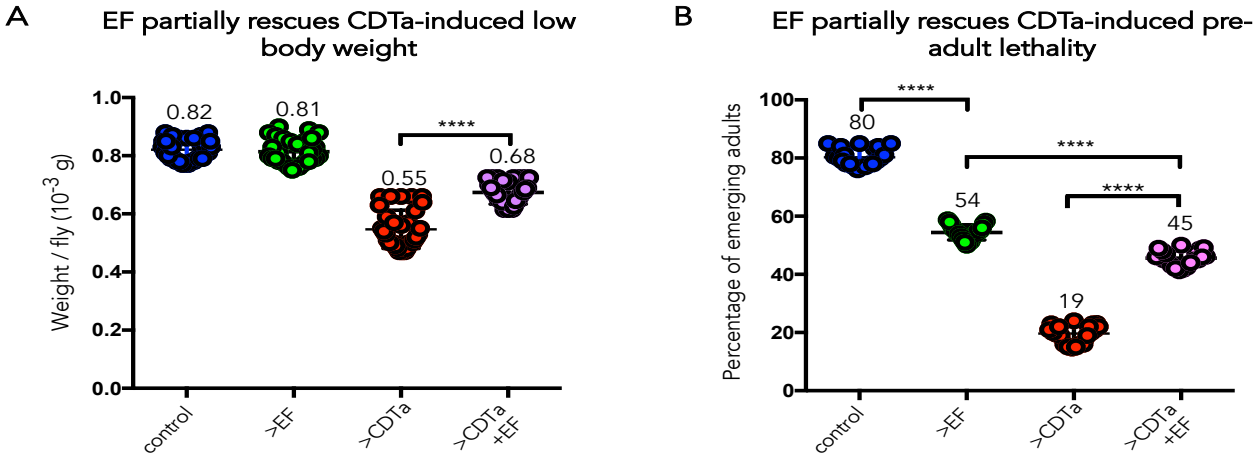

Figure S3. Anthrax EF toxin expression rescues both CDTa-induced body weight and viability to adulthood phenotypes, related to Fig 4.

(A) Comparative body weight of flies of the following genotypes: tubGAL80ts NP1/+ (control), tubGAL80ts NP1>EF (>EF), tubGAL80ts NP1>CDTa (>CDTa), and tubGAL80ts NP1>CDTa and EF (>CDTa+EF). Animals were raised at 25ºC. Data was analyzed using Prism8 ($F=249.6$, $df=3$, $p<0.0001$). (B) Comparative survival to adulthood at 25ºC. Same genotypes as in panel A are indicated. Data was analyzed using Prism8 ($F=1176$, $df=3$, $p<0.0001$).

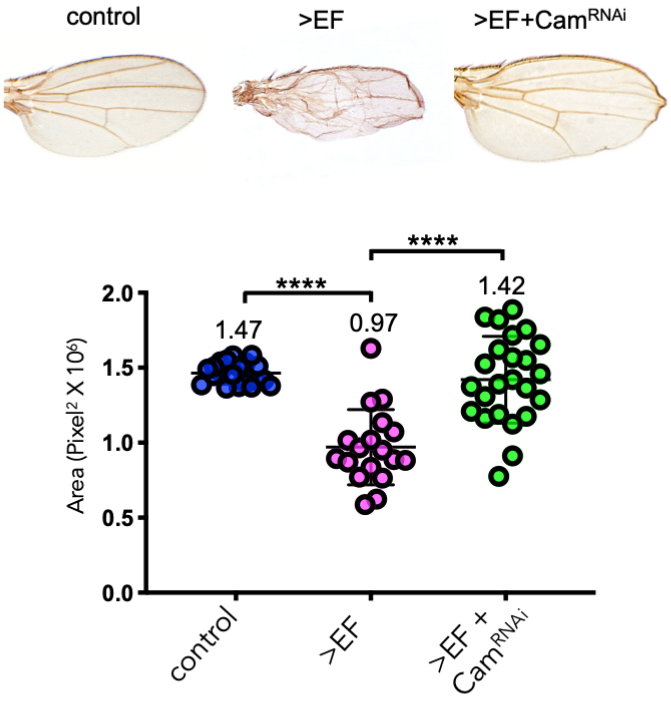

Figure S4. EF activity depends on Calmodulin, related to Figure 4.

Wings from female flies of the following genotypes: wt (control), MS1096 GAL4 tubGAL80ts > EF (>EF), MS1096 GAL4 tubGAL80ts > EF + Cam^{RNAi}, (>EF+Cam^{RNAi}). Flies were grown at 25°C. Their respective wing phenotypes were photographed, and the surface area of the wings quantified with Photoshop® and analyzed using Prism8 ($F=27.12$, $df=2$, $p<0.0001$).

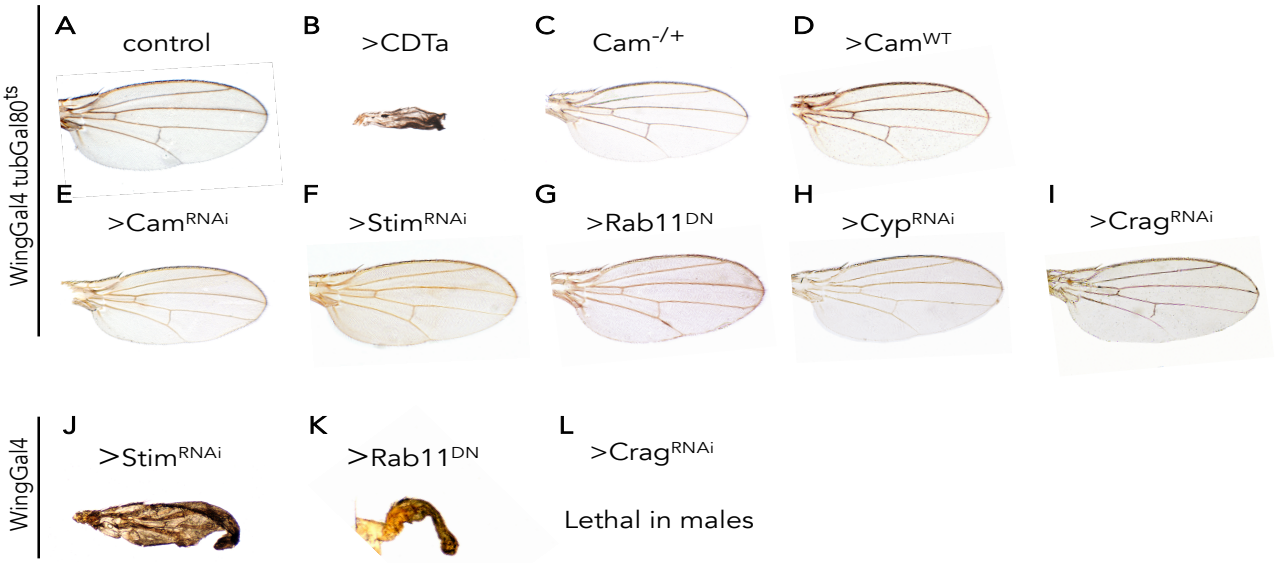

Figure S5. Wing phenotypes from flies expressing various transgenes. Related to figures 4 and 5.

Phenotypes of wings obtained from male flies grown at with 25ºC, specific genotypes are indicated. In the two upper rows, tubGAL80ts partially inhibits GAL4-dependent expression of the indicated transgenes. Except for UAS-CDTa, all transgenes in this study do not induce a phenotype when driven by MS1096 GAL4 + tubGAL80ts. On the third row, wing phenotypes in absence of tubGAL80ts, when applicable, are shown: $Stim^{RNAi}$ and $Rab11^{DN}$ induce clear wing phenotypes or lethality ($Crag^{RNAi}$) when driven by MS1096 GAL4 without tubGAL80ts suppression. All other transgenes did not induce any wing phenotype, even in the absence of tubGAL80-ts.

TRANSPARENT METHODS

Ethics statement.

All experiments were performed in strict accordance with guidelines from the National Institute of Health and the Animal Welfare Act, approved by the Animal Care and Use Committee of University of California, San Diego and the National Institute of Allergy and Infectious Diseases, National Institutes of Health (approved protocols s00227m and LPD-8E). All efforts were made to minimize suffering of animals employed in this study.

UAS-CDTa plasmid construction.

A PCR amplified cDNA fragment from *C. difficile* chromosomal DNA (strain R20291) encoding the enzymatically active CDTa moiety was inserted into the pUASt vector between the EcoR1 and Xba1 sites of the polylinker (details available upon request). The UAS-CDTa transgene was stably transformed into the *Drosophila* genome by BestGene. Two lines carrying an insertion on the second or the third chromosome were isolated.

Drosophila genetics.

Flies carrying the UAS-CDTa construct (CDTa) were crossed to various GAL4 driver stocks to drive toxin expression in specific tissues. In some experiments, timing of GAL4 activity was limited by a temperature sensitive form of GAL80 (*tubulin*-GAL80ts), which

inhibits GAL4 function below 25°C. UAS-EF was described previously (Guichard et al., 2010). Other lines used in this study: wingGal4 (MS1096-GAL4), NP1-GAL4, Hindgut-GAL4, VgGal4. Lines obtained from Bloomington *Drosophila* Stock Center (BDSC, Bloomington, IN): UAS-Cam^{RNAi} (P{TRiP.HMS01318}, # 34609), Cam^{n339}/CyO and UAS-Cam^{WT} (both derived from #6809), UAS-Stim^{RNAiE} (#41759), UAS-Stim^{RNAi}JF02567 (#27263), UAS-Crag^{RNAi} (#53261), UAS-Cyp1^{RNAi} (#33950), Rab11^{J2D1}/TM3 (#12148) and UAS-Rab11^{DNYFP} (#23261).

Survival assays.

In survival assays, expression of GAL4 activity was held by a temperature sensitive form of GAL80, which inhibits GAL4 function below 25°C. TubGAL80ts NP1/+ (control) and tubGAL80ts NP1>CDTa (>CDTa) males were grown at RT and transferred to 29^{o}C a week after hatching. Fly survival was monitored daily. Surviving flies were transferred to clean vials dusted with ground potato flakes every three days.

Immunological stains of wing imaginal discs, salivary glands and guts.

Wing imaginal discs and salivary glands were dissected, fixed in 1X PBS with 4% formaldehyde for 30 minutes, stained with either fluorescent Phalloidin or primary antibodies at 4^{o}C overnight; when using primary antibodies, fluorescent secondary antibodies were incubated at RT for two hours. Tissues were left attached to carcasses until ready to mount in SlowFade (LifeTechnologies, Carlsbad, CA, #S36936) as previously described (Guichard et al.). Double sided tape was used as a spacer to prevent tissue squashing.

Guts were dissected, fixed in 1X PBS with 4% formaldehyde for 40 minutes, stained with fluorescent Phalloidin or primary antibodies overnight; when using primary antibodies, fluorescent secondary antibodies were incubated at RT for two hours. Guts were mounted in SlowFade using double sided tape as spacer. The anterior section of the midgut, located right before the middle midgut was selected for imaging. Images were collected by confocal microscopy on a Leica TCS SP5 (Leica, Wetzlar, Germany). Images were acquired using a 63X objective, and higher magnifications were obtained using a 4X digital zoom. Antibodies and dilutions used were as follows: mouse Rab11 (1/200, BD Biosciences, San Jose, CA, #610657, RRID #AB_397984), DE-cad (1/500, DSHB, Iowa city, IA, #DCAD2, RRID #AB_2314298), D-Cat (1/500, DSHB # Dcat-1-s, RRID #AB_532377), mouse anti-Tubulin (1/100, Santa Cruz Biotechnology, Dallas, TX, sc-23948, RRID #AB_628410), Alexa Fluor 488 Phalloidin (1/100, Invitrogen, Carlsbad, CA, A12379, RRID # AB_231514), DAPI (Molecular Probes, Eugene, OR, D-1306, RRID #AB_2619482).

Data Presentation and Statistics.

Numerical data is presented as mean +/-SD. All graphs were generated and statistics calculated using the Prism-GraphPad software (San Diego, CA). T-test statistical analyses were performed whenever two conditions were compared to each other. When more than two conditions are presented in one graph, we performed ANOVA analysis. For survival assays we performed a log-rank (Mantel–Cox) test**.** All p values are provided in the figures. **** Indicates $p<0.0001$.

Wing phenotype analysis.

Measurements of the surface area of multiple wings for each genotype and condition were conducted using Photoshop® and analyzed with Prism8.