



Figures and figure supplements

Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach

Mark Austin Hanson et al

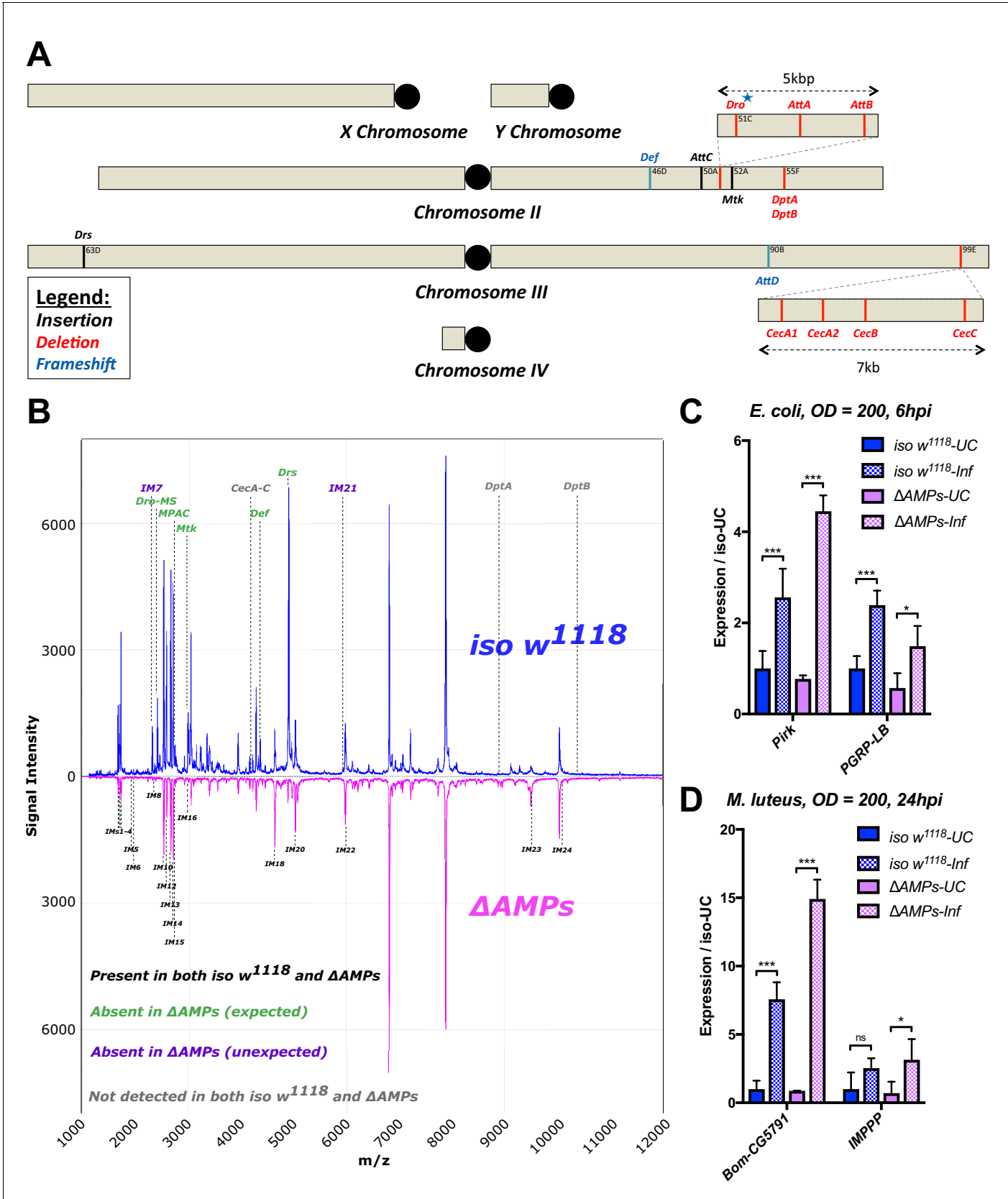


Figure 1 continued

nonsense peptide. (B) A representative MALDI-TOF analysis of hemolymph samples from immune-challenged (1:1 *E. coli* and *M. luteus* at OD600 = 200) *iso w*¹¹¹⁸ and Δ AMPs flies as described in **Uttenweiler-Joseph et al. (1998)**. No AMP-derived products were detected in the hemolymph samples of Δ AMPs flies. No signals for IM7, nor IM21 were observed in the hemolymph samples of Δ AMPs mutants suggesting that these uncharacterized immune-induced molecules are the products of AMP genes. The Imd pathway (C) and Toll pathway (D) are functional and respond to immune challenge in Δ AMPs flies. We used alternate readouts to monitor the Toll and Imd pathways: *pirk* and *PGRP-LB* for Imd pathway and *CG5791* (*Bomanin*) and *IMPPP* for Toll signaling (**De Gregorio et al., 2002; Hanson et al., 2016**). UC = unchallenged, Inf = infected. hpi = hours post-infection. Expression normalized with *iso w*¹¹¹⁸-UC set to a value of 1.

DOI: <https://doi.org/10.7554/eLife.44341.003>

		Genes affected
<i>Def</i> wt <i>Def</i> ^{SK3}	GCGCAGGCTCAGCCAGTTTCCGATGTGGATCCAATTC GCGCAGGCTCAGCC t G a a T - - - - TGTGGATCCAATTC	Defensin - Group A
<i>Cec</i> wt <i>Cec</i> ^{SK6}	GCTTGGAATCAG / 6,095bp deletion / GTCCATCAAAGG GCTTGGAAT - - - - - CATCAAAGG	Cecropin A1, Cecropin A2, Cecropin B, Cecropin C - Group A
<i>Dro</i> wt <i>Dro</i> ^{SK4}	TTGCCATGGGTGTGGCCACT - CCCGGCAAGCCACGCC TTGCCATGG c TGTGGCCACT c CCCGGCAAGCCACGCC	Drosocin - Group B
<i>Dro-AttAB</i> wt <i>Dro-AttAB</i> ^{SK2}	TCAGTTCGATTT / 4,010bp deletion / CGGTTAAATATT TCAGTTCGA - - - - - TTAAATATT	Drosocin, Attacin A, Attacin B - Group B
<i>Dpt</i> wt <i>Dpt</i> ^{SK1}	TAGATAAGGTGA / 2,137bp deletion / AGGGCACTTCAG TAGATAAGG - - - - - GCACTTCAG	Diptericin A, Diptericin B - Group B
<i>AttD</i> wt <i>AttD</i> ^{SK1}	CAACCGCCCAATGCGGAGTAAGGGTCGGTGATGATCT CAACCGCCCAATGCGG - - - - - AGGGTCGGTGATGATCT	Attacin D - Group B
<i>Mtk</i> wt <i>Mtk</i> ^{R1}	ATTCCCGCCACCGAGCTAAGATGCAACTTAATCTTGG ATTCCCGCCACCGAGCTAAG g c t a g c a c a t a t g c a g g	Metchnikowin - Group C
<i>Drs</i> wt <i>Drs</i> ^{R1}	CCGTGAGAACCTTTTCCAATATGATGCAGATCAAGTA CCGTGAGAACCTTTTCCAAT g c t a g c a c a t a t g c a g g	Drosomycin - Group C

Figure 1—figure supplement 1. Genetic description of mutations generated in this study. The *Mtk*^{R1} and *Drs*^{R1} mutations entirely replaced the CDS with an insert from the piHR vector. Non-synonymous nucleotides in mutants are given in red. Mutations are listed according to groups in **Figures 3–6** (discussed later).

DOI: <https://doi.org/10.7554/eLife.44341.004>

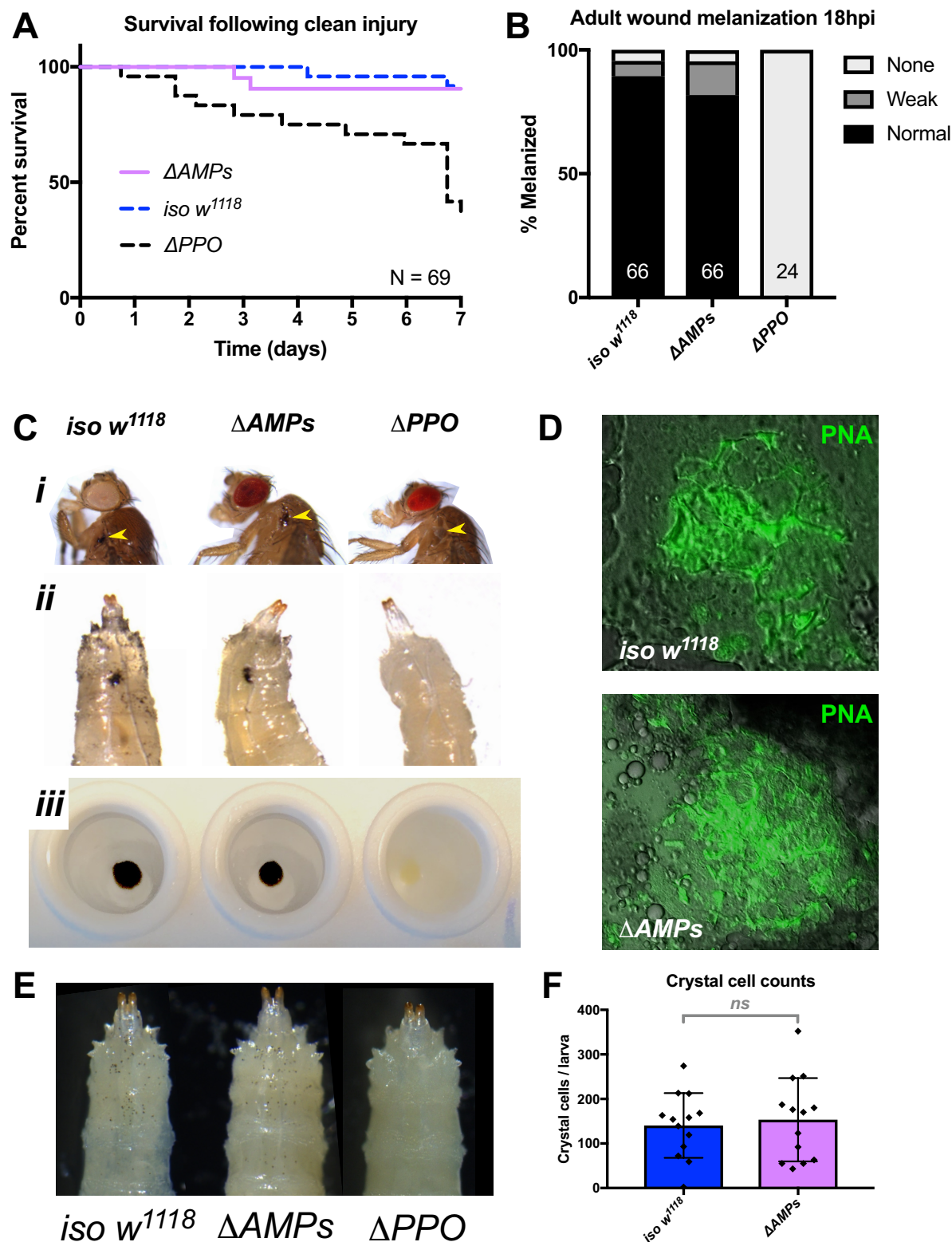


Figure 1—figure supplement 2. $\Delta AMPs$ flies have otherwise wild-type immune reactions. (A) $\Delta AMPs$ flies survive clean injury like wild-type flies, while ΔPPO mutants deficient for melanization have reduced survival over time. (B) $\Delta AMPs$ flies melanize the cuticle similar to wild-type flies following Figure 1—figure supplement 2 continued on next page

Figure 1—figure supplement 2 continued

pricking ($\chi^2 = 2.14$, $p=0.34$). Melanization categories (None, Weak, Normal) were as described in Dudzic et al (**Dudzic et al., 2018**). Sample sizes (n) are included in each bar. (C) Melanization in *iso w¹¹¹⁸*, Δ AMPs, and Δ PPO flies of the cuticle in adults (i, yellow arrowheads), larvae (ii, melanized wounds), and larval hemolymph (iii). (D) To investigate clotting ability, we used the hanging drop assay (**Scherfer et al., 2004**) with Δ AMPs larval hemolymph and visualized clot fibers with PNA staining (green). Both *iso w¹¹¹⁸* and Δ AMPs hemolymph produced visible clot fibres measured after 20 min. Hemocyte populations are normal in Δ AMPs flies, including crystal cell distribution (E) and number (F).

DOI: <https://doi.org/10.7554/eLife.44341.005>

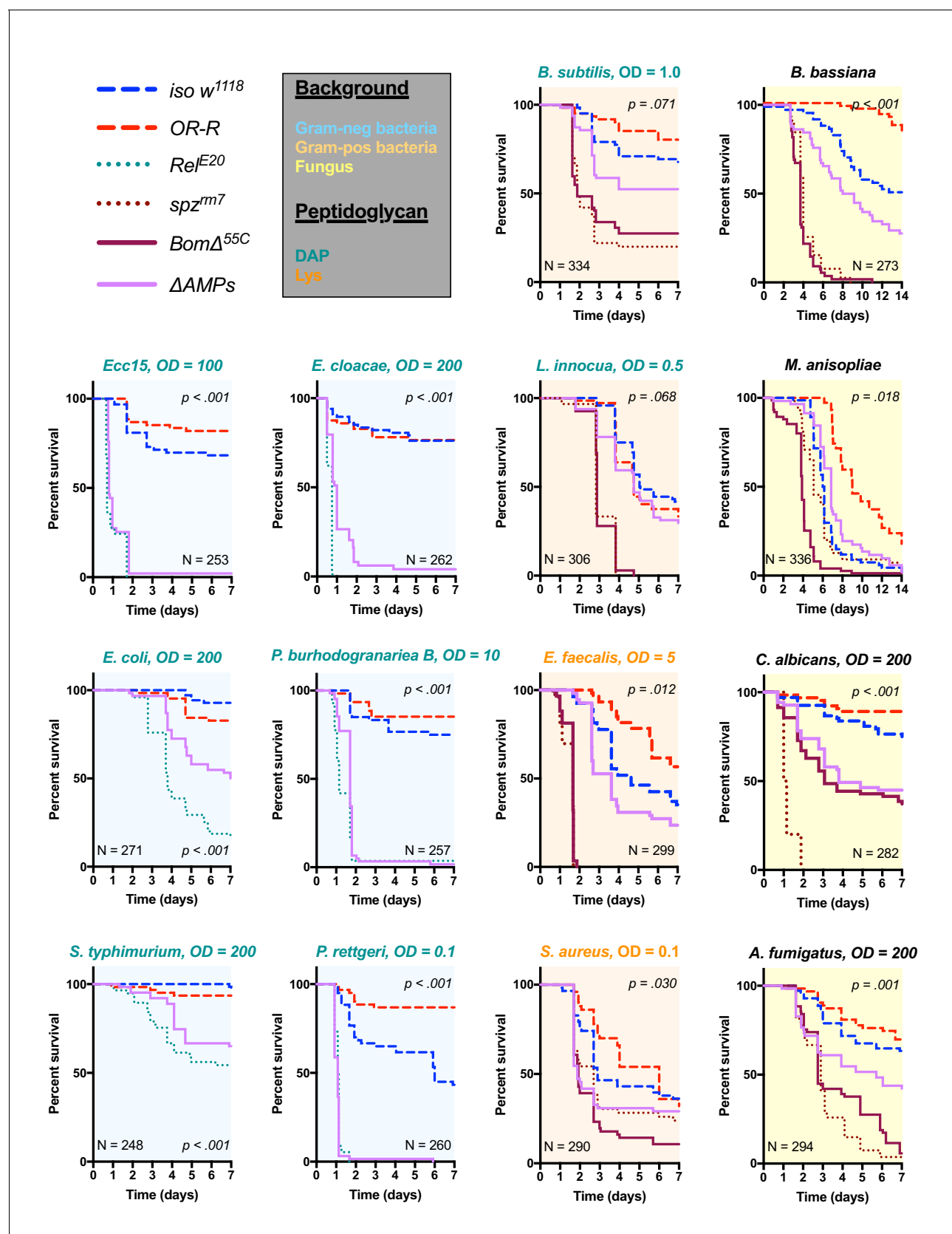


Figure 2. Survival of $\Delta AMPs$ flies to diverse microbial challenges. Control lines for survival experiments included two wild-types (w ; *Drosophila* ($iso w^{1118}$) and Oregon R (OR-R) as an alternate wild-type), mutants for the Imd response (Rel^{E20}), mutants for Toll signaling (spz^{rm7}), and mutants for Bomanins

Figure 2 continued on next page

Figure 2 continued

(*Bom*^{Δ55C}). ΔAMPs flies are extremely susceptible to infection with Gram-negative bacteria (blue backgrounds). Unexpectedly, ΔAMPs flies were not markedly susceptible to infection with Gram-positive bacteria (orange backgrounds), while *Bom*^{Δ55C} flies were extremely susceptible, often mirroring *spz*^{rm7} mutants. This pattern of *Bom*^{Δ55C} susceptibility held true for fungal infections (yellow backgrounds). ΔAMPs flies are somewhat susceptible to fungal infections, but the severity shifts with different fungi. Pellet densities are reported for all systemic infections in OD at 600 nm. p-Values are given for ΔAMPs flies compared to *iso w*¹¹¹⁸ using a Cox-proportional hazards model. N = total number of flies in experiments. A full description of p-values relative to *iso w*¹¹¹⁸ can be found in **Figure 2—source data 1**.

DOI: <https://doi.org/10.7554/eLife.44341.006>

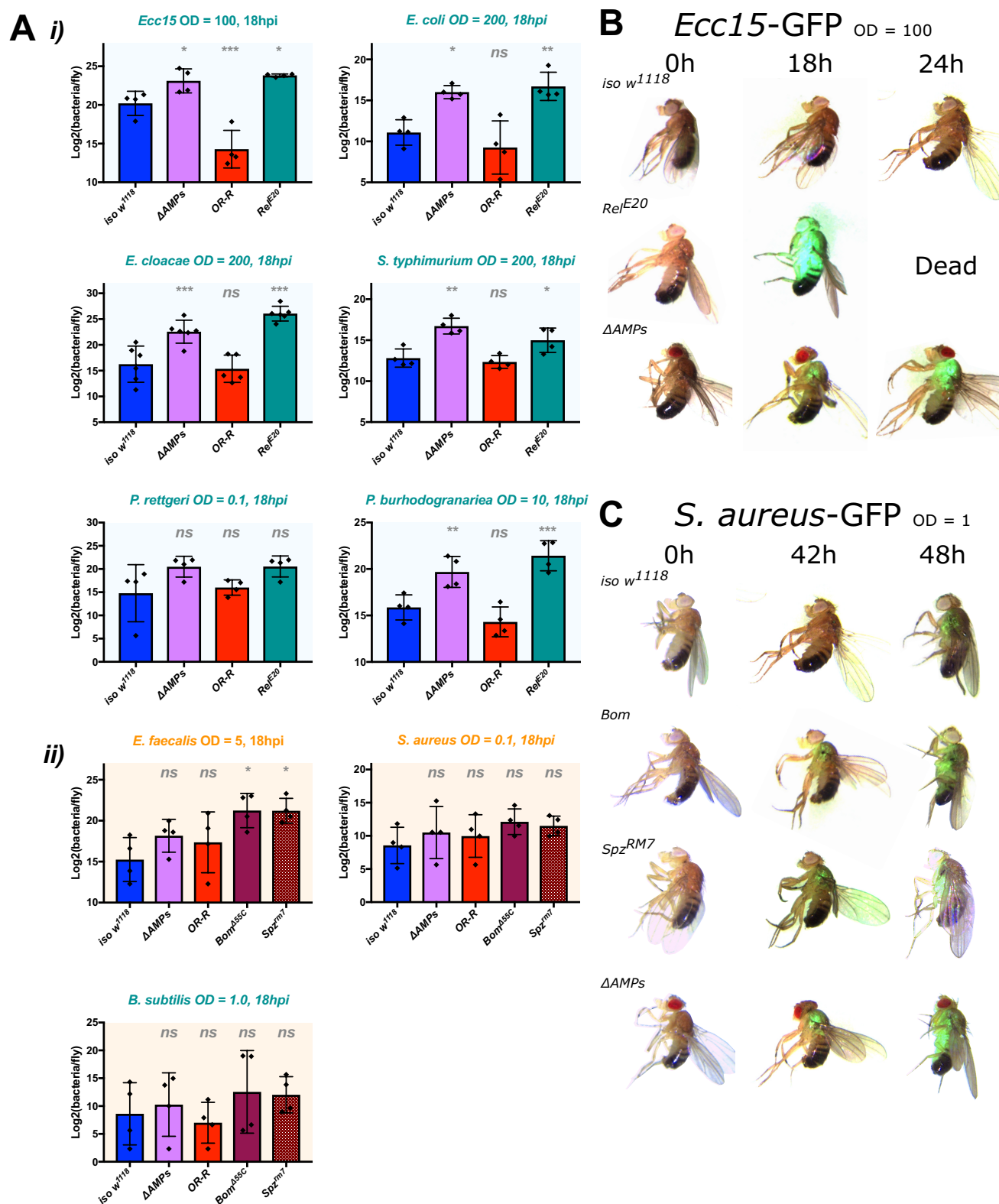


Figure 2—figure supplement 1. $\Delta AMPs$ flies fail to suppress Gram-negative bacterial growth. Colony counts were performed on pooled samples (five flies) for bacteria amenable to LB agar, a medium that avoids overnight growth of the host microbiota. (A) For Gram-negative bacterial infections, Figure 2—figure supplement 1 continued on next page

Figure 2—figure supplement 1 continued

Δ AMPs flies have significantly higher bacterial loads compared to *iso w*¹¹¹⁸ at 18 hr post-infection (hpi) (i). This is not true for any of the Gram-positive bacteria tested (ii), while *spz*^{m7} mutants carried higher bacterial loads, significantly so in *E. faecalis* infections. Gram-negative (B) and Gram-positive (C) infections with GFP-labelled bacteria spread from the wound site systemically in all genotypes tested. Thus, Δ AMPs fly mortality is likely not due to tissue-specific colonization by invading bacteria, but rather a failure to suppress bacterial growth first locally, and then systemically. One-way ANOVA: not significant = ns, $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = *** relative to *iso w*¹¹¹⁸.

DOI: <https://doi.org/10.7554/eLife.44341.007>

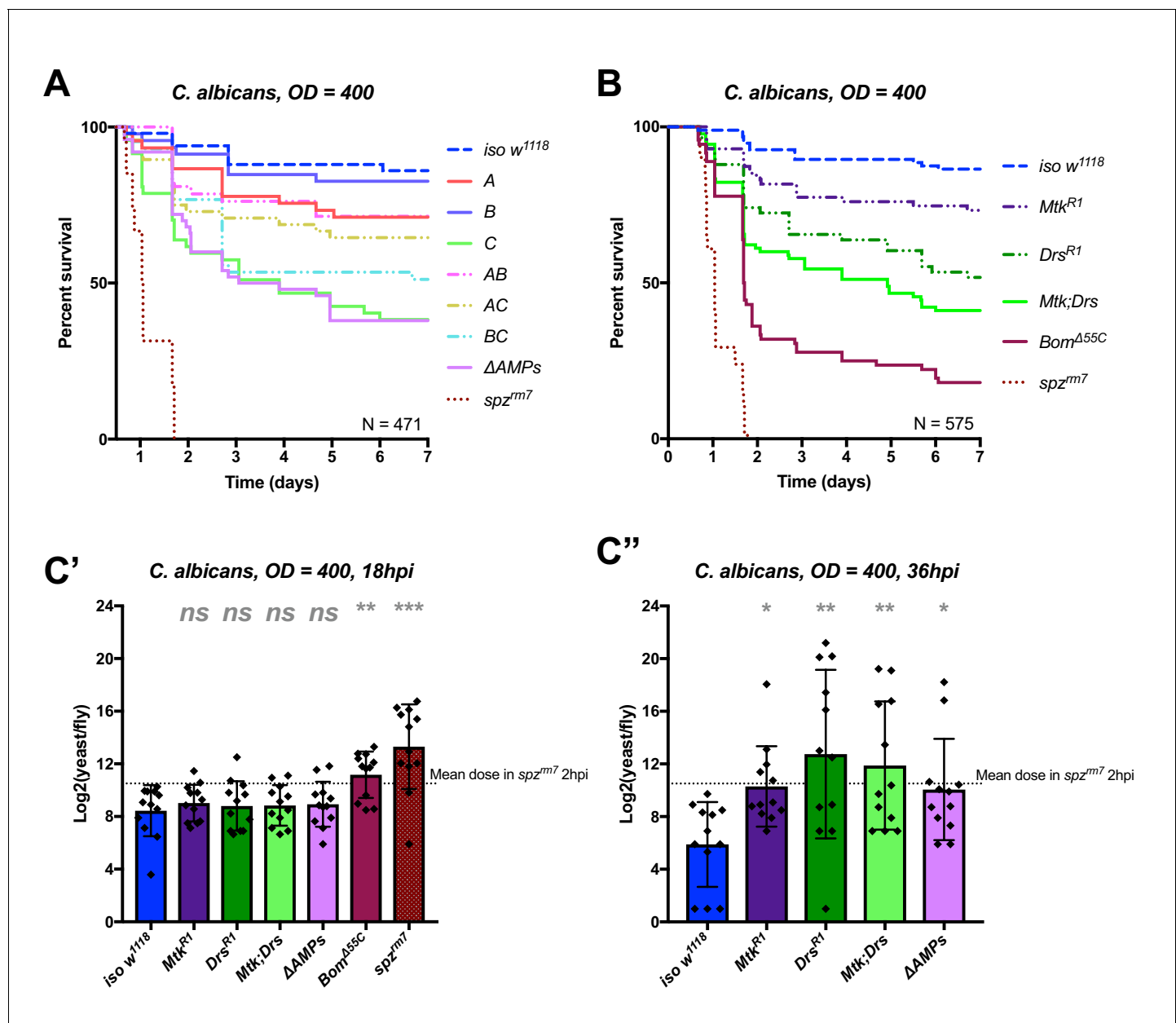


Figure 3. Identification of AMPs involved in the susceptibility of Δ AMPs flies to *C. albicans*. (A) Survival of mutants for groups of AMPs reveals that loss of only Toll-responsive Group C peptides (Metchnikowin and Drosomycin) is required to recapitulate the susceptibility of Δ AMPs flies. Co-occurring loss of groups A and C has a net protective effect (A^*C : HR = -1.71 , $p=0.002$). (B) Further dissection of Group C mutations reveals that both Metchnikowin and Drosomycin contribute to resist *C. albicans* survival ($p=0.008$ and $p<0.001$, respectively). The interaction of Metchnikowin and Drosomycin was not different from the sum of their individual effects (Mtk^*Drs : HR = -0.80 , $p=0.116$). (C) Fungal loads of individual flies at 18 hpi. At this time point, *Bom^{Δ55C}* mutants and *spz^{rm7}* flies have already failed to constrain *C. albicans* growth (C'). Fungal titres at 36 hpi (C''), a time point closer to mortality for many AMP mutants, show that some AMP mutants fail to control fungal load, while wild-type flies consistently controlled fungal titre. One-way ANOVA: not significant = ns, $p<0.05$ = *, $p<0.01$ = **, and $p<0.001$ = *** relative to *iso w¹¹¹⁸*.

DOI: <https://doi.org/10.7554/eLife.44341.009>

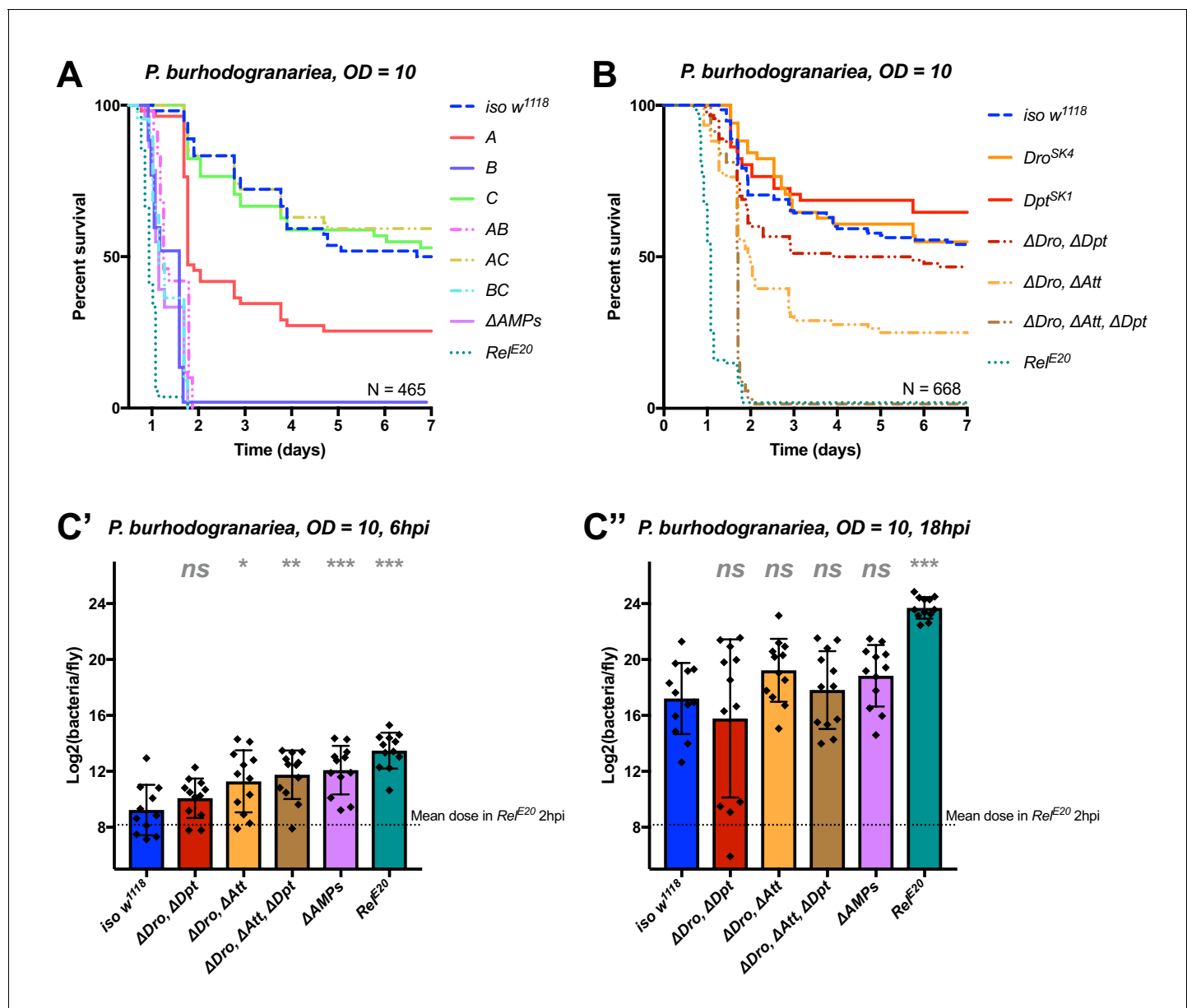


Figure 4. Identification of AMPs involved in the susceptibility of Δ AMPs flies to *P. burhododranaria*. (A) Survival of mutants for groups of AMPs reveals that loss of Imd-responsive Group B peptides (Drosocin, Attacins, and Dipterocins) recapitulates the susceptibility of Δ AMPs flies. Loss of Group A peptides also resulted in strong susceptibility ($p < 0.001$) due to additive effects of Defensin and Cecropins (Figure 4—figure supplement 4). (B) Further dissection of AMPs deleted in Group B reveals that only the loss of all Drosocin, Attacin, and Dipterocin gene families leads to susceptibility similar to Δ AMPs flies. Simultaneous loss of Attacins and Dipterocins results in a synergistic loss of resistance (Δ Att* Δ Dpt: HR = +1.45, $p < 0.001$). (C) Bacterial loads of individual flies at 6 hpi (C'). At this time point, most AMP mutants had significantly higher bacterial loads compared to wild-type flies. At 18 hpi (C''), differences in bacterial load are reduced, likely owing to the high chronic load *P. burhododranaria* establishes even in surviving flies (Duneau et al., 2017). Meanwhile *Rel^{E20}* flies succumb ~18 hr earlier than Δ AMPs flies in survival experiments, and already have significantly higher loads. One-way ANOVA: not significant = ns, $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = *** relative to *iso w¹¹¹⁸*.

DOI: <https://doi.org/10.7554/eLife.44341.010>

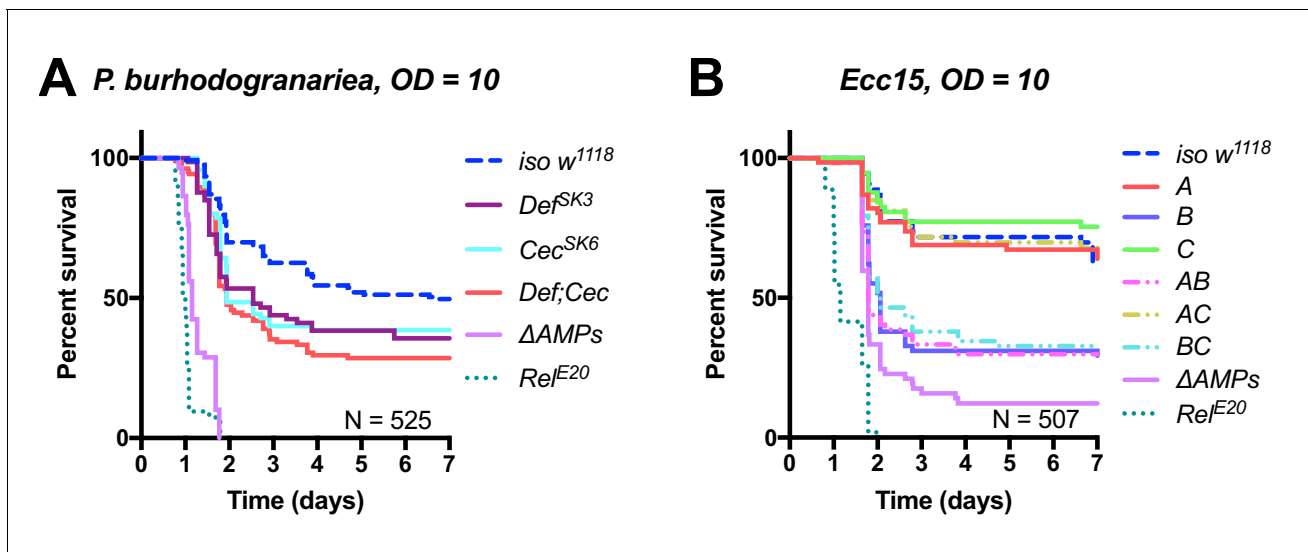


Figure 4—figure supplement 1. Further dissecting effects of AMP groups. (A) Dissection of the susceptibility of Group A flies lacking *Defensin* and *Cecropins* reveals that combined mutants have an additive loss of resistance (*Def***Cec*, HR = +0.36, p=0.342). (B) Upon infection with the Gram-negative *Ecc15*, Group B peptides (Drosocin, Attacins and Dipterocins) explain the bulk of mortality, but additional loss of other peptides in Δ AMPs flies leads to increased mortality (Log-Rank p=0.013).

DOI: <https://doi.org/10.7554/eLife.44341.011>

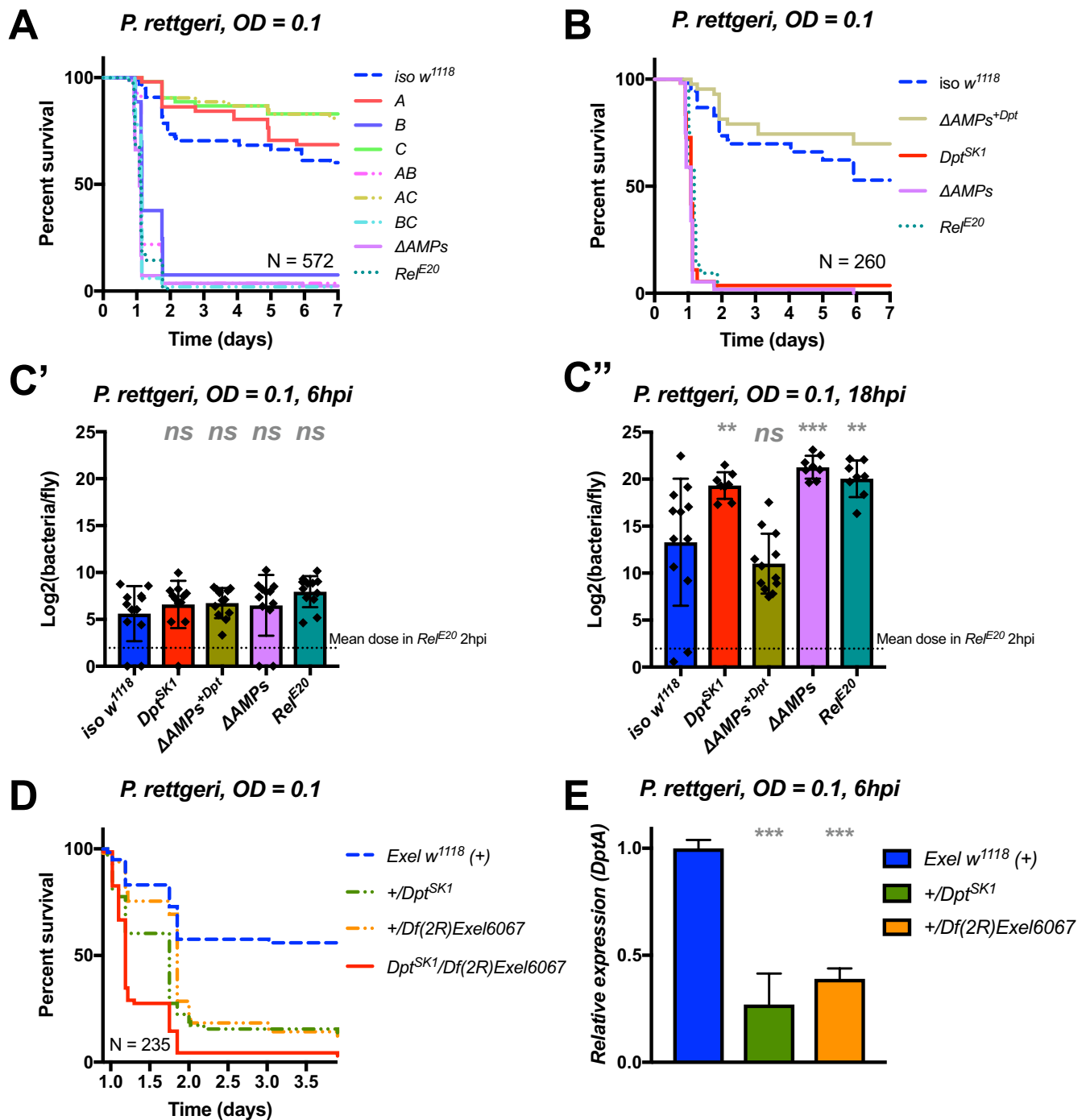


Figure 5. Identification of AMPs involved in the susceptibility of Δ AMPs flies to *P. rettgeri*. (A) Survival of mutants for groups of AMPs reveals that only loss of Imd-responsive Group B peptides (Drosocin, Attacins, and Dipterocins) recapitulates the susceptibility of Δ AMPs flies. (B) Further dissection of the mutations affected in Group B reveals that only the loss of Dipterocins (*Dpt^{SK1}*) leads to susceptibility similar to Δ AMPs flies. Remarkably, flies lacking all other AMPs (Δ AMPs^{+Dpt}) resist as wild-type. (C) Bacterial loads of individual flies are similar at 6hpi (C'), but by 18hpi (C''), *Dpt* mutants and *Rel^{E20}* flies have all failed to control *P. rettgeri* growth. (D) Heterozygote flies for *Dpt^{SK1}* and a deficiency including the *Diptericins* and flanking genes (*Df(2R) Exel6067*) recapitulates the susceptibility of *Diptericin* mutants. Intriguingly, heterozygotes with one functional copy of the *Diptericins* (Δ AMPs^{+Dpt^{SK1} or Δ AMPs^{+Df(2R)Exel6067) resist as wild-type. (E) Relative expression of *DptA* in Δ AMPs flies reveals that *Dpt^{SK1}* and *Df(2R)Exel6067* are both required for normal expression of *DptA*. Figure 5 continued on next page}}

Figure 5 continued

(2R)Exel6067) are nonetheless highly susceptible to infection. (E) *Diptericin A* transcriptional output is strongly reduced in heterozygotes 6 hpi compared to wild-type flies. One-way ANOVA: not significant = ns, $p < 0.05 = *$, $p < 0.01 = **$, and $p < 0.001 = ***$ relative to *iso w*¹¹⁸.

DOI: <https://doi.org/10.7554/eLife.44341.012>

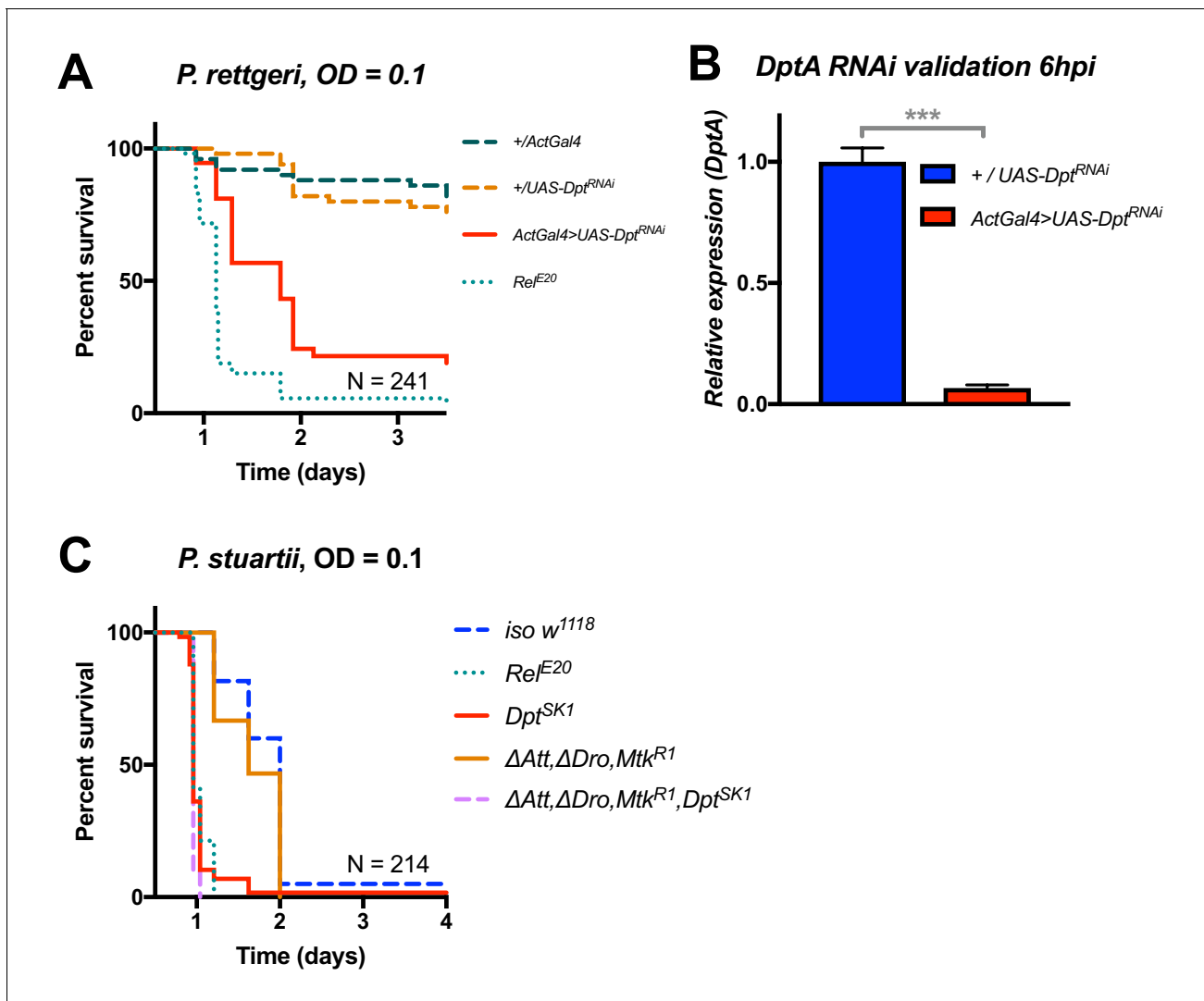


Figure 5—figure supplement 1. Additional validation of the role of *Dipteracin* in resistance to *Providencia*. (A) Silencing of *Dipteracin* by RNAi leads to higher susceptibility to *P. rettgeri* infection ($p < 0.001$). (B) Validation of the *Dipteracin* RNAi construct 6 hpi. (C) Mutants lacking multiple peptides (Attacins, Drosocin, and Metchnikowin) succumb to *P. stuartii* infection as wild-type ($\Delta\text{Att}, \Delta\text{Dro}, \text{Mtk}^{\text{R1}}$), while *Dipteracin* mutation alone (Dpt^{SK1}) or combined ($\Delta\text{Att}, \Delta\text{Dro}, \text{Mtk}^{\text{R1}}, \text{Dpt}^{\text{SK1}}$) leads to a susceptibility similar to Rel^{E20} mutants. This pattern of survival was similar to the pattern observed with *P. rettgeri*. One-way ANOVA: $p < 0.001 = ***$.

DOI: <https://doi.org/10.7554/eLife.44341.013>

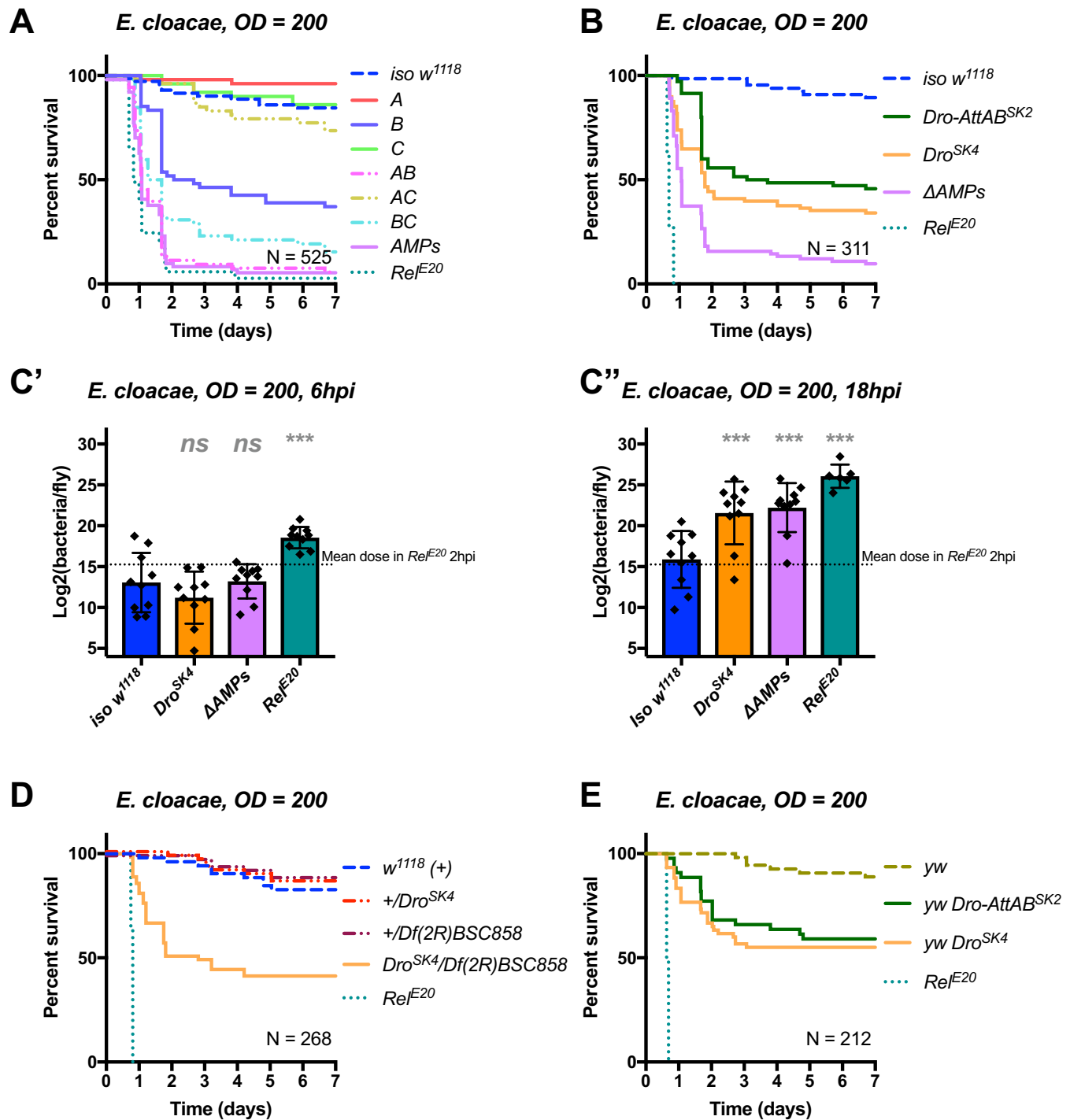


Figure 6. Identification of AMPs involved in the susceptibility of Δ AMPs flies to *E. cloacae*. (A) Survival of mutants for groups of AMPs reveals that loss of Imd-responsive Group B peptides (Drosocin, Attacins, and Dipterocins) results in a strong susceptibility to infection ($p < 0.001$), while loss of Group A or C peptides alone resists as wild-type ($p > 0.1$ each). Group AB flies were as susceptible as Δ AMPs flies, and we observed a synergistic interaction between Group A and B mutations (A*B: HR = +2.55, $p = 0.003$). (B) Further dissection of the mutations in Group B revealed that loss of Drosocin alone (*Dro^{SK4}*), or a deficiency lacking both Drosocin and Attacins AttA and AttB (*Dro-AttAB^{SK2}*) recapitulates the susceptibility of Group B flies. (C) By 18hpi, bacterial loads in individual Drosocin mutants or *Rel^{E20}* flies are significantly higher than wild-type. (D) Heterozygote flies for *Dro^{SK4}* and *Df(2R)BSC858* (a

Figure 6 continued on next page

Figure 6 continued

deficiency removing *Drosocin*, *Attacins AttA* and *AttB*, and other genes) are strongly susceptible to *E. cloacae* infection. (E) *Drosocin* mutants in an alternate genetic background (*yw*) are susceptible to *E. cloacae*. One-way ANOVA: not significant = *ns*, and $p < 0.001$ = *** relative to *iso w*¹¹¹⁸.

DOI: <https://doi.org/10.7554/eLife.44341.014>

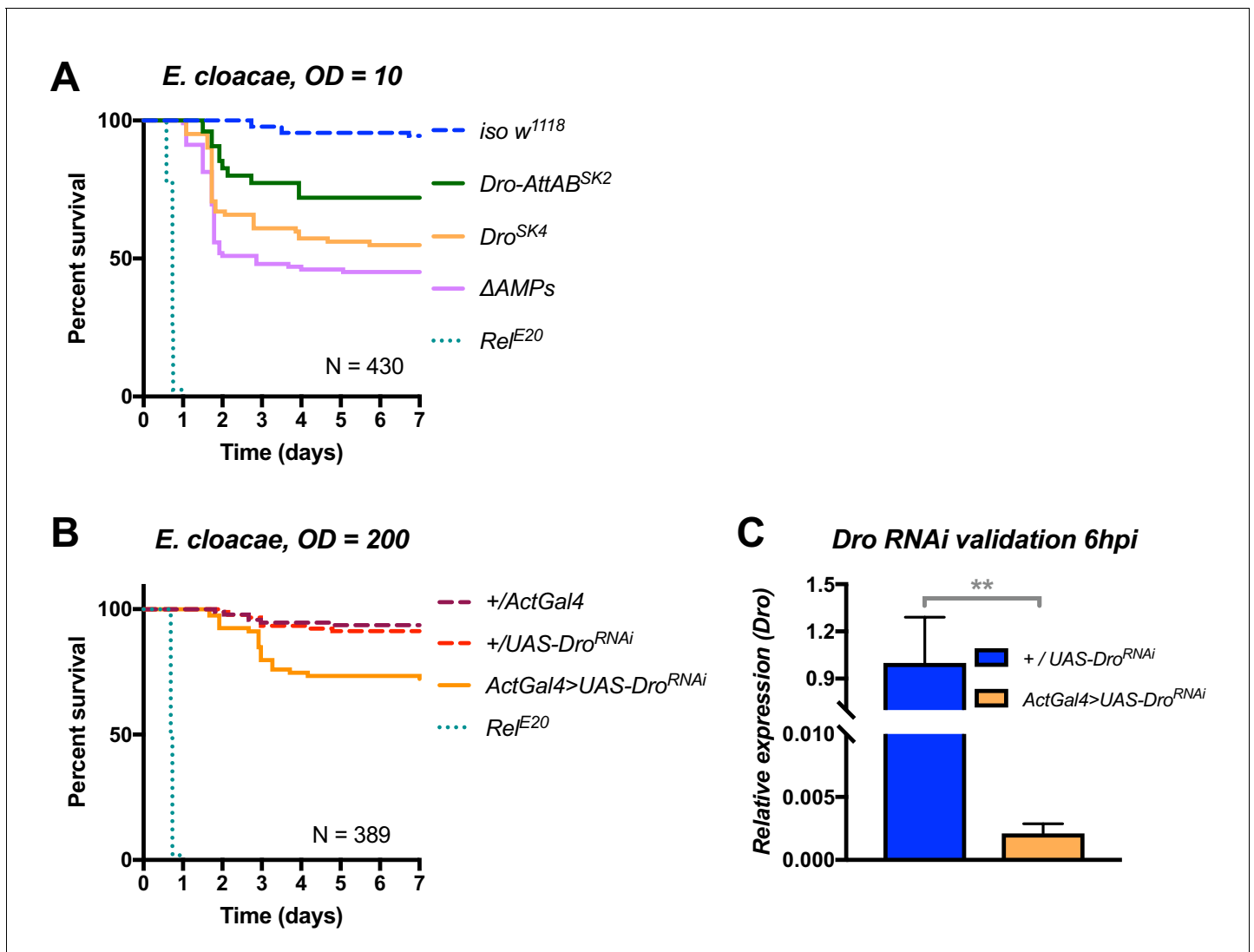


Figure 6—figure supplement 1. Additional validation of the role of *Drosocin* in defence against *E. cloacae*. (A) *Drosocin* mutant susceptibility remains even at a lower dose (OD = 10, ~7000 bacteria/fly), while *Rel^{E20}* flies succumb rapidly regardless of initial dose. (B) Silencing of *Drosocin* by RNAi leads to significant mortality from *E. cloacae* infection ($p < 0.001$). (C) Validation of the *Drosocin* RNAi construct 6hpi.

DOI: <https://doi.org/10.7554/eLife.44341.015>