Regulation of Toll Signaling and Inflammation by β-Arrestin and the SUMO Protease Ulp1

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ABSTRACT The Toll signaling pathway has a highly conserved function in innate immunity and is regulated by multiple factors that fine tune its activity. One such factor is β-arrestin Kurtz (Krz), which we previously implicated in the inhibition of developmental Toll signaling in the *Drosophila melanogaster* embryo. Another level of controlling Toll activity and immune system homeostasis is by protein sumoylation. In this study, we have uncovered a link between these two modes of regulation and show that Krz affects sumoylation via a conserved protein interaction with a SUMO protease, Ulp1. Loss of function of *krz* or *Ulp1* in *Drosophila* larvae results in a similar inflammatory phenotype, which is manifested as increased lamellocyte production; melanotic mass formation; nuclear accumulation of Toll pathway transcriptional effectors, Dorsal and Dif; and expression of immunity genes, such as *Drosomycin*. Moreover, mutations in *krz* and *Ulp1* show dosage-sensitive synergistic genetic interactions, suggesting that these two proteins are involved in the same pathway. Using Dorsal sumoylation as a readout, we found that altering Krz levels can affect the efficiency of SUMO deconjugation mediated by Ulp1. Our results demonstrate that β-arrestin controls Toll signaling and systemic inflammation at the level of sumoylation.

THE establishment of the immune system during development and subsequent maintenance of its homeostasis in the adult is under the control of several conserved signaling pathways (Evans *et al.* 2003; Lemaitre and Hoffmann 2007). A proper balance of activating and inhibitory mechanisms ensures a robust defense response to infection and at the same time prevents inappropriate activation of the immune pathways that can lead to inflammation, tissue damage, and cancer.

In both insects and mammals, Toll and related receptors are central for the function of the immune system (Lemaitre and Hoffmann 2007; Ganesan *et al.* 2011). The core components of the Toll pathway in *Drosophila* include the ligand Spätzle, the receptor Toll, the intracellular adaptors MyD88 and Tube, and the kinase Pelle. Toll signaling regulates the nuclear accumulation of the NF-κB homologs Dorsal (Dl) and Dif. Inappropriate activation of the Toll pathway often leads to a systemic inflammation phenotype, which is defined as activation of blood cells and elevated expression of innate immunity genes in the absence of infection (Paddibhatla *et al.* 2010; Ganesan *et al.* 2011).

An emerging control mechanism involved in the regulation of the core components of Toll/NF-κB signaling is sumoylation. Modification of proteins by SUMO (small ubiquitin-related modifier) has been shown to play a role in controlling multiple cellular functions, such as nucleocytoplasmic transport, transcriptional regulation, protein stability, signal transduction, and cell cycle progression (Geiss-Friedlander and Melchior 2007). In mammalian systems, the effects of sumoylation on NF-κB signaling are diverse and complex, including regulation of IκBα degradation, modulation of NEMO/IKKγ activity, and both activation and repression of transcription by NF-κB (Mabb and Miyamoto 2007). In the *Drosophila* genome, components of sumoylation machinery are mostly represented by single genes, making it an attractive system to dissect the role of sumoylation in Toll/NF-κB signaling (Talamillo *et al.* 2008a; Smith *et al.* 2012).

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Genetic analyses as well as experiments in cultured cells showed the important but conflicting roles of sumoylation in regulating Toll signaling in *Drosophila*. Loss-of-function mutations in *lesswright* (*lwr*), which encodes the *Drosophila* E2 SUMO-conjugating enzyme Ubc9, were found to cause overproliferation of hematopoietic precursors, production of abnormally high levels of the differentiated blood cells called lamellocytes, and formation of lamellocyte-containing melanotic masses (Chiu *et al.* 2005; Huang *et al.* 2005; Paddibhatla *et al.* 2010; Kalamarz *et al.* 2012). Loss of *lwr* was also associated with increased expression of *Drosomycin* (*Drs*) as well as nuclear accumulation of the Toll transcriptional effector Dl in the hemocytes, suggesting that Ubc9 exerts an inhibitory effect on Toll signaling in larvae and protects the organism from abnormal inflammation (Chiu *et al.* 2005; Huang *et al.* 2005). However, in other reports, Ubc9/Lwr was shown to bind Dl directly and promote its sumoylation, which increased Dl nuclear retention and potentiated Dl-dependent transcriptional activation in cultured cells (Bhaskar *et al.* 2000, 2002). Moreover, components of the sumoylation machinery such as SUMO/Smt3 and Ubc9/Lwr were required for expression of antimicrobial peptide genes in *Drosophila* larvae and cultured cells after exposure to microbial elicitors (Bhaskar *et al.* 2002), suggesting a positive role for sumoylation in Toll activation. Recently, sumoylation was shown to be required for antimicrobial gene expression in the second major regulator of immunity in *Drosophila*, the immune deficiency (IMD) pathway (Fukuyama *et al.* 2013). Therefore, further investigation at the genetic and molecular levels is required to elucidate the complex role of sumoylation in Toll signaling, immunity, and inflammation.

Another level of control over the Toll/NF-κB pathway is exerted by β-arrestins. β-Arrestins were initially characterized as mediators of G protein coupled receptor (GPCR) desensitization and endocytosis (Pierce and Lefkowitz 2001). More recently, our knowledge of their signaling functions has dramatically broadened to involve a wide variety of signaling pathways and modes of regulation (Kovacs *et al.* 2009). The *Drosophila* genome encodes a single ortholog of β-arrestins, Kurtz (Krz) (Roman *et al.* 2000), which has been implicated in the regulation of GPCR signaling, as well as Notch, Hedgehog, receptor tyrosine kinase, and Toll pathways (Mukherjee *et al.* 2005; Ge *et al.* 2006; Tipping *et al.* 2010; Molnar *et al.* 2011; Li *et al.* 2012).

Mammalian β-arrestin proteins can down-regulate NF-κB signaling by binding and stabilizing IκBα (Gao *et al.* 2004; Witherow *et al.* 2004) and by preventing autoubiquitination of TRAF6 (Wang *et al.* 2006). We have found that Krz limits the extent of Toll activation during embryogenesis (Tipping *et al.* 2010); however, the molecular details of this regulation are unknown. Here, we show for the first time that β-arrestin can control Toll signaling and systemic inflammation at the level of sumoylation. We demonstrate that loss of *krz* results in an up-regulation of Toll downstream effectors and that Krz exerts its functions by binding to Ulp1, a SUMO protease. Knockdown of *Ulp1* leads to an increase in global sumoylation *in vivo* and causes Toll hyperactivity phenotypes that are similar to the effects of loss of *krz*. Using Dl as a target for Ulp1-mediated desumoylation, we show that Krz is required for the optimal SUMO protease activity of Ulp1, and that both proteins are necessary for limiting the Toll pathway activity and preventing an inappropriate inflammatory response.

Materials and Methods

Plasmid construction

Drosophila Ulp1, Dorsal, SUMO/Smt3, and human SENP1 (Origene) open reading frames were amplified by PCR using tag and/or restriction site-containing primers and cloned into pMT/V5-His series vectors (Invitrogen) to generate carboxy-terminally tagged Ulp1-V5, Ulp1-SBP, and Dorsal-V5, and amino-terminally tagged Flag-SUMO^{GG} (active processed form of SUMO) and Flag-SENP1. For *in vitro* translation, HA-Krz and Ulp1-V5 full-length open reading frames and fragments were cloned into the pSP73 vector (Promega). Dl-K382R-V5 was generated using site-directed mutagenesis. HA-Krz, HA-ARRB1, and HA-ARRB2 constructs were described previously (Tipping *et al.* 2010). Ulp1-V5 was cloned into the pUAST-attB vector (Bischof *et al.* 2007) for transgenic expression in flies.

Fly stocks

The following lines were described previously: *krz^{c01503}* was isolated in a large transposition screen (Thibault *et al.* 2004), *krz^{1}* (Roman *et al.* 2000; Tipping *et al.* 2010), *Dif^{1}* (Rutschmann *et al.* 2000), *da-GAL4* (Wodarz *et al.* 1995), *Cg-GAL4* (Asha *et al.* 2003), *ppl-GAL4* (Zinke *et al.* 1999), *UAS-Dcr-2* (Dietzl *et al.* 2007), *Histone-GFP* (Tipping *et al.* 2010), *Drs-GFP*, *Dpt-lacZ* (*DD1*, provided by D. Ferrandon) (Manfruelli *et al.* 1999), and *krz5.7* (genomic *krz* rescue construct) (Tipping *et al.* 2010). For analysis in homozygous animals, the *krz^{c01503}* chromosome was extensively recombined with the *yw* stock. UAS-driven *krz* and *Ulp1* RNA interference (RNAi) lines were obtained from the Vienna *Drosophila* RNAi Center. *krz^{1}* mutant clones in the fat body were generated by crossing *FRT82B krz^{1}/TM6B* males with *HS-FLP122;;FRT82ubiGFP/TM6B* females and heat shocking the progeny embryos for 1 hr at 37°. The *UAS-Ulp1-V5* line was generated via phiC31-mediated integration into the *attP2* landing site (Bischof *et al.* 2007; Venken and Bellen 2007).

Immunostaining, antibodies, and lamellocyte counting

Antibodies used for tissue immunostaining were as follows: mouse anti-Dorsal (1:70, Developmental Studies Hybridoma Bank) and rabbit anti-Dif (Rutschmann *et al.* 2000) (1:200, provided by D. Ferrandon). For Western blotting, the following antibodies were used: guinea pig anti-Krz (Tipping *et al.* 2010), rabbit anti-pan-arrestin (Affinity Bioreagents), mouse anti-HSP70 (Sigma), rabbit anti-SUMO (provided by A. Courey), rabbit anti-beta-Gal (Molecular Probes), rabbit anti-Flag

(Sigma), mouse anti-V5 (Sigma), mouse anti-HA (Sigma), and rabbit anti-GFP (Molecular Probes). Secondary antibodies were from Jackson Immunoresearch (immunofluorescence) and GE Healthcare (Westerns). For immunoflourescence staining of fat bodies, larvae were dissected in 1× PBS and fixed in 4% formaldehyde for 30 min. Tissues were incubated in PBT (1× PBS with 0.1% Tween-20) with 1% BSA (Rockland) with primary antibody overnight at 4° and with secondary antibody for 2 hr at room temperature. Tissues were mounted with Prolong Gold antifade mounting reagent with DAPI (Invitrogen) and images were acquired with a Zeiss LSM510 confocal microscope.

For lamellocyte counting, 10 third instar larvae of each genotype were washed with 1× PBS. Hemolymph was collected into 700 μl of 1× PBS by making a small incision in the cuticle. Each sample was transferred into a well of a six-well plate with a coverslip treated with concanavalin A and incubated for 2 hr to allow cell attachment. Cells were fixed with 4% formaldehyde followed by incubation with Western blocking reagent (Roche) diluted with PBT and anti-lamellocyte primary antibody (1:10, provided by I. Ando) overnight at 4°. Coverslips were washed with PBT and incubated with fluorescent secondary antibody at room temperature. Coverslips were mounted in Prolong Gold antifade mounting reagent with DAPI (Invitrogen), and images acquired with an Olympus BX60 fluorescence microscope. A total of 20–30 fields of view were collected for each condition, and the assay was repeated in biological triplicates. The percentage of lamellocytes was determined by dividing the total number of lamellocytes observed by the total number of DAPI-positive cells.

Cell culture, immunoprecipitation, in vitro translation, and Western blots

Drosophila 529SU cells used for sumoylation assay (Bhaskar *et al.* 2002) were provided by A. Courey. Schneider 2 (S2) and 529SU cells were cultured at 25° in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 5% Pen/Strep antibiotics (Invitrogen). For DNA transfections, Effectene transfection reagent (Qiagen) was used according to manufacturer's instructions. Empty vector was added to maintain the constant amount of DNA in each transfection sample. The following day, cells were induced with 0.35 mM $CuSO_4$ overnight. Cells were then lysed in default lysis buffer [50 mM Tris pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% IGEPAL, 1.5 mM $MgCl_2$, 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA and 2× Complete protease inhibitor (Roche)]. Cell lysates were incubated with anti-HA, anti-Flag, or anti-V5 beads (Sigma) at 4° for 2.5 hr; beads were washed; and proteins were eluted with SDS sample buffer. For Western blotting, proteins were separated on 8% SDS–PAGE and transferred onto PVDF membrane (Millipore). Membranes were blocked for 1 hr with blocking solution (5% nonfat milk, 2% BSA in TTBS: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% Tween-20), incubated with primary antibodies overnight at 4°, and then with HRP-conjugated secondary antibodies (GE Healthcare) for 1 hr at room temperature. Signals were developed with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and images were acquired using the Kodak 4000R Image Station.

For *in vitro* translation, proteins were produced from pSP73 vector-based constructs using TNT high-yield wheat germ *in vitro* system (Promega) according to manufacturer's instructions. A total of 200 μl of 1× PBS was added to each reaction and samples were incubated with 20 μl anti-V5 beads at 4° for 2.5 hr. Samples were washed with 1× PBS and proteins were eluted with SDS sample buffer and analyzed by Western blotting.

Sumoylation analysis

Third instar larvae were collected in 1.5 ml Eppendorf tubes and washed with 1× PBS. Larvae were homogenized in default lysis buffer (see above) supplemented with 10 mM *N*-ethylmaleimide (NEM). Extracts were cleared by centrifugation at 15,000 rpm at 4° for 15 min, mixed with SDS sample buffer, and analyzed by Western blotting. For Dl sumoylation assay in cultured cells, appropriate constructs were transfected into 529SU cells as described above, cells were pelleted in 1.5-ml Eppendorf tubes at 500 *g* for 1 min, and washed once with 1× PBS. Cells were resuspended directly in 50 μl SDS sample buffer, and samples were heated at 90° for 5 min, followed by Western blotting (see above).

Quantitative real-time PCR

RNA was extracted from larval tissues using Absolutely RNA miniprep kit (Agilent). cDNA was prepared with Protoscript First Strand cDNA synthesis kit (New England BioLabs). RT–PCR was performed using DyNAmo SYBR Green qPCR kit (Thermo Scientific) on an Mx3005P qPCR machine (Agilent). *RpL32* signal was used for normalization. Primers used for RT–PCR were as follows: *Drs*, ACCAAGCTCCGTGAGAACCTT/TTGTATCTTCCGGACAGGCAG and *RpL32*, GCTAAGCTGTCG CACAAATG/GTTCGATCCGTAACCGATGT.

Identification of Ulp1 peptides by mass spectrometry

Krz-interacting proteins were purified from cultured cells and embryos as described in Kyriakakis *et al.* (2008). Final samples obtained after affinity purifications were analyzed by LC-MS/MS as described previously (Bouwmeester *et al.* 2004). Ulp1 peptides were detected in samples from both cultured cells and embryos.

Production of double-stranded RNA and RNAi in cell culture

DNA fragments of 500–700 bp, complementary to mRNA sequences for the targeted proteins, were amplified by using PCR. A T7 RNA polymerase promoter sequence (TAATAC GACTCACTATAGGGAGA) was included at the 5′ end of each primer. Conditions of double-stranded RNA (dsRNA) production and RNAi in *Drosophila* cell culture have been decribed previously (Clemens *et al.* 2000). PCR products

were purified with QIAquick PCR purification kit (Qiagen), transcribed with RiboMAX Large Scale RNA Production system (Promega), and 25 μg of dsRNA was added to each well of a six-well plate containing 3×10^6 cells per well. The following primers were used for making dsRNA templates: *Bla* (beta-lactamase, for control treatments), CTAATACGACTCACTATAGGGAGACCACGAGTGGGTTACATCGAACTGG/CTAATACGACTCACTATAGGGAGACCACGTTCATCCATAGTTGCCTGAC; *krz*, TAATACGACTCACTATAGGGAGACCACAGTCTCGGTATTATGGTCCAC/TAATACGACTCACTATAGGGAGACCACATTCGACACTTTCAGCTTAAACC; and *Ulp1*, TAATACGACTCACTATAGGGAGACCACCTCAAGTCACAGTGAAAAGTG/TAATACGACTCACTATAGGGAGACCACGGTGTACTACTTCCTAAGGG. DNA template for making *Ulp1* dsRNA was generated in an overlap PCR reaction from two segments of the 5′- and 3′-untranslated regions, with the following overlapping primers: GTAGCGAACACACGCGCGATGCGAATAAGG/CCTTATTCGCATCGCGCGTGTGTTCGCTAC. The sequence used for making *Ulp1* dsRNA did not overlap with the coding region and therefore did not interfere with expression of Ulp1-SBP protein.

Results

Loss of krz leads to an increased production of lamellocytes and an up-regulation of Toll signaling

To study the effects of *krz* loss of function on Toll signaling, we analyzed a homozygous lethal *P*-element insertion allele, krz^{c01503} (Thibault *et al.* 2004), as well as various conditions of dsRNA-mediated *krz* knockdown. Full-length Krz protein was not detectable in krz^{c01503} homozygous larvae, but its expression, as well as adult viability, was restored in the presence of a genomic rescue construct, *krz5.7* (Supporting Information, Figure S1A). A knockdown of *krz* in whole larvae using the *da-GAL4* driver (Wodarz *et al.* 1995), or in the fat body and the lymph gland using the *Cg-GAL4* driver (Asha *et al.* 2003), in the presence of coexpressed *Dicer-2* (*Dcr-2*) (Dietzl *et al.* 2007), also almost completely eliminated the Krz protein (Figure S1, A and B). We observed melanotic masses in homozygous krz^{c01503} third instar larvae (Figure 1B), as well as in larvae in which *krz* was knocked down using the *Cg-GAL4* driver (Figure 1C), consistent with a previous report showing melanotic mass formation in krz^{1} homozygotes (Roman *et al.* 2000). Melanotic masses were detected not only next to the fat body cells, but also in and near the lymph gland, nervous system, dorsal vessel, brain stem, and in free circulation. Melanotic mass formation is often associated with increased numbers of lamellocytes, which normally constitute only a small proportion of all blood cells (Minakhina and Steward 2006). Using a lamellocyte-specific antibody (gift of I. Ando), we observed a ninefold increase in the percentage of circulating lamellocytes in krz^{c01503} homozygous third instar larvae and a threefold increase in *Cg-GAL4*-driven RNAi knockdown animals, compared to controls (Figure 1D). Lamellocyte counts returned to normal levels when a genomic *krz* rescue construct was combined with krz^{c01503}, confirming that this defect was specifically attributable to loss of *krz* (Figure 1D).

Formation of melanotic masses and an increased differentiation of lamellocytes were reported for several mutations that cause hyperactivation of Toll signaling (Gerttula *et al.* 1988; Roth *et al.* 1991; Lemaitre *et al.* 1995), suggesting that Krz may be required for limiting the activity of the Toll pathway. To determine whether Toll pathway activity is responsible for the melanotic mass phenotype in *krz* mutants, we combined the krz^{c01503} allele with loss of *Dif*, which is a Toll effector protein involved in lamellocyte differentiation (Huang *et al.* 2005). Lamellocyte counts were significantly lower in Dif^{1}; krz^{c01503} larvae, compared to krz^{c01503} mutants (Figure 1E), suggesting that this phenotype in *krz* mutant animals is dependent on downstream Toll pathway activation.

We next studied the effects of *krz* loss on Toll target gene expression. We used a fly line (*DD1*) that carries two immunity-related reporters: *Drosomycin* (*Drs*) promoter-GFP, which responds to Toll activation, and *Diptericin* (*Dpt*) promoter-LacZ, which is downstream of IMD signaling (Manfruelli *et al.* 1999). Live imaging of *DD1*; krz^{c01503} larvae revealed a strong up-regulation of *Drs*-GFP expression (Figure 1F), which was also confirmed by Western blotting (Figure 1G). In contrast, *Dpt*-LacZ expression was not affected (Figure 1G). These results indicate that loss of *krz* function specifically affects Toll signaling, without altering the IMD pathway.

For a more direct measurement of endogenous *Drs* expression, we performed quantitative RT–PCR. Analysis of expression in second instar larvae showed that *Drs* levels were modestly but significantly higher in krz^{c01503} homozygotes, compared to controls, and that this effect could be reversed when a genomic rescue construct was present (Figure 1H). Importantly, *Drs* levels were similar to controls in Dif^{1}; krz^{c01503} double mutant second instar larvae, suggesting that Dif is required for the observed overexpression of *Drs* in *krz* mutants. In krz^{c01503} third instar larvae, however, we did not detect significant changes in *Drs* expression (data not shown). It is possible that *krz* mutants accumulate multiple developmental defects by the third instar stage, which may have some adverse effects on gene expression. Consistent with such a possibility, overall level of Dl was lower in krz^{c01503} third instars (Figure S1C), and clonal analysis showed a significantly lower level of Dl and Dif in krz^{1} mutant fat body cells (Figure S1, D–E′′′).

Expression of downstream targets is induced by Dl and Dif that accumulate in the nuclei in response to activated Toll signaling (Ip *et al.* 1993; Lemaitre *et al.* 1995, 1996). We asked whether localization of Dl and Dif was affected by loss of *krz* function. In the fat bodies of control *yw* third instar larvae, both Dl and Dif were evenly distributed throughout the cell (Figure 2, A–A′′ and C–C′′). In contrast, both proteins showed predominantly nuclear localization in third instar larval fat bodies expressing *krz* dsRNA under the control of the *Cg-GAL4* driver (Figure 2, B–B′′ and D–D′′). Consistently, staining of fat bodies from krz^{c01503} second instar larvae showed that Dl was nuclear in most cells (Figure

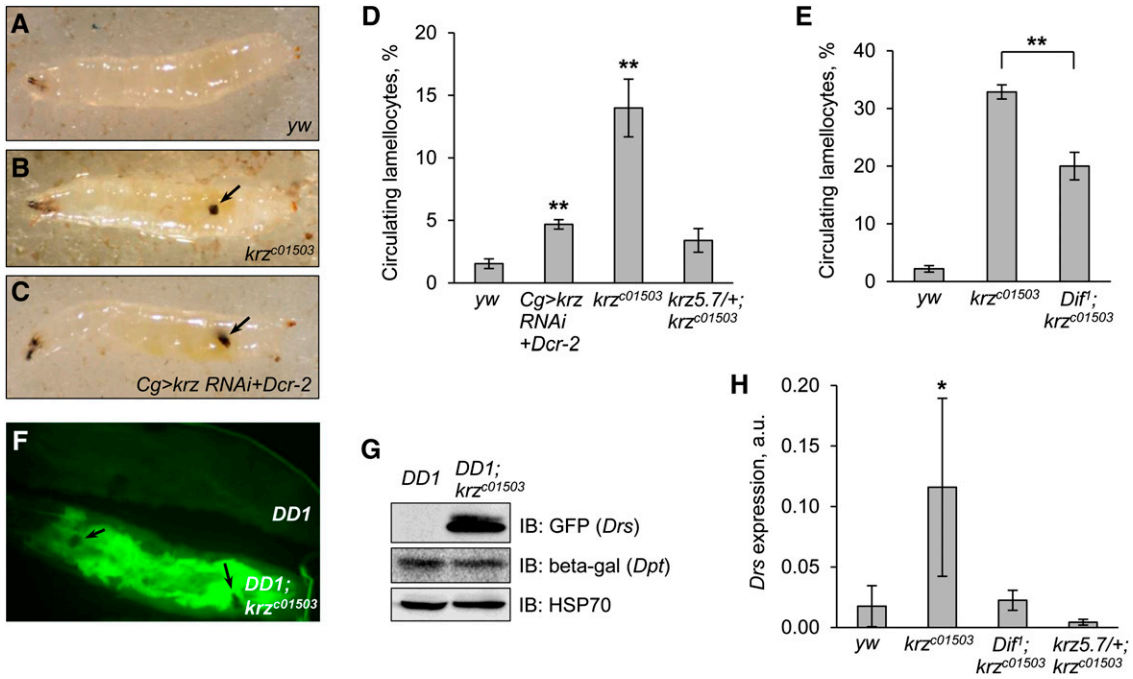

Figure 1 Loss of *krz* leads to an increased production of lamellocytes and activation of Toll signaling. (A–C) Melanotic mass formation in control and *krz* loss-of-function larvae (arrows). (D and E) Quantification of circulating lamellocytes in the hemolymph of third instar larvae as percentage of all hemocytes. (D) Proportion of lamellocytes increased approximately ninefold in krz^{c01503} homozygous larvae and threefold in RNAi knockdown animals, relative to *yw* controls. (E) Proportion of lamellocytes was significantly lower in Dif^{1}; krz^{c01503} animals, compared to krz^{c01503} homozygotes. (F) Live fluorescence image of third instar larvae carrying *Drosomycin-GFP* reporter as part of the *DD1* chromosome. *Drs-GFP* was highly expressed in krz^{c01503} homozygotes. Arrows indicate melanotic masses. (G) Western blot analysis of expression from *Drs-GFP* and *Dpt-lacZ* (*beta-gal*) reporters in whole third instar larvae. *Drs-GFP* expression was not detected in the *DD1* line but was highly elevated in *DD1*; krz^{c01503} animals, whereas *Dpt-LacZ* levels were not affected. (H) Quantitative RT–PCR of endogenous *Drosomycin* gene expression in whole second instar larvae. *Drs* levels were higher in krz^{c01503} homozygous larvae but were reduced when krz^{c01503} was combined with either loss of *Dif* or genomic *krz* rescue (*krz5.7*). $^{**}P < 0.01$; $^{*}P < 0.05$. Error bars represent standard deviation. a.u., arbitrary units.

S1, F–G′′′), and in krz^{1} mutant clones residual Dl and Dif proteins accumulated in the nuclei, though the latter effect could also be due to a more rapid degradation of Dl and Dif in the cytoplasm (Figure S1, D–E′′′). Taken together, formation of melanotic masses, increased levels of lamellocytes, nuclear accumulation of transcriptional effectors, and hyperactivation of a downstream target gene in *krz* mutants suggest that Krz is necessary to prevent inappropriate activation of Toll signaling in *Drosophila* larvae. Loss of *krz* function results in Toll pathway hyperactivity, which can contribute to systemic inflammation phenotypes observed in *krz* mutants, even in the absence of infection by pathogens.

Krz binds to the Ulp1 protein

To gain insight into possible molecular mechanisms of Krz involvement in controlling Toll signaling and systemic inflammation, we carried out a proteomic analysis of Krz interacting proteins in embryos and cultured cells (Kyriakakis *et al.* 2008). Among the interactors identified in both cells and embryos was Ulp1, a SUMO protease (Figure S2A) (Bhaskar *et al.* 2002; Smith *et al.* 2004). To validate the binding between Krz and Ulp1, we expressed the HA-Krz and Ulp1-V5 proteins in *Drosophila* S2 cells and were able to co-immunoprecipitate (co-IP) them in both directions (Figure 3, A and B), confirming that Krz and Ulp1 form a complex. The binding between these proteins appears to be direct, as Ulp1-V5 could also be efficiently immunoprecipitated by HA-Krz when both proteins were produced using *in vitro* translation (Figure S2C). Mapping of a possible Ulp1-Krz interaction region identified a carboxy terminal domain (part C) of Ulp1 as the most likely interaction surface, with the middle portion of the Ulp1 protein (part B) contributing to binding (Figure S2, B and D). Part C in this study corresponds to the conserved catalytic domain in Ulp1, which is responsible for its desumoylation activity.

To determine whether the interaction between Krz and Ulp1 that we discovered is evolutionarily conserved, we tested whether human homologs of Krz (β-arrestins 1 and -2) and Ulp1 (SENP1) can bind each other. HA-tagged β-arrestin 2 (HA-ARRB2), but not β-arrestin 1 (HA-ARRB1), was immunoprecipitated with Flag-SENP1 when expressed in *Drosophila* S2 cells (Figure 3C). These experiments have therefore identified a novel, conserved interaction between Krz/β-arrestin and Ulp1/SENP1.

Knockdown of Ulp1 results in Toll pathway hyperactivity

Based on our observation of excessive Toll pathway activity in *krz* mutants, we asked whether loss of *Ulp1* function would also affect Toll signaling. A single *P*-element insertion

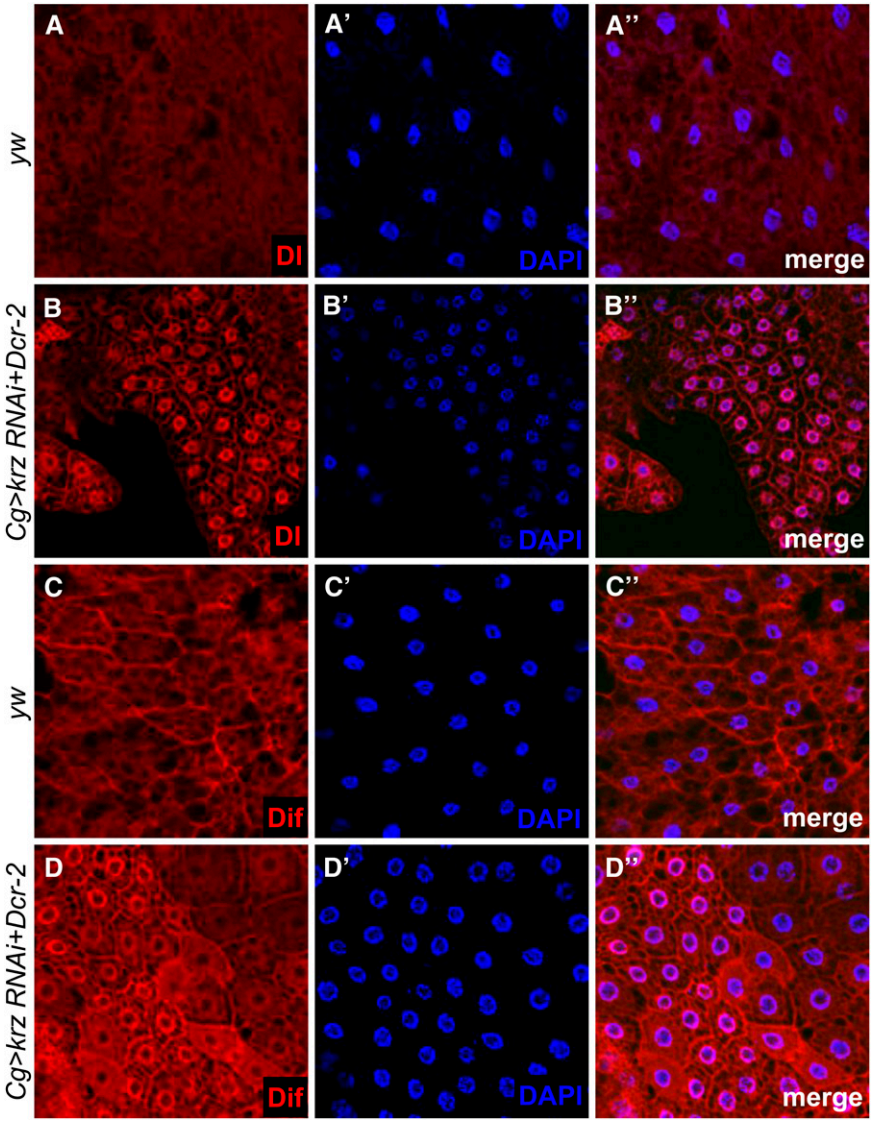

Figure 2 Loss of *krz* results in nuclear accumulation of Dorsal and Dif. (A–D′′) Antibody staining of Dorsal and Dif proteins (red) in the fat bodies of third instar larvae. DAPI (blue) marks the nuclei. Dl (A–A′′) and Dif (C–C′′) staining of the fat bodies from control *yw* larvae showed diffuse subcellular distribution. In the fat bodies of larvae coexpressing *krz* dsRNA and *Dcr-2* with the *Cg-GAL4* driver, both Dl (B–B′′) and Dif (D–D′′) were preferentially localized in the nuclei.

that disrupts the *Ulp1* gene, *P{lacW}Ulp1*^{G0026} (Peter *et al.* 2002), has been lost (U. Schäfer, personal communication), and the next nearest *P*-element insertion is located outside of the Ulp1 transcript and is homozygous viable without an apparent phenotype (A. Veraksa, unpublished observation). As a genetic lesion in the *Ulp1* gene is not available, we analyzed larvae in which the function of *Ulp1* was reduced by expression of *Ulp1* dsRNA and *Dcr-2* using the ubiquitous *da-GAL4* (Wodarz *et al.* 1995) driver. *Ulp1* RNAi knockdown larvae developed extensive melanotic masses located throughout the body and did not survive beyond third instar stage (Figure 3D). The proportion of circulating lamellocytes was increased ~15-fold in *Ulp1* RNAi third instars, compared to *yw* controls (Figure 3E). Quantitative RT–PCR showed that the endogenous *Drs* gene was up-regulated by ~60-fold in the *Ulp1* knockdown animals (Figure 3F). The Toll pathway transcriptional effector Dl was predominantly nuclear in many fat body cells of *Ulp1* RNAi third instar larvae (Figure 3, G–H′′). These phenotypes together suggest that, like loss of *krz*, reduction in *Ulp1* function leads to hyperactivation of Toll signaling and an inflammatory syndrome in the absence of infection, manifested in the differentiation of lamellocytes, formation of melanotic masses, and increased expression of antimicrobial peptide genes.

Ulp1 desumoylates Dorsal and other proteins

Because Ulp1 functions as a SUMO protease (Smith *et al.* 2004), we asked whether reduced levels of *Ulp1* would affect sumoylation in larvae. Knockdown of *Ulp1* using *da-GAL4* and *Dcr-2* resulted in a strong increase of global sumoylation in third instars (Figure 4A), consistent with previous *Ulp1* RNAi experiments performed in cultured cells (Smith *et al.* 2004, 2011). Knockdown of *krz* using the same driver did not change the pattern of overall sumoylation in third instar larvae (Figure 4A).

Dl is sumoylated on lysine 382 and desumoylated by Ulp1 in cultured cells (Bhaskar *et al.* 2002). Our attempts to detect Dl sumoylation *in vivo* were not successful, so we

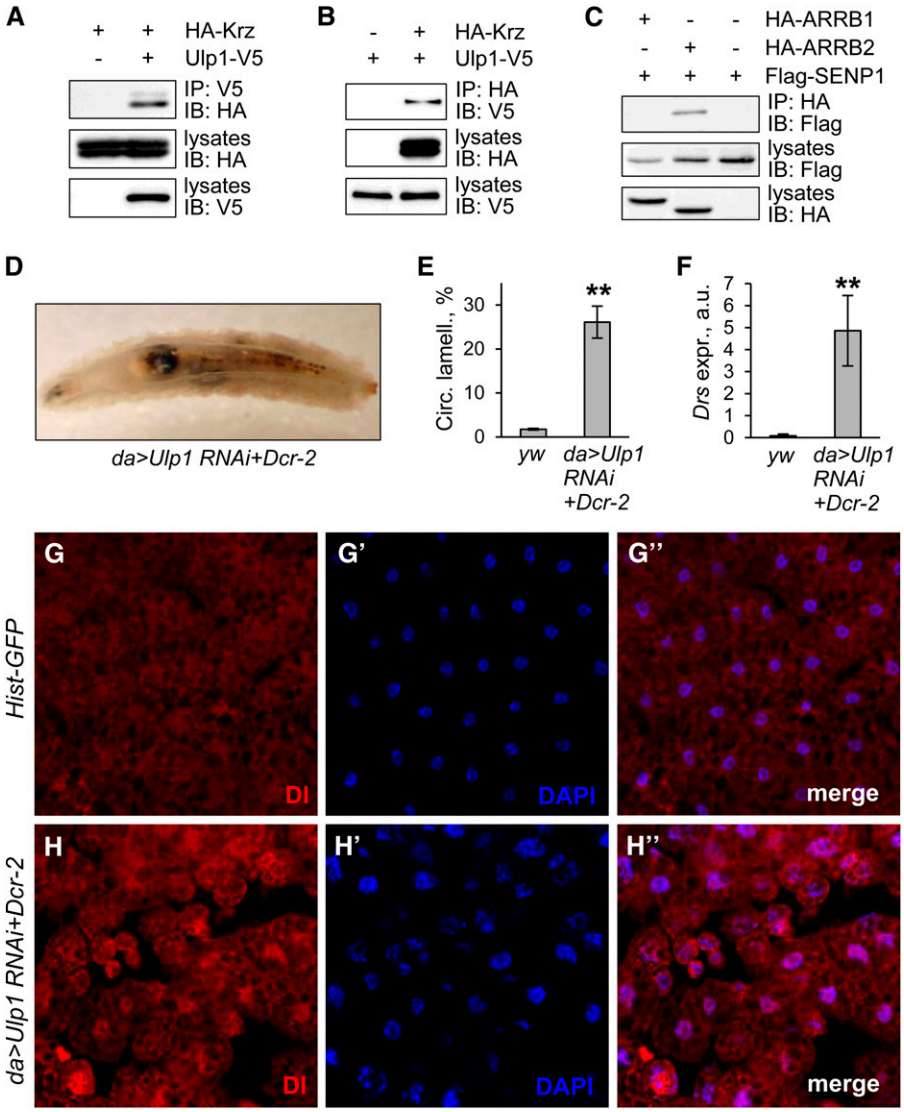

Figure 3 Ulp1 is a Krz-interacting protein, and its knockdown results in Toll pathway activation. (A and B) Co-immunoprecipitation of Krz and Ulp1 from *Drosophila* S2 cells. HA-Krz and Ulp1-V5 were transfected in the indicated combinations, immunoprecipitated with V5 or HA affinity resin, and immunoblotted with the corresponding antibodies. Binding between HA-Krz and Ulp1-V5 was observed in both directions of co-IP. (C) Co-immunoprecipitation of mammalian β-arrestins and SENP1 from *Drosophila* S2 cells. HA-ARRB1, HA-ARRB2, and Flag-SENP1 were transfected in the indicated combinations, immunoprecipitated with HA affinity resin, and immunoblotted. Flag-SENP1 formed a complex with β-arrestin 2 but not β-arrestin 1. (D) Melanotic mass formation in a third instar larva in which *Ulp1* dsRNA and *Dcr-2* were expressed using the *da-GAL4* driver. Melanotic masses are visible throughout the body. (E) Quantification of circulating lamellocytes in the hemolymph of third instar larvae as percentage of all hemocytes. Proportion of lamellocytes increased ~15-fold in *Ulp1* knockdown animals. (F) Quantitative RT–PCR of endogenous *Drosomycin* gene expression in whole third instar larvae. *Drs* levels were 60-fold higher in *Ulp1* knockdown animals, compared to controls. (G–H'') Immunostaining of Dorsal protein (red) in the fat bodies of third instar larvae. DAPI (blue) marks the nuclei. Compared to the *Hist-GFP* controls (G–G''), Dl nuclear levels were higher in the *Ulp1* RNAi larvae (H–H''). $^{**}P < 0.01$. Error bars represent standard deviation. a.u., arbitrary units; IB, immunoblot; IP, immunoprecipitation.

studied the effects of Krz and Ulp1 on Dl sumoylation using a *Drosophila* 529SU cell line (gift of A. Courey), in which sumoylation is enhanced by coexpression of Smt3 (*Drosophila* SUMO) and Ubc9/Lwr (an E2 SUMO conjugation enzyme) (Bhaskar *et al.* 2002). As would be expected from changes in global sumoylation observed *in vivo* (Figure 4A), knockdown of *krz* did not appreciably alter the level of Dl sumoylation in 529SU cells, whereas *Ulp1* RNAi resulted in a significant increase in Dl sumoylation (Figure 4B, quantified in Figure 4C). Overexpression of Ulp1 tagged with a streptavidin binding peptide (Ulp1-SBP) completely eliminated Dl sumoylation in both *krz* and *Ulp1* RNAi conditions, as well as reduced global sumoylation. A nonsumoylatable form of Dl, Dl-K382R (Bhaskar *et al.* 2002), was not sumoylated in this assay, confirming the identity of the Dl-SUMO band (Figure 4B). Taken together, these results show that reduction of *Ulp1* function resulted in an increase in global sumoylation *in vivo* and in cultured cells, whereas a knockdown of *krz* alone did not have a detectable effect. Sumoylation of Dl, which is one of the Ulp1 targets, followed this overall pattern.

Synergistic effects of Krz and Ulp1 on sumoylation and systemic inflammation

We then asked whether *krz* and *Ulp1* interact genetically. When either *krz* or *Ulp1* was singly knocked down using *Cg-GAL4* (without *Dcr-2*, for a weaker knockdown effect), no melanotic masses were observed in third instar larvae (Figure 5, A and B). Remarkably, when *krz* and *Ulp1* dsRNAs were coexpressed with *Cg-GAL4*, multiple melanotic masses formed in third instar larvae (Figure 5C). Correspondingly, the levels of circulating lamellocytes were similar to those in controls in single *krz* or *Ulp1* knockdown conditions, but were 10-fold higher when *krz* and *Ulp1* were knocked down together (Figure 5D). Simultaneous knockdown of *krz* and *Ulp1* using *Cg-GAL4* also led to a 25-fold up-regulation of *Drs* (Figure S5A). Moreover, when the *da-GAL4* driver was used for single knockdowns without *Dcr-2*, *krz* and *Ulp1* RNAi animals survived to adulthood, but a simultaneous knockdown of both *krz* and *Ulp1* with *da-GAL4* resulted in 100% lethality at the larval stages. The phenotype of a combined weak knockdown

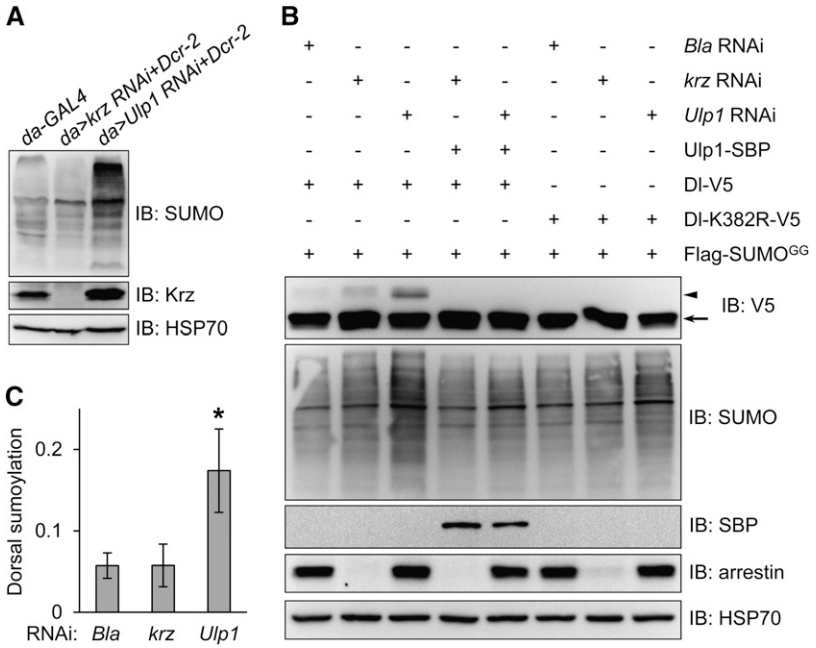

Figure 4 Desumoylating activity of Ulp1. (A) Western blot analysis of overall sumoylation levels in whole third instar larvae. Knockdown of *Ulp1* significantly increased global sumoylation *in vivo*, whereas knockdown of *krz* did not. (B) Sumoylation of Dorsal in 529SU cells. Cells were treated with control (*Bla*, beta-lactamase), *krz*, or *Ulp1* dsRNA and transfected with the indicated Dl constructs together with Flag-SUMO^{GG}. Total cell extracts were immunoblotted with the indicated antibodies. Knockdown of *Ulp1* resulted in an increased sumoylation of Dl. Transfection of high levels of Ulp1-SBP completely eliminated Dl sumoylation. A mutated form of Dl (K382R) was not sumoylated under any condition. Arrow indicates the nonsumoylated form of Dl; arrowhead marks the position of the sumoylated form. (C) Quantification of Dl sumoylation in three independent experiments. A ratio of sumoylated to a nonsumoylated form is shown. $*P < 0.05$. Error bars represent standard deviation.

of both *krz* and *Ulp1* was therefore similar to a strong knockdown of either *krz* or *Ulp1* alone (compare Figures 1, 3, and 5), suggesting that these genes are involved in the same developmental pathway whose disruption results in an increased production of lamellocytes and formation of melanotic masses.

The *Cg-GAL4* driver is expressed both in the fat body and in the lymph gland (Asha *et al.* 2003), and it is possible that the phenotypes in double knockdown animals involved some communication between these organs. To test for the specificity of the observed effects, we used the *ppl-GAL4* driver, which is expressed exclusively in the fat body (Zinke *et al.* 1999). Double knockdown of both *krz* and *Ulp1* at 29° resulted in a significant up-regulation of endogenous *Drs* expression, suggesting that the loss of these two genes specifically in the fat body is sufficient to generate a mutant phenotype (Figure 5E).

To gain insight into a possible functional significance of Krz and Ulp1 interaction, we studied Dl sumoylation in 529SU cells under the conditions of single or joint knockdown of *krz* and *Ulp1*. As expected from the results described above (see Figure 4B), knockdown of *krz* alone did not increase Dl sumoylation, whereas knockdown of *Ulp1* did (Figure 5F, lanes 2 and 3). However, when cells were treated with dsRNA for both *krz* and *Ulp1*, Dl sumoylation was further increased to levels that were significantly higher than those observed for *Ulp1* RNAi alone (Figure 5F, lane 7). These results were confirmed in three independent experiments (Figure 5G). Furthermore, we could titrate out Dl sumoylation by expressing small increasing amounts of Ulp1-SBP in the background of *Ulp1* knockdown (Figure 5F, lanes 4–6). When both *krz* and *Ulp1* were knocked down, Dl sumoylation was more persistent and required higher levels of Ulp1-SBP to achieve the same degree of reduction (Figure 5F, lanes 8–10). A similar pattern could be seen for overall protein sumoylation levels in this experiment (Figure 5F, SUMO blot). Taken together, these results suggest that Krz is a cofactor for the Ulp1 desumoylation activity, and that this role of Krz becomes apparent when Ulp1 is present in limiting amounts.

Discussion

In this study, we present evidence that reveals a novel function of the β-arrestin Krz in controlling sumoylation and preventing systemic inflammation in *Drosophila* larvae. Our results can be summarized in the model presented in Figure 5H: at least part of Krz protein function is carried out via its direct association with Ulp1, a SUMO protease. Krz and Ulp1 synergistically regulate the level of sumoylation of multiple proteins, including the Toll transcriptional effector Dorsal. Sumoylation can regulate nuclear localization, transcriptional activity, and other protein functions that converge to control the Toll pathway and possibly other regulators of the larval immune system. The effects of Ulp1 and Krz counterbalance the function of Ubc9/Lwr, which promotes sumoylation. A proper balance of sumoylation is important for preventing inappropriate inflammatory responses in the absence of pathogens.

Modulation of Ulp1 activity by Krz was most easily revealed in sensitized backgrounds of partial *Ulp1* loss of function. While loss of *krz* by itself did not have a detectable effect on sumoylation, it showed a clear synergism with loss of *Ulp1*, which was manifested in greater sumoylation of Dl and other proteins and stronger phenotypes in joint *krz* and *Ulp1* knockdown conditions (Figure 5). Our data suggest that normally the Krz protein facilitates the SUMO deconjugation function of Ulp1, but the molecular details of this interaction are currently unknown. One possibility is that Krz affects the binding of Ulp1 to its substrates. In transfected cells, the binding between

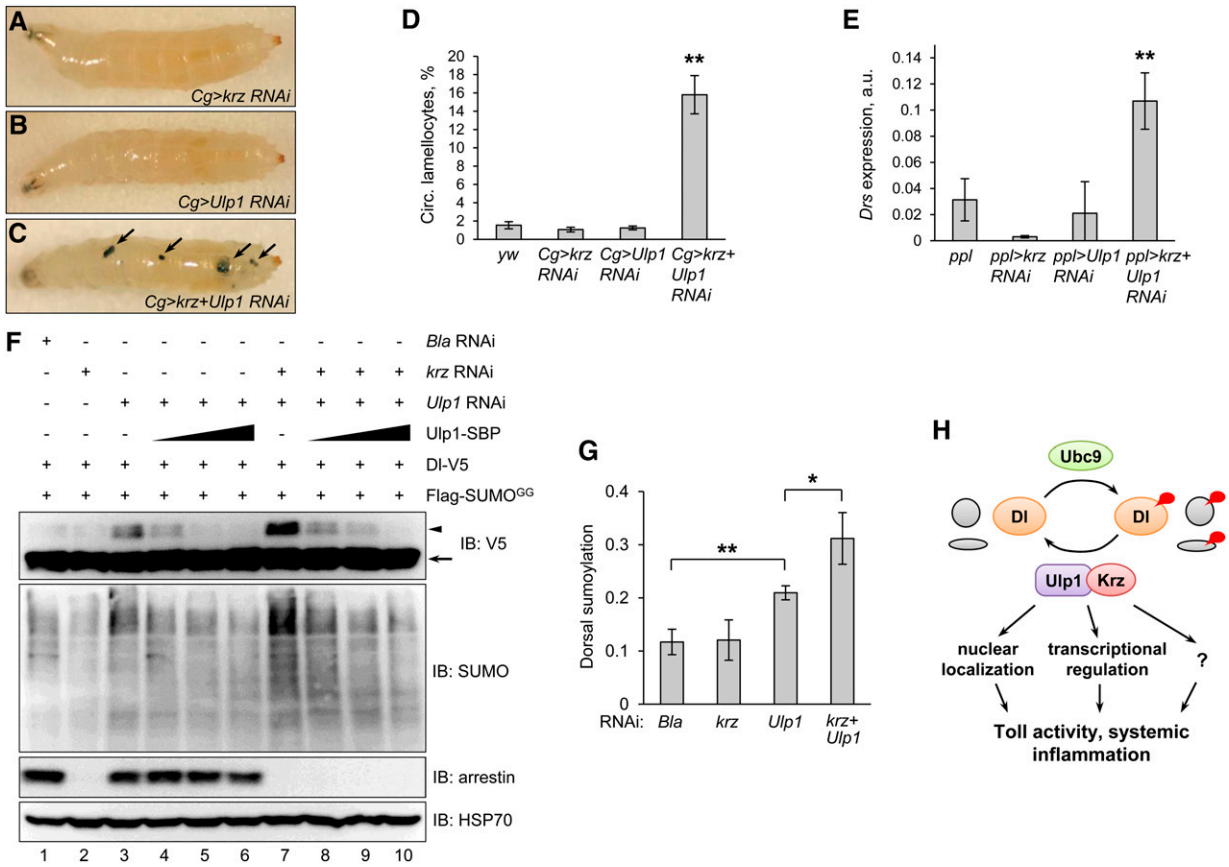

Figure 5 Krz and Ulp1 synergistically control sumoylation and Toll pathway activity. (A–C) Formation of melanotic masses in third instar larvae. When expressed without *Dcr-2* using the *Cg-GAL4* driver, knockdown of neither *krz* nor *Ulp1* alone resulted in melanotic mass formation (A and B). However, extensive melanotic masses were observed when *krz* and *Ulp1* dsRNAs were coexpressed (C, arrows). (D) Quantification of circulating lamellocytes in the hemolymph of third instar larvae as percentage of all hemocytes. Proportion of lamellocytes increased ~10-fold in the double knockdown animals, compared to *yw* controls. (E) Quantitative RT–PCR of endogenous *Drosomycin* gene expression in whole third instar larvae grown at 29°, using the *ppl-GAL4* driver. *Drs* levels were 3.5-fold higher in the double knockdown animals, compared to controls. (F and G) Synergistic effects of Krz and Ulp1 on Dl sumoylation in 529SU cells. (F) Cells were treated with control (*Bla*, beta-lactamase), *krz*, and/or *Ulp1* dsRNA and transfected with the Dl-V5 construct together with Flag-SUMO^{GG} and increasing (but small) amounts of Ulp1-SBP. Total cell extracts were immunoblotted with the indicated antibodies. Arrow indicates the nonsumoylated form of Dl; arrowhead marks the position of the sumoylated form. (G) Quantification of Dl sumoylation in three independent experiments. Knockdown of both *krz* and *Ulp1* resulted in a significantly higher level of Dl sumoylation, compared to a knockdown of *Ulp1* alone. (H) Model summarizing the activity of Krz and Ulp1 in controlling sumoylation and Toll pathway activity. Krz promotes desumoylating activity of Ulp1 toward Dorsal and other targets. A proper balance of sumoylation is important for limiting Toll pathway activity and preventing an inappropriate inflammatory response in the absence of pathogens. $^{**}P < 0.01$; $^{*}P < 0.05$. Error bars represent standard deviation.

Ulp1 and Dl was not affected by a knockdown of *krz* (Figure S3), making this a less favorable mechanism. It is also possible that Krz alters Ulp1 enzyme activity. We speculate that this is the more likely scenario given that Krz binds to Ulp1 in or near its catalytic domain (Figure S2).

The Toll pathway is one of the signaling systems controlled at the level of sumoylation. Our findings extend previous reports on the role of sumoylation in regulating Toll signaling and systemic inflammation in *Drosophila* and underscore the complexity of this regulation. It was previously shown that a reduction in global sumoylation in *lwr* (Ubc9) mutants resulted in hyperactivation of Toll signaling and induced melanotic mass formation (Chiu *et al.* 2005; Huang *et al.* 2005; Paddibhatla *et al.* 2010; Kalamarz *et al.* 2012). In this study, knockdown of *Ulp1*, which causes an increase in overall sumoylation, was surprisingly found to result in a similar systemic inflammation phenotype. Moreover, we found that overexpression of Ulp1-V5, which is predicted to decrease global sumoylation, also led to an abnormally high *Drs* expression and accumulation of Dl in the nucleus (Figure S4). Previous studies using assays in cultured cells showed that sumoylation generally increases the activity of Dl as a transcription factor and also increases its nuclear retention (Bhaskar *et al.* 2000, 2002), and it is likely that these factors contribute to the hyperactivation of Toll signaling in *Ulp1* knockdown animals. However, since mutations in general sumoylation components such as Ubc9/Lwr and Ulp1 affect sumoylation of many targets in addition to Dl, it seems that the resulting phenotypes cannot be attributed solely to changes in the activity of Dl, but likely involve additional regulators.

When analyzing the effects of loss of *krz* and *Ulp1* on Toll signaling, we noted that certain aspects of the mutant

phenotypes were stronger than others. Thus, lamellocyte differentiation and Dl and Dif nuclear localization were altered more readily compared to the induction of antimicrobial peptides, particularly when the latter was compared to the condition of overexpression of *Toll*^{10b}, an extreme gain-of-function Toll allele (Erdelyi and Szabad 1989) (Figure S5A). Correspondingly, a simultaneous knockdown of *krz* and *Ulp1* had a modest effect on the ability of larval hemolymph to reduce the titer of pathogenic bacteria (Figure S5B). We speculate that this may be explained by sumoylation affecting only a subset of Toll functions, whereas the activated Toll receptor is capable of inducing all possible downstream effects, achieving maximum activation of its target genes.

At present, it is not known whether all of the observed effects in *krz* and *Ulp1* mutant conditions are cell autonomous, though our clonal analysis suggests that most of the defects such as the accumulation of residual Dl in the nucleus were observed only in the cells mutant for *krz* (Figure S1), and a simultaneous knockdown of *krz* and *Ulp1* specifically in the fat body resulted in *Drs* induction (Figure 5E). It is still possible that a communication between the fat body and other tissues contributes to systemic inflammation phenotypes observed in the *krz* and *Ulp1* mutants, as was suggested for other mutants affecting sumoylation (Paddibhatla *et al.* 2010). Notwithstanding, the requirement for Toll pathway activation in this process is clear, as demonstrated by significant decreases in the severity of the phenotypes observed in the background of *Dif*^{1} mutation (Figure 1).

In addition to Toll, other signaling pathways may also be affected by global changes in sumoylation and thus contribute to the overall *Ulp1* (and by extension, *lwr*) loss of function phenotypes. For example, sumoylation can control the activity of c-Jun N-terminal kinase (JNK) and Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathways in *Drosophila*, both of which are known to be involved in the regulation of immune system homeostasis (Gronholm *et al.* 2010; Huang *et al.* 2011). Other pathways controlled by sumoylation, including Ras (Nie *et al.* 2009), ecdysteroid signaling (Talamillo *et al.* 2008b), and the IMD pathway (Fukuyama *et al.* 2013), may also contribute to the observed effects such as systemic inflammation.

Our data have revealed a new regulatory mechanism for β-arrestin-mediated control of cellular signaling through modulation of sumoylation. Because the interaction between Krz/β-arrestin and Ulp1/SENP1 is conserved, it is likely that this mode of regulation is general and exists in other organisms. It will be of interest to determine whether mammalian β-arrestins can regulate Toll/NF-κB and other signaling pathways at the level of sumoylation.

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Supporting Information
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Regulation of Toll Signaling and Inflammation by β-Arrestin and the SUMO Protease Ulp1

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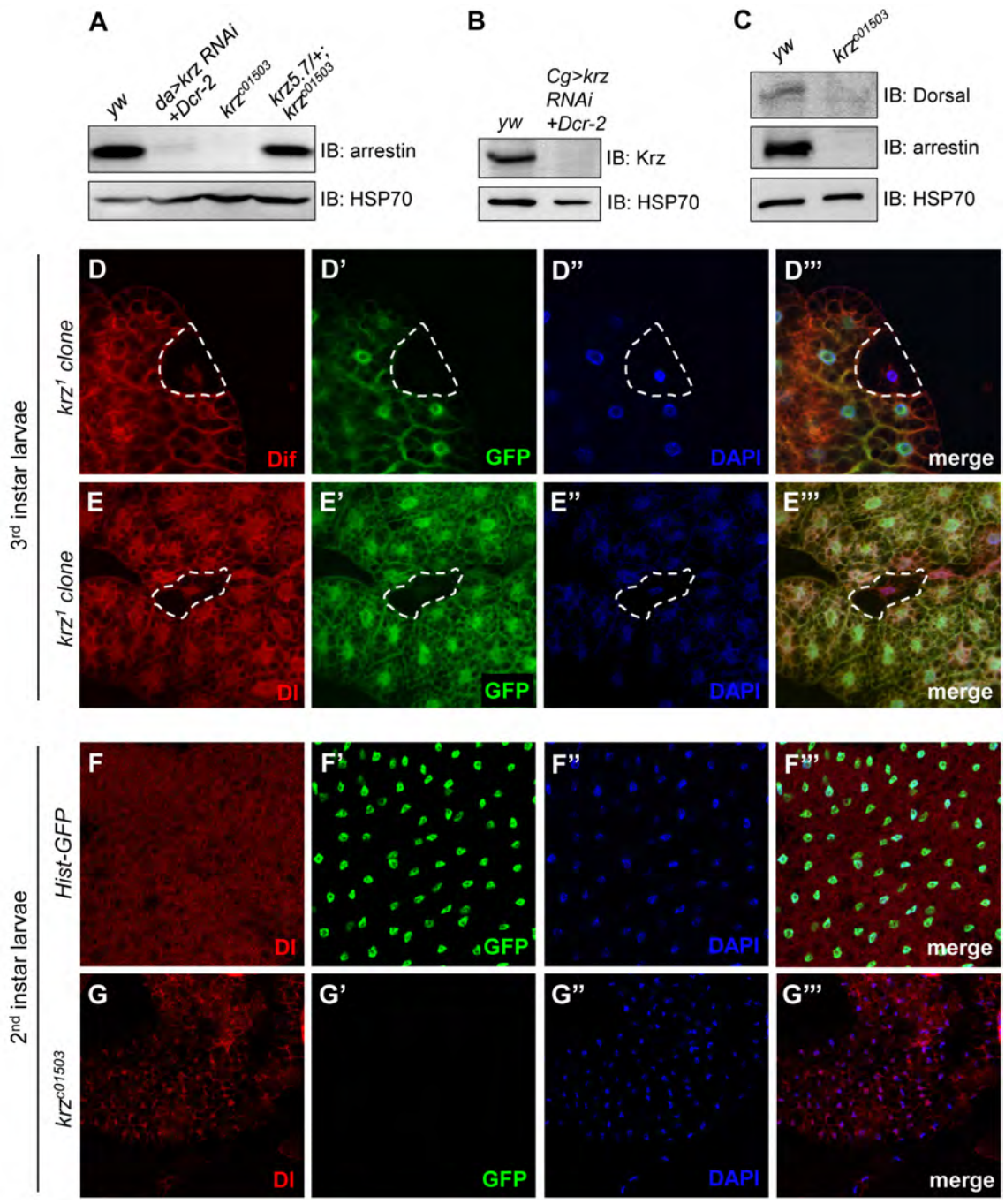

Figure S1 Expression of Krz, Dorsal and Dif in various *krz* loss of function conditions. (A) Western blot analysis of Krz protein levels using pan-arrestin antibody in whole third instar larvae. Krz expression was not detectable in *krz*^{c01503} homozygous animals but was restored by an introduction of a genomic rescue construct, *krz5.7*. HSP70 was used as a loading control. (B) Western blot analysis of Krz protein levels using anti-Krz antibody in the fat bodies of third instar larvae. Krz was undetectable in the fat bodies of larvae coexpressing *krz* dsRNA and *Dcr-2* using the *Cg-GAL4* driver. (C) Western blots of extracts from late third instar larvae probed with the indicated antibodies. The level of the Dorsal protein was significantly decreased in *krz*^{c01503} homozygous animals. HSP70 was used as a loading control. (D-E''') Expression of Dorsal and Dif in *krz*^{1} mutant clones in the fat bodies of third instar larvae. *krz*^{1} mutant cells are marked by the absence of GFP and are outlined with a white dotted line. Dl and Dif levels were lower than in surrounding cells, with residual protein located mostly in the nuclei. (F-G''') Localization of Dorsal in the fat bodies of second instar larvae. In Hist-GFP controls, Dl showed diffuse localization throughout the cell (F-F'''). In *krz*^{c01503} homozygotes, a significant proportion of cells had predominantly nuclear localization of Dl (G-G''').

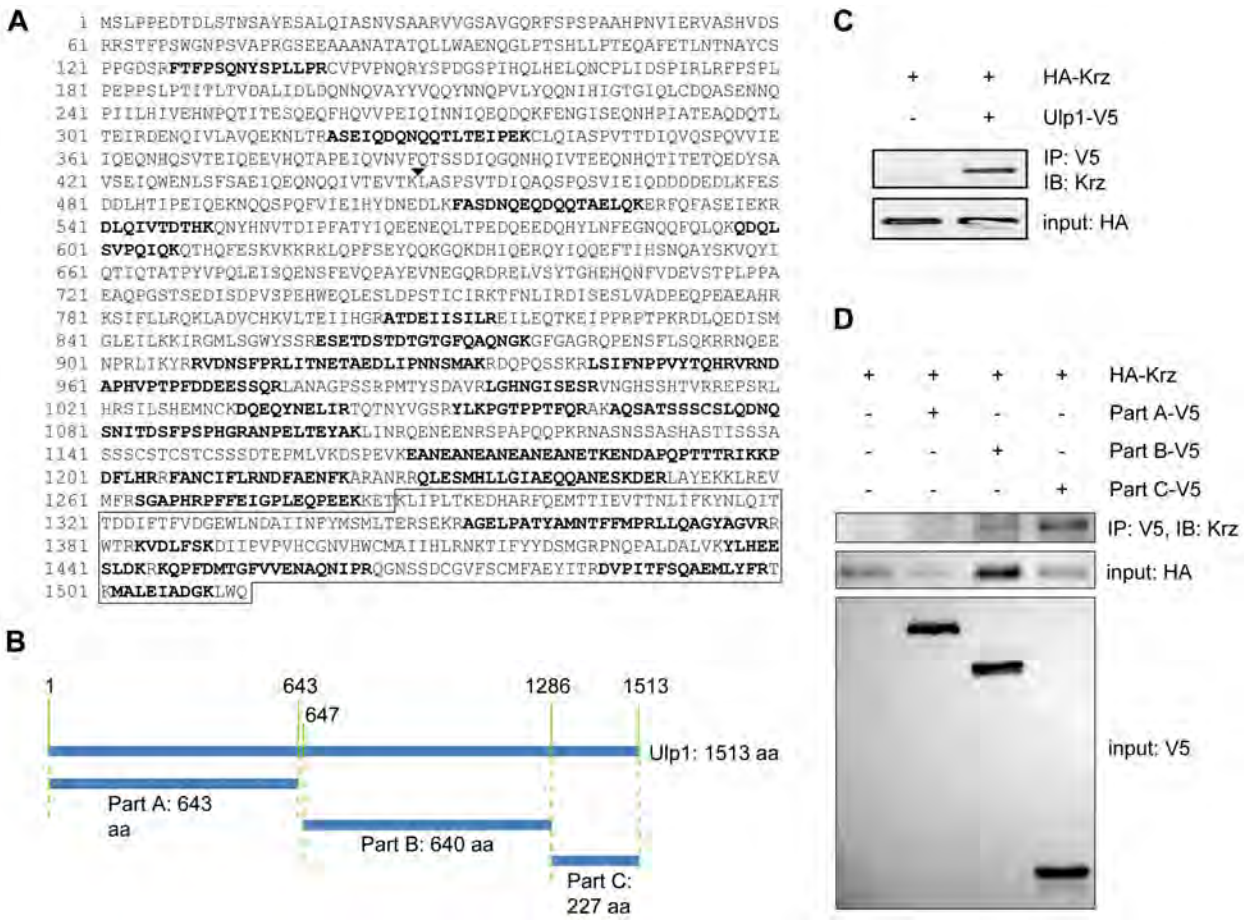

Figure S2 Identification of Ulp1 as a direct Krz interactor. (A) Amino acid sequence of the Ulp1 protein, showing an aggregate peptide coverage identified in Krz pull-downs by mass spectrometry (sequences in bold). Our mass spectrometry data confirmed that the predicted annotation of the coding sequence of Ulp1 in FlyBase is correct, as we observed two peptides from the extended amino terminus beyond the previously reported isoform (whose amino terminus is indicated by a triangle), as well as other peptides located throughout the length of the protein. A conserved carboxy terminal catalytic domain, responsible for the desumoylating activity of Ulp1, is boxed. (B) Domains of Ulp1 used for binding studies in (C) and (D). Part C corresponds to the conserved catalytic domain. (C) HA-Krz and full-length Ulp1-V5 were translated in vitro and immunoprecipitated using anti-V5 affinity resin. HA-Krz was immunoprecipitated only in the presence of Ulp1-V5. (D) HA-Krz and parts of Ulp1 were translated in vitro and immunoprecipitated using anti-V5 affinity resin. Part C of Ulp1 showed the strongest interaction with HA-Krz, followed by Part B, whereas Part A did not bind to HA-Krz.

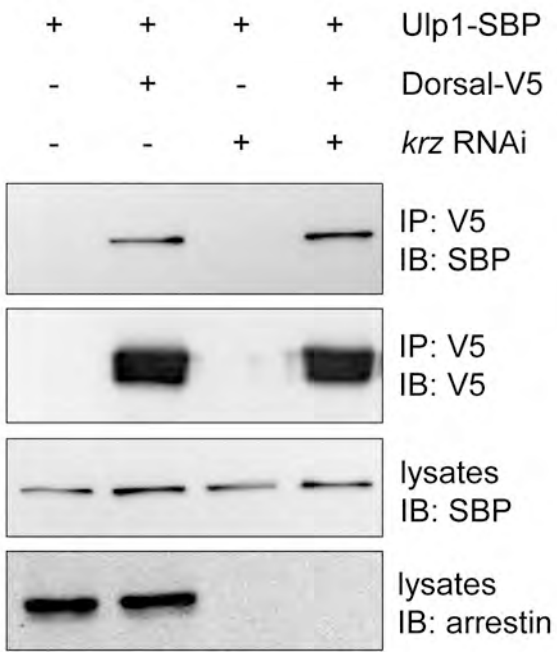

Figure S3 Loss of *krz* does not affect the binding between Ulp1 and Dorsal. Ulp1-SBP and Dl-V5 were transfected into S2 cells that were untreated or treated with *krz* dsRNA, immunoprecipitated with anti-V5 affinity resin, and immunoblotted with the indicated antibodies. Knockdown of *krz* did not affect the degree of binding between Ulp1 and Dl.

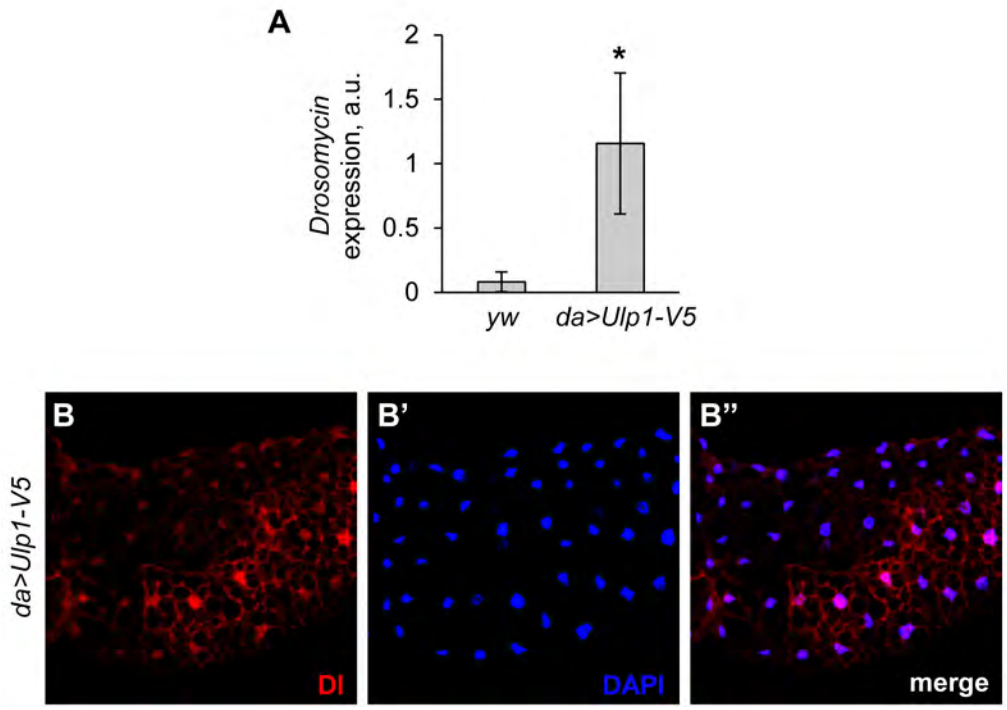

Figure S4 Overexpression of Ulp1 activates Toll signaling. (A) Quantitative RT-PCR of endogenous *Drosomycin* gene expression in whole third instar larvae. *Drs* levels were increased by approximately 14-fold when Ulp1-V5 was expressed using the *da-GAL4* driver, compared to *yw* controls. (B-B'') Localization of Dorsal protein in the fat bodies of larvae expressing Ulp1-V5 under the control of the *da-GAL4* driver. Dl showed preferential nuclear localization in a subset of cells. *, $p < 0.05$. Error bars represent standard deviation.

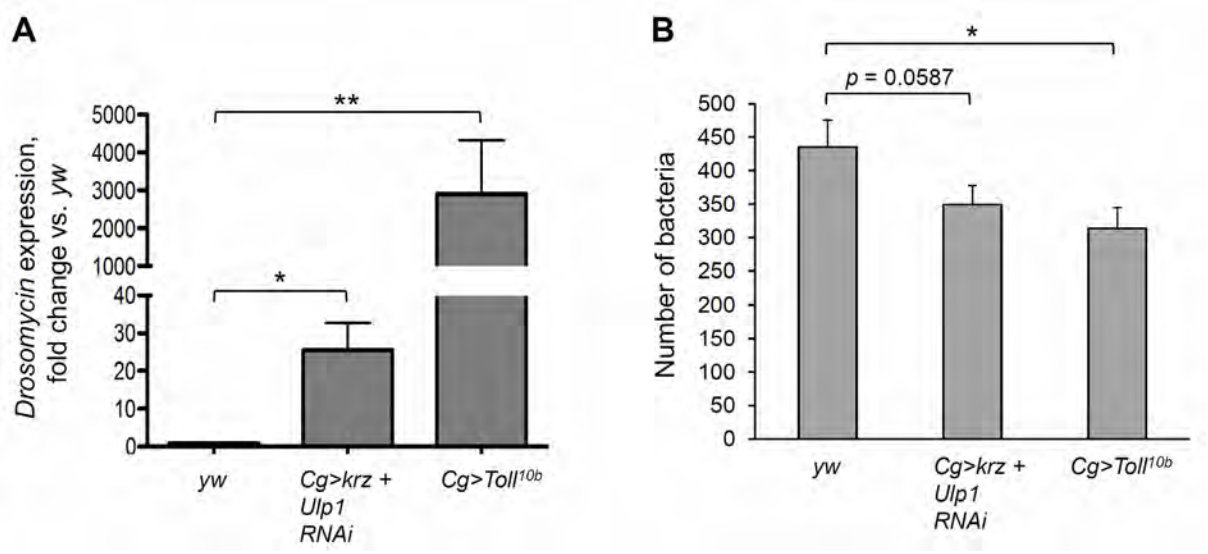

Figure S5 Effects of combined knockdown of *krz* and *Ulp1* on *Drs* expression and larval immune function. *Cg-GAL4* was used to drive the indicated *UAS* transgenes in third instar larvae. (A) Quantitative RT-PCR of endogenous *Drs* gene expression in whole third instar larvae. *Drs* levels were increased approximately 25-fold in a double knockdown of *krz* and *Ulp1*, and 3000-fold when *Toll*^{10b} was overexpressed. Note that *Toll*^{10b} is the strongest gain of function mutant reported. (B) Bacteria killing assay. Five microliters of diluted cultures of *E. faecalis* in 2xYT media containing approximately 450 bacteria were mixed with hemolymph from 10 opened third instar larvae. The mixture was incubated for 30 min, plated on 2xYT agar plates and the number of colonies counted after 18 hours at 37^{o}C. Overexpression of *Toll*^{10b} resulted in a moderate but significant decrease in bacterial numbers. Knockdown of *krz* and *Ulp1* also led to a modest decrease, with the *p* value indicated. Five independent experiments were performed. *, $p < 0.05$; **, $p < 0.01$. Error bars represent standard error.