Supporting Information

Yamamoto-Hino et al. 10.1073/pnas.1424514112

SI Materials and Methods

Generation of *senju* KO Flies. *senju* KO flies were generated using ends-out technology (1). A targeting vector was designed to replace the entire *senju* gene with the *white* gene (contained within the vector). DNA fragments of ∼3 kb, which corresponded to the 5′-upstream and 3′-downstream regions of the *senju* gene, were PCR-amplified from genomic DNA and inserted into the *white* gene-flanking polylinker sites within the pW35 vector [obtained from the *Drosophila* genomics research center (DGRC)]. The flies used for gene targeting were obtained from the Bloomington *Drosophila* Stock Center (BDSC, Bloomington, IN).

Generation of *SPE* Mutant Flies Using CRISPR/Cas9-Mediated Gene Targeting. To construct a gRNA vector, the *SPE*-specific oligonucleotides, SPE-1-F and SPE-1-R (the sequences of which are listed in Table S1), were annealed and introduced into the U6-gRNA vector. The U6-SPE-gRNA vector was then integrated into the attP40 landing site using phiC31 integrase, and the offspring (in which the transgene was balanced) were collected to establish a stock. The SPE-gRNA strain was then crossed with the nos-Cas9 strain, which expresses the Cas9 protein in germ cells. Obtained founder flies were crossed with WT flies. The SPE sequence of each offspring was checked using SPE-CHK-263F and SPE-CHK+273R primers. The primers are listed in Table S1.

Fly Stocks. The fly strains used in this study are listed in Table S2. Myc-tagged *senju*-expressing flies (*UAS-senju-myc*) have been described previously (2). To generate *UAS-nec-myc*, *UAS-spz-myc*, and *UAS-SPE-myc* flies, *nec* cDNA (GH10112), *spz* cDNA (SD20537), and *SPE* cDNA (GH28857) were obtained from DGRC. A DNA fragment encoding the 3*Myc* sequence was ligated to the 3′end of the *nec*, *spz*, and *SPE* coding regions, and each was inserted into the pUAST vector. The vectors were then injected into w^{1118} embryos to generate transgenic flies. The primers used are listed in Table S1.

Generation of Germ-Free Larvae. Germ-free larvae were generated as previously described (3).

Immunostaining. The following primary antibodies were used: mouse anti-Nimrod (1:100; provided by I. Ando, Hungarian Academy of Sciences, Szeged, Hungary), mouse anti-L1 (1:100; provided by I. Ando), and mouse anti−β-Gal [40a-1, 1:100; Developmental Studies Hybridoma Bank (DSHB)]. For the immunofluorescence experiments, the lymph glands and fat body were dissected from *Drosophila* third-instar larvae, fixed in 4% (wt/vol) paraformaldehyde in PBS, incubated in 0.5% BSA/0.1% Triton X-100 in PBS, and then incubated with the appropriate primary antibodies. The specimens were then stained with an Alexa 488-conjugated anti-mouse IgG secondary antibody and DAPI. Fluorescence images were obtained using a fluorescence microscope (BX35; Olympus). To examine the localization of the Senju protein, a rat polyclonal anti-Senju antibody was generated against a synthetic peptide corresponding to the C-terminal 18 amino acid residues (D371–V388). Lymph glands were harvested from larvae carrying *GFP-GalT*, fixed, and stained with the anti-Senju antibody, followed by a Cy3-conjugated anti-rat IgG antibody and TOPRO-3. Fluorescence images were captured under a laser-scanning confocal microscope (FV500; Olympus).

Infection Experiments. *M. luteus* or *B. subtilis* strains were used for the infection experiments. Thirty adult flies were pricked in the thorax with a thin needle dipped in a concentrated pellet of microbial culture. Larval challenge was performed by microinjection of a bacterial pellet via a glass capillary. Larvae (12 h after infection) and adult flies (24 h after infection) were collected and used for qRT-PCR and lectin binding analysis.

qRT-PCR. To obtain staged larvae, a red household food dye was added to the food to permit visualization of the gut contents. The emptying of the gut marks the difference between early- and late-wandering third-instar larvae (4). Ten larvae or 10 flies were frozen at −80 °C and then ground up. Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's protocol. Superscript reverse transcriptase (Invitrogen) and oligo(dT) primers were used for the reverse transcription reaction. Real-time PCR was performed using a 7500 HT Fast Real-Time PCR system (Applied Biosystems) and Power SYBR Green (Applied Biosystems). The amount of amplified transcript was normalized against an internal control [*ribosomal protein 32* (*rpl32*)]. The primers used are listed in Table S1.

Western Blotting and Immunoprecipitation. To generate the anti-Spz C106 antibody, a GST fusion protein containing amino acids V144–G243 of *Drosophila* Spz was expressed in *E. coli* via the pET vector. The recombinant protein was then purified and injected into rats. A mouse anti-Myc (9E10) antibody was obtained from Santa Cruz Biotechnology. PNGase F was purchased from New England Biolabs and used according to the manufacturer's protocol. Myc-tagged Spz and Myc-tagged SPE were immunoprecipitated with anti-Myc agarose beads (Sigma). For protein analysis, lysates of hemolymph were collected from 10 larvae by pricking with an empty capillary, and the proteins immunoprecipitated from 100 larvae were subjected to SDS/PAGE. The proteins were transferred to PVDF membranes (Millipore), which were then blocked with PBS containing 0.05% Tween 20 and 5% (wt/vol) skim milk, followed by an overnight incubation with the appropriate primary antibodies. The primary antibodies were detected with HRP-conjugated anti-mouse and anti-rat antibodies (Jackson Immunoresearch), and the signals were visualized using the Supersignal West Pico Chemiluminescent Substrate (Thermo).

Nucleotide Sugar Transport Assay. *senju* cDNA, His-tagged at the C terminus, was subcloned into the copper-inducible expression vector, pYEX-BX, and then introduced into the *Saccharomyces cerevisiae* strain CB001L. The in vitro nucleotide sugar transport assay was then performed as previously described (5).

Lectin Array. Thirty lymph glands isolated from *Drosophila* larvae, or hemolymph collected from 30 adult flies by pricking with an empty capillary, were lysed in PBS containing 1% Triton X-100 and complete protease inhibitor mixture (Roche). Extracted proteins (1 μg) were labeled with a Cy3 Monoreactive Dye (GE Healthcare) and sent to Glycotechnica (www.glycotechnica.com/) for glycan profile analyses using LecChip lectin array chips, which comprise 45 selected lectins fixed on a glass slide (Dataset S1). A solution containing glycoproteins labeled with Cy3 glycoprotein was applied to the lectin chip and incubated overnight. A fluorescence image of the array was then acquired using an evanescent-field fluorescence scanner (Glycostation Reader 1200; SC-Profiler). The net intensity value for each spot was calculated by subtracting the background value from the signal intensity. The

net signal intensity values from three spots were then averaged. Because the CV associated with this method is 10% (Glycotechnica), a change in signal intensity of >20% (twice the CV) was considered significant.

Survival Experiments. Thirty WT and *senju*-overexpressing flies (overexpression driven by *da-Gal4* or *srp-Gal4*) were challenged by septic injury using a thin needle dipped into a concentrated pellet of *B. subtilis* culture. The vials were put in an incubator at 25 °C, and the surviving flies were counted every 6 h. Results are expressed as the percentage of infected flies present at different time points after infection. Data are representative of three independent experiments.

Hemolymph Transfer Experiments. Thirty WT and *senju*[1] larvae were sterilized by washing three times in 70% (vol/vol) ethanol. The larvae were then pricked with an empty capillary to collect the hemolymph. The collected hemolymph was suspended in 30 μL of PBS and divided into two tubes. One tube was boiled at 95 °C for 5 min to denature the proteins, and the other was kept on ice. After centrifugation at 17,000 × *g* at 4 °C for 5 min, 50 nL of supernatant was injected into the abdomen of WT recipients. Flies were then incubated for 18 h at 25 °C and used for qRT-PCR.

Statistical Analysis. Data represent the mean ± SD of three independent experiments. Statistical analysis was performed by the Student *t* test using Microsoft Excel.

1. Gong WJ, Golic KG (2004) Genomic deletions of the Drosophila melanogaster Hsp70 genes. *Genetics* 168(3):1467–1476.
2. Yamamoto-Hino M, et al. (2012) Cisterna-specific localization of glycosylation-related proteins to the Golgi apparatus. *Cell Struct Funct* 37(1):55–63.
3. Ryu JH, et al. (2008) Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. *Science* 319(5864):777–782.
4. Andres AJ, Thummel CS (1994) Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol* 44:565–573.
5. Muraoka M, Kawakita M, Ishida N (2001) Molecular characterization of human UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter, a novel nucleotide sugar transporter with dual substrate specificity. *FEBS Lett* 495(1-2):87–93.

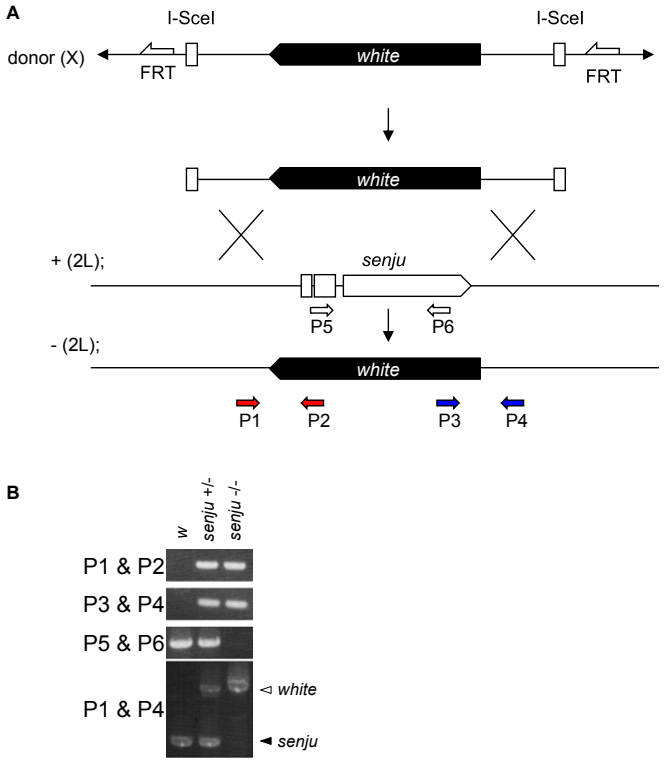

Fig. S1. KO of the *senju* gene by ends-out recombination. (*A*) Donor DNA from the X chromosome was generated by FLP and I-Sce-I (*Top*). Homologous recombination with the endogenous *senju* region replaced most of the *senju* coding sequence (opened box) with the white gene (filled box; *Middle*). The final structure (*Bottom*) was verified by PCR using four sets of primers (P1–P6). (*B*) The expected bands were amplified by four sets of primers specific for chromosomal DNA from *white* (*w*), *senju*$^{-/+}$, *and senju* $^{-/-}$ flies.

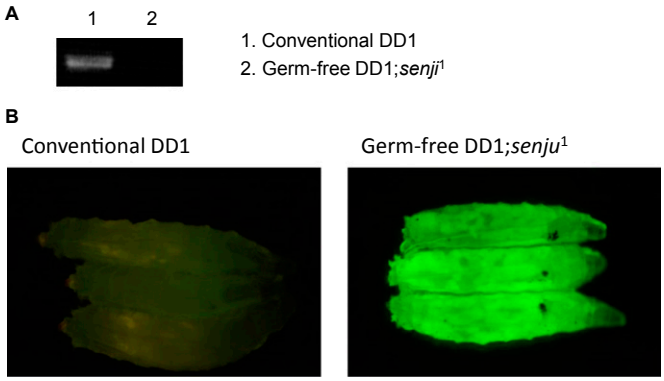

Fig. S2. *Drs* mRNA was up-regulated in germ-free *senju*[1]. (*A*) Bacterial contamination was examined by performing PCR analysis using the bacterial gene encoding the 16S rRNA of DD1 (*Drs*-GFP, *Dpt*-lacZ) larvae cultured under conventional conditions and of DD1;*senju*[1] larvae cultured under germ-free conditions. (*B*) *Drs*-GFP fluorescence induced in DD1;*senju*[1] under germ-free conditions.

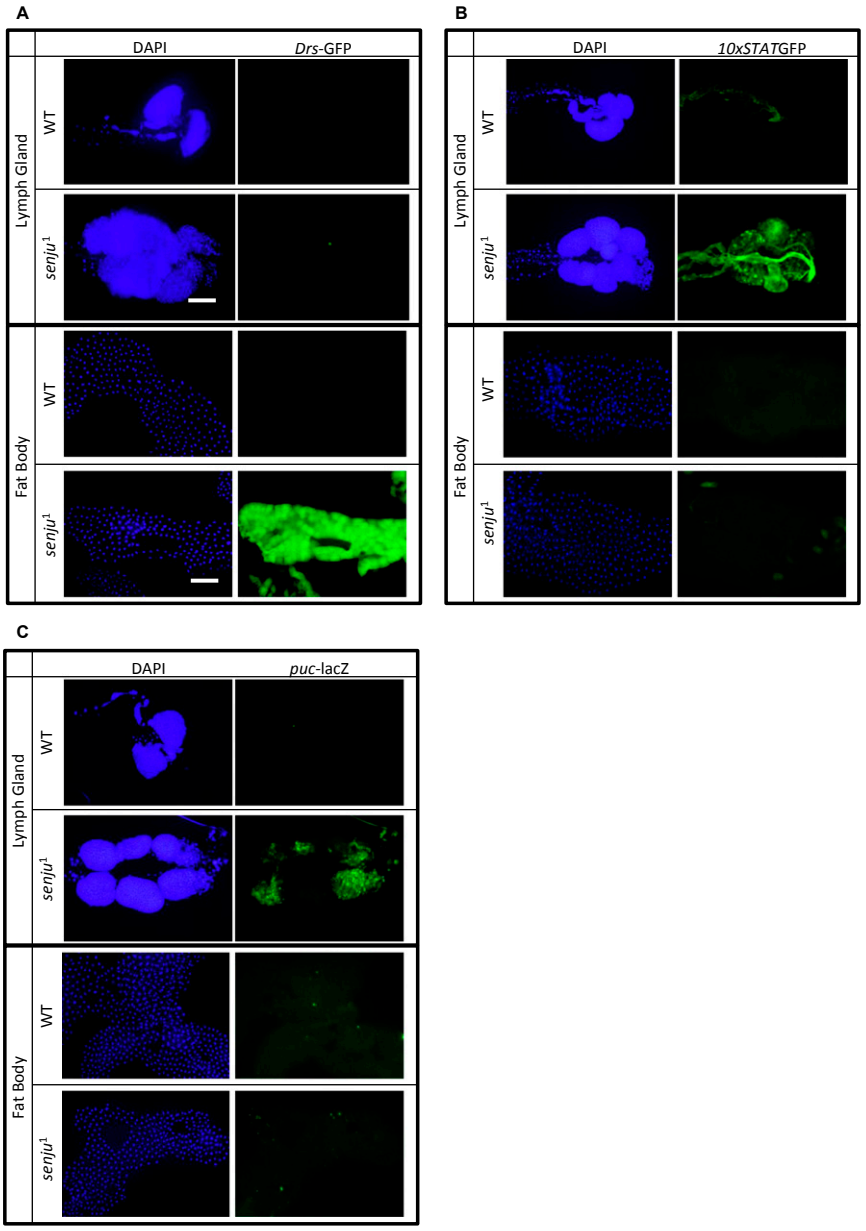

Fig. S3. Loss of *senju* activates the Toll, JAK/STAT, and JNK pathways in immune organs. (*A*) Expression of the *Drs*-GFP fluorescent reporter was induced predominantly in the *senju*^{1} fat body. (*B*) Expression of the *10xSTAT*-GFP fluorescent reporter was induced predominantly in the *senju*^{1} lymph gland. (*C*) Immunostaining revealed predominant expression of the *puc*-lacZ reporter in the *senju*^{1} lymph gland. (Scale bars, 200 μm.)

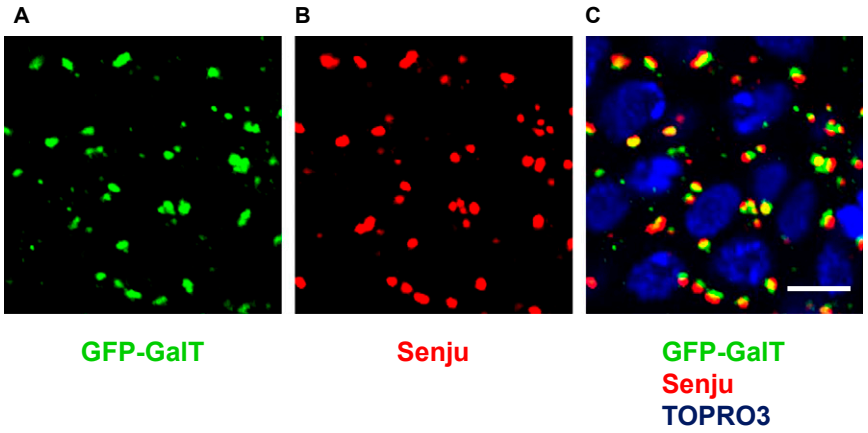

Fig. S4. Localization of the Senju protein. (*A*–*C*) Lymph glands from late third-instar larvae carrying the *GFP-GalT* transgene as a marker for the Golgi apparatus (green in *A* and *C*) were stained with an antibody against Senju (red in *B* and *C*). Nuclei were stained with TOPRO-3 (blue in *C*). (Scale bar, 5 μm.)

A

```
Wild type   106:ACGCAGCGGAGATCAGCTTTGGCAGTTGTACGCCCCAGCAGAGCGATGAGCGCGGCCAGTGCGTTCA
SPE^sk6     106:ACGCAGCGGAGATCAGCTTTGGCAGTTGTACGCCCCAGC--------GAGCGCGGCCAGTGCGTTCA
```

B

```
Wild type     1:MASTERNFLLLSLVVSALSGLVHRSDAAEISFGSCTPQQSDERGQCVHITSCPYLANLLM 60
SPE^sk6       1:MASTERNFLLLSLVVSALSGLVHRSDAAEISFGSCTPQRARPVRSHHQLPLPGQPADGGA 60

Wild type    61:VEPKTPAQRILLSKSQCGLDNRVEGLVNRILVCCPQSMRGNIMDSEPTPSTRDALQQGDV 120
SPE^sk6      61:KDSRPANPSLQEPVWPGQQG*------------------------------------  80
```

Fig. S5. CRISPR/Cas9-induced mutation at the *SPE* locus. (*A*) Sequence alignment of *SPE* from WT flies and flies harboring the *SPE*^{SK6} allele [which has an 8-bp deletion (from nt 116 to nt 123) in the coding region that causes a frameshift]. (*B*) Alignment of translated SPE protein sequences from WT and *SPE*^{SK6} flies. SPE^{SK6} contains amino acids 1–38 of SPE (indicated by the red box) but lacks amino acids 39–400, which includes the major protease domain (amino acids 135–400).

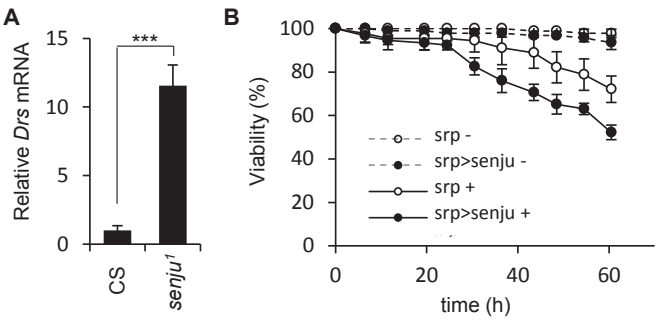

Fig. S6. Toll activation in *senju*^{1} adult flies and increased susceptibility to infection induced by Senju overexpression. (*A*) qRT-PCR analysis of *Drs* expression in unchallenged WT (CS) and *senju*^{1} adult flies. The values are normalized to the expression of *ribosomal protein 32* (*rpl32*) and expressed as the mean ± SD relative to the expression in WT flies, which was set at 1 (control). Data are representative of three similar experiments. **P* < 0.05 (Student *t* test). (*B*) Survival of *senju*-overexpressing (*srpGal4* > *UAS-senjumyc*, srp > senju) and control (*srpGal4*>, srp) flies after immune challenge with *B. subtilis* (+) or no challenge (–).

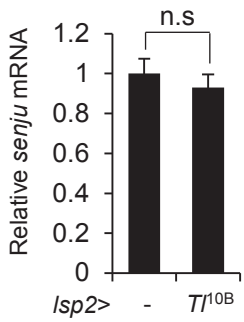

Fig. S7. *senju* expression in *lsp2> Tl*^{10B} flies. *senju* transcription (as measured by qRT-PCR) was unchanged when the Toll pathway was activated by over-expressing of *Tl*^{10B}.

Table S1. Oligonucleotide sequences used in this study

Purpose	Gene	Orientation	Primer sequence
qPCR	*rpl32*	Forward	GCAAGCCCAAGGGTATCGA
		Reverse	CGATGTTGGGCATCAGATACTG
	Drs	Forward	TTGTTCGCCCTCTTCGCTGTCCT
		Reverse	GCATCCTTCGCACCAGCACTTCA
	Tep1	Forward	TGAGGAACGGGAGAGTATCG
		Reverse	GCAAGCTTTGAACCCAATTC
	puc	Forward	GGCCTACAAGCTGGTGAAAG
		Reverse	AGTTCAGATTGGGCGAGATG
	Dpt	Forward	GTTCACCATTGCCGTCGCCTTAC
		Reverse	CCCAAGTGCTGTCCATATCCTCC
	senju	Forward	CTTCACGGCAGTCCTATGCT
		Reverse	TTTGGTCGTGGCATCACTTA
Transgenic fly	*nec*	Forward	ataagaatgcggccgcATGGCGAGCAAAGTCTCGATCC
		Reverse	ccgctcgagGACGCTCATGGGCGTGGGATAC
	spz	Forward	ataagaatgcggccgcATGATGACGCCCATGTGGATATCG
		Reverse	acgcgtcgacCCCAGTCTTCAACGCGCACTTGCAG
	SPE	Forward	gaagatcTAGCGACGAGATGGCTTCTACG
		Reverse	ccgctcgagTGGCTCCAATTTCTGCTTTATC
Generation of *SPE* mutant	SPE-1-F	Forward	CTTCGCCGCGCTCATCGCTCTGCT
	SPE-1-R	Reverse	AAACAGCAGAGCGATGAGCGCGGC
	SPE-CHK-263F	Forward	CTAAGCACTTGACCTTGTTGATTGTGAG
	SPE-CHK+273R	Reverse	GTGCTGCCTGGAAATGACGTCTGCGGC
Bacterial contamination	8FE	Forward	AGAGTTTGATCMTGGCTCAG
	1492R	Reverse	GGMTAGCTTGTTACGACT

The sequences in lowercase letters represent additional sequences containing restriction enzyme sites.

Table S2. Fly stocks used in this study

Types of fly strains	Gene name	Allele	Obtained from	Reference
Mutant	*Dif/Dl*	J4	Shoichiro Kurata	(1)
	PGRP-SA	seml	Julien Royet	(2)
	GNBP3	hades	Dominique Ferrandon	(3)
	grass	Herrade	Dominique Ferrandon	(4)
	ModSP	1	Bruno Lemaitre, Takayuki Kuraishi	(5)
	psh	1	Jean-Marc Reichhart	(6)
	SPE	SK6	Generated in this study	
	spz	rm7	Shoichiro Kurata	(7)
Gal4 line	*srp-Gal4*		Michele Crozatier	(8)
	lsp2-Gal4		*Drosophila* Genetic Research Center, Kyoto	
	da-Gal4		Bloomington	
UAS line	UAS-*Tl*^{10B}		Tony Ip	(9)
	UAS-*senju-myc*			(10)
	UAS-*nec-myc*		Generated in this study	
	UAS-*spz-myc*		Generated in this study	
	UAS-*SPE-myc*		Generated in this study	
Others	*Dome*-MESO		James Cast-G Hombria	
	2×STAT92E-GFP		Baeg, GyeongHun	(11)
	10×STAT92E-GFP		Baeg, GyeongHun	(11)
	puc-lacZ	E69	*Drosophila* Genetic Research Center, Kyoto	
	GFP-GalT		Konrad Basler	(12)
	DD1 (*Drs*-GFP, *Dpt*-lacZ)		Dominique Ferrandon	(13)

1. Meng X, Khanuja BS, Ip YT (1999) Toll receptor-mediated Drosophila immune response requires Dif, an NF-kappaB factor. *Genes Dev* 13(7):792–797.
2. Michel T, Reichhart JM, Hoffmann JA, Royet J (2001) Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* 414(6865): 756–759.
3. Gottar M, et al. (2006) Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. *Cell* 127(7):1425–1437.
4. El Chamy L, Leclerc V, Caldelari I, Reichhart JM (2008) Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll. *Nat Immunol* 9(10):1165–1170.
5. Buchon N, et al. (2009) A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. *Proc Natl Acad Sci USA* 106(30):12442–12447.
6. Ligoxygakis P, Pelte N, Hoffmann JA, Reichhart JM (2002) Activation of Drosophila Toll during fungal infection by a blood serine protease. *Science* 297(5578):114–116.
7. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86(6):973–983.
8. Crozatier M, Ubeda JM, Vincent A, Meister M (2004) Cellular immune response to parasitization in Drosophila requires the EBF orthologue collier. *PLoS Biol* 2(8):E196.
9. Hu X, Yagi Y, Tanji T, Zhou S, Ip YT (2004) Multimerization and interaction of Toll and Spätzle in Drosophila. *Proc Natl Acad Sci USA* 101(25):9369–9374.
10. Yamamoto-Hino M, et al. (2012) Cisterna-specific localization of glycosylation-related proteins to the Golgi apparatus. *Cell Struct Funct* 37(1):55–63.
11. Bach EA, et al. (2007) GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. *Gene Expr Patterns* 7(3):323–331.
12. Port F, et al. (2008) Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nat Cell Biol* 10(2):178–185.
13. Manfruelli P, Reichhart JM, Steward R, Hoffmann JA, Lemaitre B (1999) A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. *EMBO J* 18(12):3380–3391.

Dataset S1. Affinity of control (*J4*) and *senju*^{1}, *J4* lymph gland lysates for lectins

Dataset S1

Significantly reduced values [*senju*^{1};*J4 vs. J4* (control)] are highlighted in red.