Supplementary Methods

Signaling pathway reporter

The *mtk* luc reporter was generated by fusing a 343bp long promoter containing several Rel binding sites (Senger et al., 2004) from the *Metchnikowin* gene fragment to the *firefly* luciferase gene. Primers were designed to contain SpeI and HindIII sites

forward: 5'-AGGATTACTAGTCAGAAAAACCAACAGGGCGCTAAA

reverse: 5'-CACATGAAGCTTTAGCTCGGTGGCGGGAATTGATTGA

The region was amplified by PCR from *Drosophila* genomic DNA and subsequently cloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA).

Genome-wide RNAi screen of the IMD pathway

All screening experiments were performed in 96-well tissue culture plates (Cellstar, Greiner, Frickenhausen, Germany). Screening plates were pre-loaded with an average of 1µg dsRNA per well in 10µl of 1mM Tris pH7 with a Biomek FX robot (Beckmann-Coulter, Fullerton, CA, USA). To transfect the reporter constructs, 5 million SL2 cells were seeded per 35mm dish (Cellstar, Greiner, Frickenhausen, Germany) and cultured for 24h. 2µg plasmid DNA (1µg *mtk luc* and 1µg pIZ (Invitrogen, Carlsbad, CA USA) *Renilla* luciferase expression plasmid) was co-transfected using Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 16h after transfection, cells were resuspended in serum free medium. 50,000 cells in 50µl were subsequently seeded onto the dsRNA per well in 96-well tissue culture plates using a liquid dispensing unit (Multidrop, Thermo Labsystems, Egelsbach, Germany). After 45 minutes of starvation, 50µl of serum containing medium was added to each well. Cells were incubated in sealed assay

plates at 25°C. 4 days after RNAi treatment, cells were stimulated by adding heat-inactivated *E.coli* (DSM498) to a final concentration of 20µg/ml. 16h after induction, cells were lysed and the lysate was split to read firefly and *Renilla* luciferase activities independently in separate assay plates (white 96-well LIA plates, Greiner) using a Mithras LB940 plate reader (Berthold Technologies, Bad Wildbad, Germany). The screen was performed in duplicate.

Computational analysis

To identify candidate genes that modified IMD signalling pathway activity, the raw luciferase results were normalized by median centering of each 96well plate separately for FL and RL channels. *z*-scores were calculated as the number of median adjusted standard deviations (mad; implemented in *R*) that a particular well differed from the median of the 96well plate. We filtered dsRNA treatments with a minimum z-scores > 2.4 for negative regulators or < -2.4 for positive regulators in both replicates, respectively. Selected candidates were retested in secondary assays.

Cell-based assay for Toll signaling

5 million SL2 cells in a 35mm dish were transiently transfected using Cellfectin. Toll pathway was induced with a constitutive active Toll expression vector (0.1µg) which was obtained by subcloning an EcoRV-ApaI fragment from pMT-TollΔLRR (Sun et al., 2002) into the EcoRV and ApaI sites of pACV5His (Invitrogen, Carlsbad, CA, USA) downstream of the actin promoter. In addition, a *Drosomycin* firefly luciferase reporter (0.9µg) (Tauszig et al., 2000) and a plasmid constitutively expressing *Renilla* luciferase under the control of a ribosomal protein (RNA polymerase III 128kD subunit protein) promoter (1µg) (RP128-RL, Kerstin Bartscherer unpublished) were

co-transfected. 16h after transfection, cells were collected and resuspended in serum free medium. 20,000 cells in 20ml were seeded onto assay plates (384 well polystyrene plates from Greiner) preloaded with 0.3μg of dsRNA per well. After an incubation of 45 minutes, 30μl of complete medium was added; plates were sealed and incubated 5 days at 25°C to allow RNA depletion.

Cell viability assay

CellTiter Glo (Promega) assay of cell viability was performed in SL2 cells following treatment with dsRNA against GFP, DIAP1 and IAP2 as triplicates in 96-well tissue culture plates (Falcon, BD Biosciences, Heidelberg, Germany). Cells were incubated for 5 days at 25°C to allow protein depletion. CellTiter Glo reaction was preformed following manufacturers instructions.

Cell-based assay for JAK/STAT signaling

$S2R^+$ cells were transfected using Effectene with 0.5μg of *6x3xDrafLuc* JAK/STAT signaling reporter, 0.5μg of *pAc5.1-UpdGFP* to induce the pathway (Mueller et al., 2005), 0.25μg of *RP128-RL Renilla* luciferase plasmid as a co-reporter and 0.75μg of *pAC5.1* plasmid (Invitrogen) to adjust the DNA amount to 2μg. RNAi and luciferase assays were performed in 96-well plates as described above.

Epistasis Analysis

For epistasis experiments, SL2 cells grown in 6-well plates were transfected with 0.7μg pPac-mycIMD-HA (Georgel et al., 2001) or RelΔPEST (Stoven et al., 2003) expression vectors to stimulate pathway activity. To monitor pathway activity *mtk luc* (0.7μg) and pIZ-RL (0.7μg) were cotransfected. 24h after transfection, cells were

collected and treated with dsRNA against *GFP*, *PGRP-LC*, *IMD*, *Tak1*, *Dredd*, *Key*, *Rel* or *IAP2* in 96-well plates as described above. All epistatic combinations were performed in quadruplicate. Luciferase activity was measured after 96h.

RNA extraction and quantitative real-time RT-PCR

2 million SL2 cells were seeded in 1.5ml serum free medium per well of a 6-well tissue culture plate onto 15µg dsRNA (*GFP, IMD, IAP2* and *Mkk4/hep*). After a 1h starvation step, 2ml serum-containing medium was added. Cells were then incubated for 84h to ensure protein depletion. To induce an immune response, cells were stimulated for 60 minutes by adding heat-inactivated *E. coli* (20 µg /ml) or peptidoglycan (50 µg /ml; a kind gift of Bruno Lemaitre) to the medium. qPCR data was processed as described in (Pfaffl, 2001).

Determination of cell number by Neubauer hemocytometer count

Per well of a 24-well plate (Greiner), 0.5 million SL2 cells were seeded in 0.5ml serum free medium and 2µg of dsRNA (*GFP*, *DIAP1* or *IAP2*) was added. After 1h starvation, 0.5ml serum containing medium was added per well, plates were sealed and incubated at 25°C. On the days 2 to 5, cells were removed from the well bottom by carefully pipetting up and down. The samples were stained with Trypan Blue (Sigma, Munich, Germany), transferred to a Neubauer hemocytometer chamber (Brand) and live cells counted by light microscopy. For each dsRNA, two independent samples were counted each day.

Supplementary Figure 1 Cell-based assay system employed to monitor IMD/Rel signaling.

(**A**) Schematic representation of reporter constructs. To monitor IMD pathway activity, a reporter gene consisting of the firefly luciferase gene under control of the promotor of the IMD/Rel target gene *metchnikowin* (*mtk*) was used. As a secondary assay, an *attacin* (*attA*) reporter was employed (middle panel). A constitutively expressed *IZ Renilla* luciferase reporter construct allows monitoring of cell growth and viability (bottom panel).

(**B**) Luciferase assay system. In SL2 cells, the firefly luciferase reporter is 22-fold induced after stimulation with heat-inactivated *E.coli* for 18h (FL, black bars). The constitutive expression of the *Renilla* luciferase reporter is independent of an immune stimulus (RL, white bars). Error bars represent standard deviations of 4 independent replicates. See also Supplementary Figure 1 for *mtk* reporter specificity.

(C) **(D)** and **(E)** Cell-based assay demonstrating the specificity of *mtk luc* reporter construct for IMD/Rel signaling in SL2 cells.

The *mtk luc* reporter is based on promotor sequences that have been previously shown to contain NF-κB binding sites specific for Relish, but not the related NF-κB factors Dorsal and Dif (Senger et al., 2004). Consistently, the reporter is not activated by a dominant active Toll$^{\Delta LRR}$ (**C**, Toll$^{\Delta LRR}$), which activates the Toll pathway reporter Drs luciferase. We also did not observe any effect of knock-down of *Tube*, a Toll signaling component on the *mtk* reporter induction (**D**, *Tube*) although its knock-down has an effect on the Drs reporter induction by Toll$^{\Delta LRR}$ (**E**, *Tube*). Although *Metchnikowin* activation requires both Toll and IMD pathways *in vivo* (Levashina et al., 1995), we

are confident that the mtk reporter does only monitor IMD activity, but not Toll signaling activity.

Supplementary Figure 2 ATP-viability assay after *IAP2* depletion.

To test *IAP2* requirement in cell viability, we used a luciferase based CellTiter-Glo (Promega) assay to measure intracellular ATP levels after 5 days of RNAi against *GFP*, *DIAP1* and *IAP2*. Luciferase activity following dsRNA treatment against GFP was used for normalization. Depletion of DIAP1 led to a significant reduction of intracellular ATP levels and indicative of a strongly reduced viability. In contrast, RNAi against *IAP2* again did not show any viability phenotype in this assay.

Supplementary Figure 3 Epistasis analysis of *IAP2*

qPCR analysis of IAP2 function in JNK signaling in SL2 cells. mRNA levels of the JNK target gene *puckered* (*puc*) increase after stimulation with heat-inactivated *E.coli* for 1h (*GFP*, +). Knock down of JNK transcription factors (*AP-1*), blocks induction of the IMD/JNK cascade (*AP-1*, +). Similar to *AP-1* RNAi, *IAP2* knock down suppresses induction of IMD/JNK signaling (*IAP2*, +). Error bars represent standard deviation of two replicates.

References

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Supplementary Table 1: Candidate modifiers of IMD/Rel signaling

gene		dsRNA ID[a]	mtk-reporter (z-score)	attA-reporter[b] [%]	IMD[b] Epistasis	Rel[b] Epistasis	Gene Ontology	Interpro 8.0 evidence	GO Evidence
PGRP-LC	CG4432	HFA10461	-7.0	22	n.d.	n.d.	defense response		GO:0006952
IMD	CG5576	HFA05928	-7.5	20	+	-	signal transduction; defense response	IPR000719	GO:0007165; GO:0006952
Tak1	CG18492	HFA20561	-5.2	53	+	-	signal transduction; defense response	IPR000719	GO:0007165; GO:0006952
Dredd	CG7486	HFA18461	-5.0	29	+	-	apoptosis, defense response		GO:0007165; GO:0006952
Key	CG16910	HFA04285	-6.2	42	+	-	signal transduction		GO:0008063; GO:0006952
FADD	CG12297	HFA14122	-2.7	n.d.	n.d.	n.d.	signal transduction; defense response	IPR000488	GO:0007165; GO:0006952
IKK	CG4201	HFA16714	-6.5	17	+	-	signal transduction	IPR000719	GO:0008063
Rel	CG11992	HFA16819	-6.9	5	+	+	signal transduction	IPR000451	GO:0007165
IAP2	CG8293	HFA07444	-2.5	37	+	-	anti-apoptosis; protein ubiquitination	IPR001370	GO:0006916; GO:0016567
Ush	CG2762	HFA00843	-4.8	62	+	+	signal transduction	IPR007087	GO:0008293
Hel25E	CG7269	HFA03342	-6.7	36	+	+	mRNA-nucleus export; nuclear mRNA splicing		GO:0006406; GO:0000398
CG6197	CG6197	HFA06967	-4.3	21	+	+	RNA processing and translation	IPR003107	GO:0006396
AGO1	CG6671	HFA05912	-5.3	26	+	-	RNA processing and translation	IPR003165	GO:0016246
CG8435	CG8435	HFA07179	-2.8	37	+	+	unknown	IPR007590	
CG6905	CG6905	HFA08577	-4.2	37	+	+	RNA processing and translation	IPR001005	GO:0000398
CG2469	CG2469	HFA08562	-4.2	37	+	+	unknown	IPR001440	
CG5605	CG5605	HFA11779	-5.4	20	+	+	RNA processing and translation	IPR004403	GO:0006415
Spt6	CG12225	HFA18836	-5.0	22	+	+	signal transduction	IPR006641	GO:0007242
CG17498	CG17498	HFA10274	-3.8	16	n.d.	n.d.	cell cycle		GO:0007049
Gap1	CG6721	HFA11172	-3.5	36	+	-	signal transduction	IPR001936	GO:0000165
Ttk	CG1856	HFA17091	-3.0	45	n.d.	n.d.	Transcription regulator	IPR007087	GO:0000122
Arf102F	CG11027	HFA17195	-3.3	36	+	-	signal transduction	IPR006689	GO:0007166
l(1)10Bb	CG1639	HFA20346	-4.0	35	+	-	Transcription regulator	IPR001748	GO:0030528
Cactin	CG1676	HFA20576	-2.5	68	n.d.	n.d.	signal transduction		GO:0007165

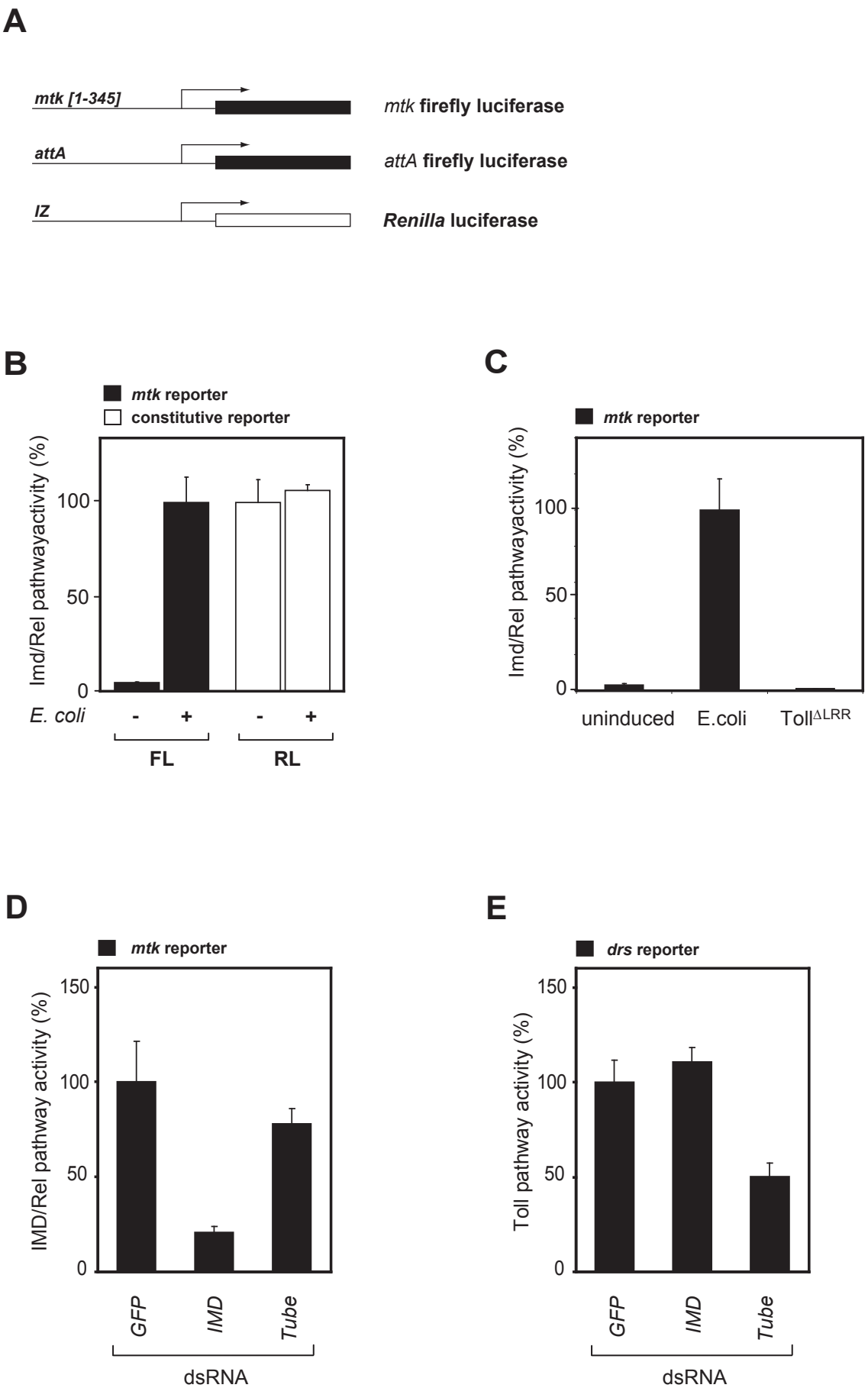
[a] **Complete amplicon information and details on score specificity can be obtained at http://rnai.dkfz.de**

[b] **Percent reporter activity after E. coli treatment as compared to an unrelated control (GFP). (+) denotes a decrease with $p < 0.05$**

Supplementary Table 2: dsRNA template and qPCR primer sequences

Gene	Primer 1	Primer 2	
Gene-specific primers for RNAi probes			
Tube	GGGACAATGACATTTAC	GGCACCAGAAGTTGGAG	
Pelle	GGGGCGTCAGCAACTCA	GCCGAGGCTGATGCTAA	
GFP	ACCCTCGTGACCACCCTGACCTAC	GGACCATGTGATCGCGCTTCTCGT	
DIAP1	CCGGCATGTACTTCACACAC	GAATCGGCACTGACTTAGCC	
Tak1	CCGTGGAACGCTAAGTATCC	AGGCACAGTTAGCCAGCAAT	
PGRP-LC	GACCAAAGATCGGGGGAC	GCTTATCACCGAACGTCAC	
IMD	GAGAATGTCAAAGCTCAGGAACCT	GAGAAATGCTGACCGTTTTGCGCG	
Key	GCCTGCGTTCACAGTCGTAAA	GCTTCAGGACATCGGATTGAA	
Dome	GCGTCTGCGCAGTGATCC	GTGGGCTCCGATGGATAGA	
Stat92E	GCTTGCCCAAAACTACAGTTAC	GCGACTGTGGGTGGATTGTT	
IAP2	GGAGCACAAGCGCTTTTTTC	GACAACTCCTCCATCGATACC	
Fos	GGCAACGCGAATACCTCAAAT	GGCTTGAGATCCAAGGGTGAA	
Jun	GGGTAAACACACCCGATTTGG	GCTAGCCAGGTCGACGTTCTC	
Hep	CGCAGTCGGTTATCCTTGAT	GGATCGGGTATCAGCATAGC	
Mkk4	CTGCTCGAAAACAGAGTCCC	AAATTGGACAAGGTGATGGC	
Rel	GTGGAACACATGGATCGCTAA	GCCGACTTGCGGTTATTGATT	
Dredd	TCGTACGCCTCCACATTGTA	CCGCTTGCTCCTAGATGTTC	
qPCR primer sequences			**UPL***
IAP2	CACGCTTATGCAAGGTATGC	GGGACAATTGGCTACACTGG	153
Metchnikowin	CCACCGAGCTAAGATGCAA	TCTGCCAGCACTGATGTAGC	125
IMD	CCTTTCGAGAAGGCACAGTT	TGCCTTTGTGTTTCTTTGCTC	77
Rp49	CGGATCGATATGCTAAGCTGT	GCGCTTGTTCGATCCGTA	105
Puckered	GCCACATCAGAACATCAAGC	CCGTTTTCCGTGCATCTT	79
Mmp-1	CCGATTTGCTGTTGACTCG	GAGGGCGTGTTCTTGCAG	131
Cecropin A2	GGACAATCGGAAGCTGGTT	TGTGCTGACCAACACGTTC	137

[a] **Probe Numbers according to updated Roche UniversalProbeLibrary Numbers for qPCR.**
(Steckel and Boutros, Biochemica, 2005 ; Mouritzen et al.; Nature Methods, 2005; Mauritz et al.; Biochemica, 2005)
http://www.universalprobelibrary.com



Supplementary Figure 1
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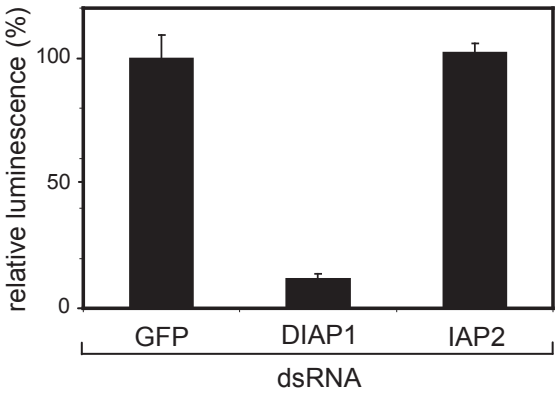

Supplementary Figure 2
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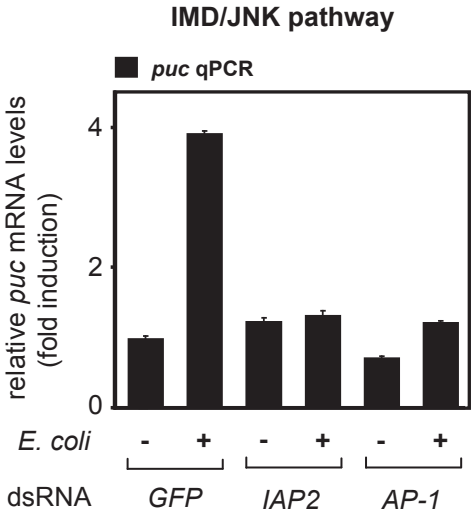

Supplementary Figure 3
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