

A Large-Scale RNAi Screen Identifies *Deaf1* as a Regulator of Innate Immune Responses in *Drosophila*

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Key Words

Functional genomics • Innate immune responses • *Drosophila* • RNA interference • Toll signalling

Abstract

Innate immune signalling pathways are evolutionarily conserved between invertebrates and vertebrates. The analysis of NF- κ B signalling in *Drosophila* has contributed important insights into how organisms respond to infection. Nevertheless, significant gaps remain in our understanding of how the activation of intracellular signalling elicits specific transcriptional programs. Here we report a genome-wide RNA interference survey for transcription factors that are required for Toll-dependent immune responses. In addition to the NF- κ B homologs Dif, Dorsal and factors of the general transcription machinery, we identified Deformed Epidermal Autoregulatory Factor 1 (*Deaf1*) to be required for the expression of the Toll target gene *Drosomycin* in cultured cells and in *Drosophila* in vivo. We show that *Deaf1* is required for the survival of flies after fungal, but not *E. coli*, infection. We determine that *Deaf1* acts downstream of the NF- κ B factors Dorsal and Dif. These results indicate that *Deaf1* is an important contributor to innate immune responses in vivo.

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Introduction

All metazoans have developed immune responses that protect the organism against pathogenic challenges. Among the most ancient of these processes are innate immune responses that recognize pathogens through specific receptors and initiate signalling pathways leading to cellular or humoral responses. Important insights into immune signalling were discovered in the model organism *Drosophila melanogaster*, which is devoid of adaptive immunity and solely relies on innate immune defences. Genetic studies have identified two NF- κ B related pathways essential for immune function in *Drosophila*, which are commonly referred to as the Toll and Immune Deficiency (*Imd*) pathways. Both relay the signal through intracellular cascades culminating in the nuclear translocation of NF- κ B proteins Dorsal-related immunity factor (*Dif*), Dorsal and Relish, followed by the activation of target genes [reviewed in 1].

When pathogens succeed in bypassing the local immune mechanisms of barrier epithelia, systemic infections by fungi or Lys-type Gram-positive bacteria trigger

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the Toll signalling module through activation of soluble pattern-recognition receptors in the hemolymph [2]. Activation of these receptors sets off a proteolytic cascade which culminates in the activation of the Toll ligand Spätzle by the protease Spätzle processing enzyme [3]. In a pattern recognition receptor independent pathway, proteases secreted by bacteria or fungi can induce Spätzle processing enzyme-dependent Spätzle activation through cleavage of the protease Persephone [4–7]. In either case, binding of the activated cytokine Spätzle to the Toll receptor leads to a conformational change in the extracellular receptor domains that facilitates receptor dimerization. On the intracellular side, the Toll/interleukin-1 receptor domains of the dimerized receptors serve as sites for the formation of a multiprotein complex composed of the proteins MyD88, Tube and Pelle, which interact via their death domains. Through steps that are not yet fully understood, the signalling complex induces the phosphorylation of Cactus, the *Drosophila* homolog of I κ B. In the absence of signalling, Cactus is bound to NF- κ B transcription factors Dif or Dorsal and retains them in the cytosol. Upon phosphorylation, Cactus dissociates from Dif or Dorsal and is subsequently targeted for proteasomal degradation. Once released from Cactus inhibition, Dif and Dorsal translocate to the nucleus and drive expression of target genes [2]. Stimulation of the Imd pathway leads to the activation of another NF- κ B molecule, Relish, which induces expression of factors required for defence against diaminopimelic acid-type Gram negative bacteria (e.g. *Escherichia coli*) [1]. Among genes specifically induced by the Toll or Imd pathways are the antimicrobial peptides (AMPs) *Drosomycin* (*Drs*) and *Diptericin* (*Dpt*), which are commonly used as specific marker genes for the activation of Toll or Imd pathways, respectively [8].

Dif, Dorsal and Relish bind to conserved NF- κ B binding motifs in immune gene enhancers. Their affinity to bind varying κ B motif sequences directs the expression of differential genetic programs after Toll or Imd pathway activation [9]. Although necessary for the expression of immune genes, NF- κ B factors alone are not sufficient to mediate a temporal and tissue-specific immune response [10]. Most immune regulatory DNA elements harbour additional conserved sequence motifs: GATA transcription factor binding sites were found to mediate the tissue-specific expression of immune genes in the larval fat body and the mid gut [11–13]. Furthermore, constitutive AMP expression in epithelial tissues is regulated by Hox and POU transcription factors. In particular the Hox transcription factor Caudal inhibits local AMP ex-

pression in the gut and therefore protects intestinal commensal bacterial populations [14]. In addition, reports indicate that Dif needs the co-regulator module dTrap80 to activate the expression of immune responsive genes in vitro [15]. In Imd mediated immune responses, the nuclear protein Akirin was found to be necessary for Imd target gene expression in *Drosophila* and, moreover, for NF- κ B mediated target gene expression in mice [16]. Although many components of the extra- and intracellular Toll signalling routes are known, significant gaps remain in our understanding of how Toll-dependent gene transcription is regulated.

An important advance for the functional identification and characterization of genes has been the systematic use of RNA interference (RNAi) to silence gene expression. In metazoans, the RNAi pathway can be triggered through introduction of double-stranded RNAs that are homologous to endogenous mRNAs [17, 18]. Several genome-scale RNAi screens in *Drosophila* have discovered novel components of signalling pathways [19, 20]. Using quantitative cell-based assays in a high-throughput screening format, such approaches can identify components which might have been missed in previous classical genetic screens due to early lethality or complex phenotypes [21].

In this study, we took a genomic approach to identify transcription factors required for Toll-dependent target gene expression by performing an RNAi screen. The analysis of 1,033 dsRNAs directed against putative transcription factors revealed 32 significant phenotypes, including both novel modulators and components of the general transcription machinery. Specifically, we identified the DNA-binding domain SAND (for Sp100 AIRE-1 NucP41/75 Deaf1) containing the protein Deformed Epidermal Autoregulatory Factor 1 (Deaf1), as being required for Toll dependent *Drs* expression.

Recently Reed et al. [22] identified Deaf1 as a factor binding to the *metchnikowin* (*mtk*) and *Drs* promoter in vitro using affinity chromatography. Deaf1 binding motifs within both promoters were found as being required for proper *Drs* and *mtk* luciferase reporter induction. In addition, over-expression of Deaf1 lead to reporter activation, while coexpression with Dif and Dorsal resulted in synergistic reporter activation [22]. Here we show, by the analysis of Deaf1 loss of function phenotypes, that Deaf1 is required for immune-induced expression of *Drs* and *mtk*. Depletion of *Deaf1* in adult tissues reveals that it is requisite for the expression of anti-fungal response genes and required to combat fungal infections in vivo. For *Drs* expression, we map the function of Deaf1 down-

stream or in parallel to Dif and Dorsal using cell-based epistasis analysis. Site directed mutagenesis of the SAND DNA-binding motif in the *Drs* promoter indicates that Deaf1 functions as a cofactor of transcription for immune-regulated genes. Thus, our findings suggest a function for SAND domain-containing DNA-binding proteins like Deaf1 in defining the appropriate expression level of immune response genes.

Materials and Methods

RNA Synthesis

The RNAi library and secondary dsRNA probes, which were generated by in vitro transcription as described previously [23], are shown in table 1 of the online supplementary material for this article (all online supplementary material can be found at www.karger.com/doi/10.1159/000248649). Complete primer and amplicon sequence information can be found at <http://rnai.dkfz.de>.

Cell Culture, Transfection, RNAi and Luciferase Assays

Drosophila SL2 cells were cultured in Schneider's *Drosophila* Medium (Invitrogen), supplemented with 10% fetal calf serum (PAA) and 1% penicillin-streptomycin (Invitrogen) at 25°C. High-throughput screening in SL2 cells was performed as described previously (detailed in the extended Methods section of the online supplementary materials) [20]. Briefly, SL2 cells were transiently transfected with pAct-EGFR-Toll [24], a *Drs* promoter firefly luciferase reporter (*Drs-luc*) [25] and a plasmid constitutively expressing *Renilla* luciferase (pIZ; Invitrogen). Sixteen hours after transfection, cells were resuspended in serum-free medium and seeded in 96-well tissue culture plates pre-spotted with 1 µg of dsRNA. After 45 min of starvation in serum-free media, complete medium was added, plates were sealed and incubated at 25°C. Four days after dsRNA treatment, the medium was exchanged with serum-free medium containing hEGF (Biomol) to a final concentration of 0.2 ng/µl. Sixteen hours after induction, cells were lysed and the lysate was split to read firefly and *Renilla* luciferase activities independently. The screen was performed in duplicate. Validating experiments for Toll were performed as described previously [20]. All luciferase assays were performed in quadruplicate and independently reproduced at least once, if not indicated otherwise. For the *mtk*-reporter luciferase assay, S2 cells were seeded in a 384-well plate pre-loaded with 250 ng of dsRNA, with 30,000 cells/well in 15 µl serum-free media. After 1 h incubation, 20 µl of serum-containing media was added. 24 h after seeding, cells were transfected with 20 ng of the *mtk*-reporter and 10 ng of the *Renilla* reporter using Effectene (Qiagen). After 4 days of incubation, cells were induced with *E. coli* at a final concentration of 10 µg/ml for 16 h prior to luciferase measurement [20]. For over-expression of Toll^{ΔLRR} cells were transfected as above with the addition of 20 ng of pAc- Toll^{ΔLRR}.

qRT-PCR Experiments

Three million SL2 cells were seeded in 1 ml serum-free medium per well of a 6-well tissue culture plate onto 15 µg total dsRNA (directed against either *GFP*, *Deaf1*, *Dif*, *Dorsal*, or *cactus*). For double depletion, 7.5 µg dsRNA for each probe was used. Af-

ter a 1 h starvation step, 1 ml serum-containing medium was added. Cells were incubated for 84 h to ensure protein depletion. Total RNA from SL2 cells was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA per sample was digested by DNaseI before reverse transcription using SuperScript III (Invitrogen) according to the manufacturer's description. For the in vivo infection experiments, total RNA from whole flies was extracted using Trizol, purified and DNaseI digested on columns using the RNeasy kit (Qiagen). Two micrograms of total RNA were reverse transcribed using SuperScript III. qRT-PCR data was processed as described [26]. qRT-PCR primer sequences are listed in online supplementary table 2. Experiments were performed in 2 biological and 2 technical replicates and at least once independently reproduced, if not indicated otherwise.

Generation of Stable Cell Lines Expressing pMT-Toll^{ΔLRR}

Stable cell lines expressing Toll^{ΔLRR} under the control of the metallothionein (pMT-Toll^{ΔLRR}) were generated using DES-Inducible Kit with pCoBlast (Invitrogen) following manufacturer's instructions. Expression of the construct was induced by addition of CuSO₄ to a final concentration of 500 µM for 48 h.

Ecopic Expression of Deaf1, Dif and Dorsal in SL2 Cells

Deaf1 full-length and deletion constructs were generated using standard PCR amplification from reverse transcribed total RNA of SL2 cells. Primer sequences are listed in the extended Methods online. Full length, ΔC, ΔN, ΔCN constructs of Deaf1 were subcloned into pAc5.1-V5/His (Invitrogen) expression plasmid using *EcoRI/XbaI* restriction sites. To express Deaf1 constructs, cells were transiently transfected with Effectene (Qiagen) according to the manufacturer's instructions. To express Dif and Dorsal in cells we transiently transfected pSH-Dif and pSH-Dorsal (a kind gift of Tony Ip) in SL2 cells, as described above. Expression was induced by addition of CuSO₄ to a final concentration of 500 µM for 48 h.

Protein Extraction, Western Blotting and Immunocytochemistry

Cells were seeded in 6-well dishes with or without dsRNAs and transfected after 24 h. To check for expression of cloned Deaf1 full length and deletion constructs, cells were harvested 3 days after transfection. For experiments with RNAi treatment, we allowed the cells to grow for 5 days after transfection before harvesting. Protein extraction, SDS-Page and Western blot assays were performed using standard protocols. To detect V5/His-tagged Deaf1 protein on Western blots we used an HRP-conjugated mouse α-His6 antibody (Roche) diluted 1:2,000. Detection of signals was performed using ECL plus (Amersham). Mouse anti-tubulin (a gift from U. Euteneuer) was used at a dilution of 1:1,000. For immunocytochemistry cells treated with either *GFP* or *cactus* dsRNA were transfected with the full-length Deaf1 expression construct. After 5 days, cells were seeded on cover slips coated with poly-L-lysine (Sigma). Fixation and permeabilization was performed as described previously [27]. DNA was stained using Hoechst (Invitrogen), the actin cytoskeleton was visualized by phalloidin - FITC (Sigma) treatment. Dorsal protein was detected using the mouse α-dorsal primary antibody [28] and goat α-mouse IgG TRITC (Dianova), His/V5-tagged Deaf1 proteins using a rabbit α-V5 primary antibody (MBL) and goat α-rabbit

TRITC secondary antibody (Dianova). Images were captured with a Perkin Elmer spinning disc confocal ERS-FRET on a Nikon TE2000 inverted microscope using a $\times 63$ objective at the Nikon Imaging Center Heidelberg, with a binning set to 1. Images were assembled and processed in Adobe Photoshop.

Drosophila Stocks and in vivo Infection Assays

Deaf1 RNAi transgenic *Drosophila* lines were generated by cloning a 500 bp sequence of Deaf1 exon 3 (for detailed cloning procedure see extended Methods online) into the pWiz P-element expression vector [29]. Constructs were injected in w^{1118} embryos and multiple homozygous viable transgenic insertion lines were tested. The driver lines *daughterless-Gal4* (*da-Gal4*), *actin-Gal4* (*act-Gal4*) and *Cg-Gal4* (expression in hemocytes and the fat body) were obtained from the Bloomington Stock Center. *key* ($y, w, P [ry+, dpt- LacZ], P [w+, drs- GFP]; cn bw key [1]$) and *Dif* ($y, w, P [ry+, dpt- LacZ], P [w+, drs- GFP]; Dif [1]$) mutant stocks were a kind gift of Dominique Ferrandon. To increase RNAi efficiency, third instar larvae were incubated at 29°C and allowed to develop. Adults were infected 7–10 days after hatching. Infection experiments to measure AMPs were performed by pricking flies with a sharp needle dipped into a concentrated culture of *Micrococcus luteus* (*M. luteus*) or *E. coli*. For each replicate, 10 flies were infected for 6 h with *E. coli* or 24 h with *M. luteus*. RNA was extracted as described above from at least 4 biological replicates per genotype per experiment. For survival assays, 120 flies were infected with *E. coli* by pricking or with *Beauveria bassiana* by shaking anesthetized flies for 30 s in a Petri dish containing a sporulating fungal culture. The number of surviving flies was counted every 24 h after infection.

Results

RNAi Survey for Regulators of Toll-Pathway Targets

To identify novel regulators of Toll signalling, we performed a large-scale RNAi survey analyzing the effect of silencing 1,033 transcription factors encoded in the *Drosophila* genome. To this end we transiently transfected *Drosophila* SL2 cells with a chimeric Toll-EGFR (Toll Epidermal Growth Factor Receptor) which induces Toll pathway activity upon addition of recombinant human EGF (hEGF) to the cell culture medium [24]. Pathway activity after RNAi treatment was then monitored with a *Drs* promoter firefly luciferase reporter (*Drs-luc*) [25], while cell viability and transfection efficiency was monitored using a constitutively expressed *Renilla* luciferase co-reporter [20]. We first confirmed the assay's applicability by targeting the Toll pathway components MyD88, Dif and Dorsal. The *Drs-luc* reporter was induced 13-fold upon stimulation with hEGF. As shown in figure 1a, dsRNA directed against MyD88 led to a >95% reduction in reporter activity compared to the negative control (GFP). In contrast, *Dif* and *Dorsal* dsRNAs led only to marginally reduced reporter signals, while silencing both

NF- κ B factors simultaneously resulted in significant phenotypes. These results support work showing that Dif and Dorsal act synergistically to activate *Drs* expression in S2 cells [30, 31].

To screen for novel transcriptional modulators of Toll target gene expression, we analyzed a subset RNAi library covering all *Drosophila* genes which encode putative site specific transcription factors based on their gene ontology annotation [32, 33]. Probes targeting the known pathway components Pelle and MyD88 were included to serve as internal positive controls in the screen. The screening procedure is depicted in figure 1b and is described in detail in the extended Methods online. Duplicate data sets were analyzed using the Bioconductor software package *cellHTS* [34]. The results were filtered to exclude any dsRNA that induced cell growth and viability defects [23] or phenotypes that were not reproducible between replicates. We considered a result as reproducible when the standard deviation of both replicates did not exceed 50% of their mean value. Using these criteria we selected 32 dsRNA probes that significantly altered the expression of the *Drs* reporter (fig. 1c and online suppl. table 1). It is interesting to note that Relish, which is known to be required for Imd pathway signalling, scored as a strong regulator of *Drs* reporter activity. Indeed, this finding supports previous reports that Relish can form heterodimers with Dif or Dorsal and binds to *Drs* promoter regions [31]. However, as this result suggests a significant rate of Relish activation in the cells, we cannot exclude the possibility of artificial Imd pathway activation by *E. coli* peptidoglycan contamination in the recombinant hEGF reagent. We further found the transcription initiation factor TFIID isoform Trf2 and the TFIIA isoforms TFIIA-L and TFIIA-S to be required for *Drs* reporter activation. Among the negative regulators, we identified dsRNAs targeting Brahma and Dalao, which both are components of the Brahma chromatin remodelling complex [35].

One of the strongest phenotypes was observed for dsRNA targeting the gene *Deaf1*. Bioinformatic analysis revealed that *Deaf1* had not been found in any other RNAi screens published to date [36], suggesting that it is a potential new component of the Toll pathway and not a general modulator of the transcriptional machinery.

Deaf1 RNAi Leads to Loss of Drs Expression in Response to Toll Signalling

To exclude the possibility that Deaf1 RNAi phenotypes are caused by unintended off-target knock-downs due to unspecific dsRNA sequence matches in secondary

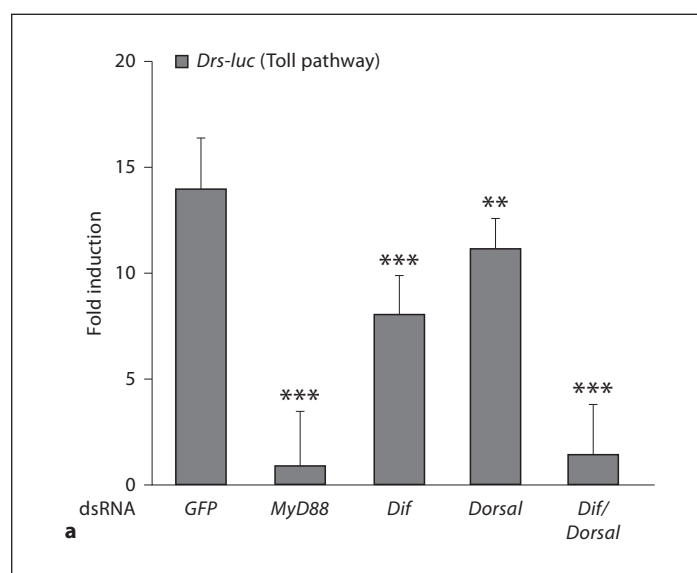
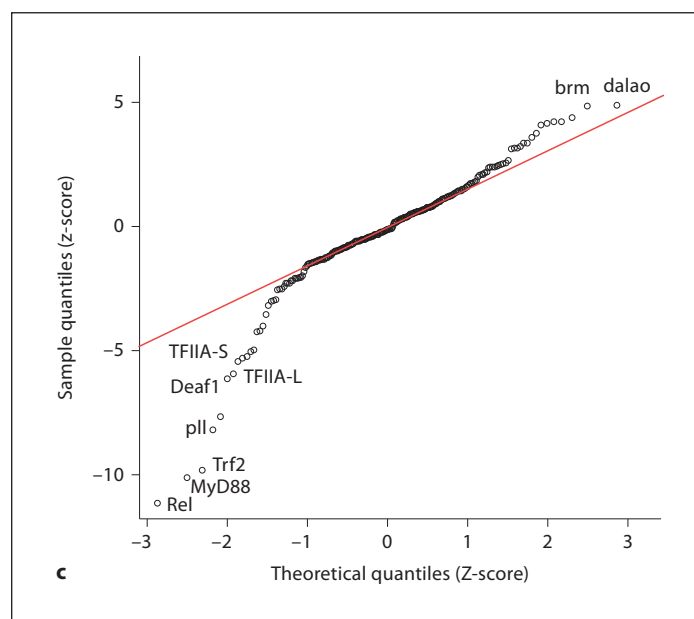
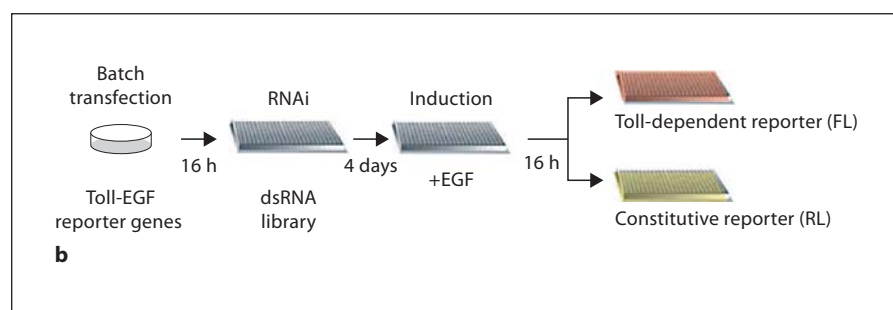


Fig. 1. An RNAi screen for transcriptional regulators of Toll signalling in SL2 cells.

a Proof of principle experiment. SL2 cells transfected with *Toll-hEGFR*, *Drs* firefly luciferase (*Drs-luc*) and *Renilla* luciferase co-reporter underwent RNAi against *GFP*, *MyD88*, *Dif*, *Dorsal* and a double knock-down of *Dif/Dorsal*. Error bars indicate the standard deviation of 8 replicates. ** $p = 0.0039$; *** $p < 0.0001$, determined using 1-way ANOVA. The *Drs-luc* reporter was induced 13-fold upon stimulation with hEGF. The data are representative of 3 independent experiments. **b** Schematic representation of the screening procedure. SL2 cells were transfected in batch with *Drs-luc*, *Toll-hEGFR* and a constitutively active *Renilla* co-reporter. After 16 h, transfected cells were seeded on pre-spotted dsRNA 96-well plates to mediate RNAi, incubated for 4 days and induced with hEGF for 16 h. To assay luciferase activity, cells were lysed and subsequently split to measure firefly and *Renilla* activity independently. FL = Firefly luciferase; RL = renilla luciferase. **c** Quantile-quantile plot of phenotype score distribution. Deviation from the line represents extreme values unexpected under a normal distribution. A threshold of 2.5 for negative regulators and -2.5 for positive regulators was chosen to select candidate modifiers.



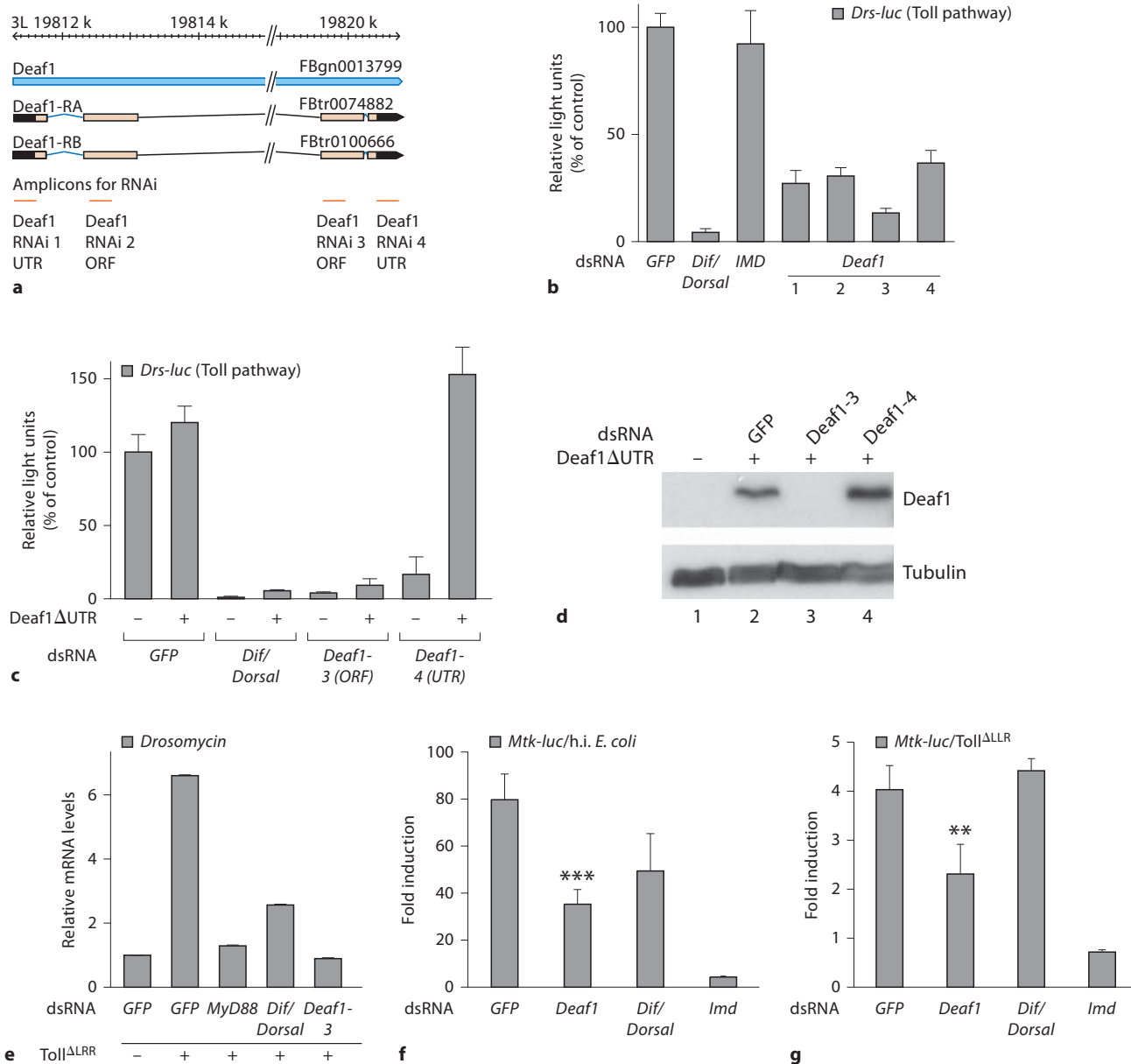


Fig. 2. Specificity of Deaf1 RNAi induced phenotypes. **a** RNAi probes targeting Deaf1 (located on chromosome 3L). Exons are highlighted in orange, untranslated regions in black. Bars at the bottom depict the locations of 4 independent dsRNA probes targeting Deaf1 (Deaf1 1-4). **b** RNAi in SL2 cells targeting different regions of the Deaf1 transcript. SL2 cells transfected with *Drs-luc*, *Toll*ΔLRR and *Renilla* co-reporter were treated with GFP, *Dif/Dorsal*, *Imd* and independent *Deaf1* dsRNAs. Results were normalized to GFP control. The relative induction of *Drs-luc* by *Toll*ΔLRR is 43 fold. Error bars indicate the standard deviation of 8 replicates. The data are representative of 3 independent experiments. **c** Rescue of Deaf1 RNAi induced phenotype. SL2 cells were trans-

ected with *Drs-luc*, *Toll*ΔLRR, *Renilla* co-reporter and either empty expression vector or full length Deaf1 cDNA lacking its 3' UTR (Deaf1ΔUTR) and treated with the indicated dsRNA. All samples were normalized to GFP control in absence of *Deaf1* cDNA. Error bars represent standard deviations from 4 samples. The data are representative of 2 independent experiments. **d** Detection of Deaf1 protein levels after RNAi by Western blot using HRP-coupled mouse α-His6 antibody. The loading control was β-tubulin. SL2 cells transfected with full length Deaf1 (Deaf1ΔUTR) were treated with the indicated dsRNAs. **e** Quantitative RT-PCR (qRT-PCR) of endogenous *Drs* mRNA levels after RNAi treatment. A stably transfected SL2 cell line with the *Toll*ΔLRR truncated recep-

genes, we designed 3 independent dsRNAs in addition to the probe contained in the library. The probes were designed to target the 5' UTR of *Deaf1* (fig. 2a; Deaf1-1), the second and the third exons [Deaf1-2 (library probe), Deaf1-3] as well as the 3' UTR (Deaf1-4). All probes were calculated to exclude any 21mer matches against any other transcripts in the *Drosophila* genome [37]. The pathway was activated by the overexpression of a dominant active form of Toll, Toll^{ΔLR}. As shown in figure 2b, all 4 independent *Deaf1* dsRNA sequences reduced the expression of the *Drs* reporter to a similar extent, confirming the specificity of the Deaf1 phenotype.

To further verify that the loss of *Deaf1* is responsible for the observed phenotype, we performed a genetic rescue by expression of a *Deaf1* cDNA that lacks the endogenous 5' and 3' UTRs and is therefore not targeted by Deaf1-1 and Deaf1-4 RNAi probes. Following RNAi against *GFP*, *Dif/Dorsal*, *Deaf1-3* and *Deaf1-4* cells were transfected with Toll^{ΔLR}, *Drs-luc* and *Renilla* reporter genes in combination with either the Deaf1 rescue construct or empty expression plasmid as a control. We observed a strong reduction of reporter signals with RNAi against *Dif/Dorsal* and exonic regions of *Deaf1* (Deaf1-3) in the presence or absence of the rescue construct (fig. 2c). In contrast, RNAi targeting the 3' UTR (Deaf1-4) exhibited a strong signalling phenotype that was completely rescued in cells ectopically expressing *Deaf1* cDNA. To test the efficiency of RNAi on protein levels, we checked the expression of the rescue construct after RNAi against *GFP*, *Deaf1-3* and *Deaf1-4* by Western blot. RNAi against the third exon of *Deaf1* resulted in a complete loss of detected protein (fig. 2d). Taken together, these experiments demonstrate that *Deaf1* RNAi induced phenotypes are specific and that Deaf1 is required for Toll-dependent *Drs* induction in hemocyte-like SL2 cells.

tor was treated with the depicted dsRNAs. To induce Toll^{ΔLR} expression, cells were treated with 500 μM CuSO₄ four days prior to RNA extraction. *Drs* mRNA levels were normalized to *Rp49* mRNA levels. Error bars indicate the standard deviation of 2 replicates. Data shown are representative of 3 independent experiments. **f, g** Effect of Deaf1 depletion on *mtk*-luciferase (*mtk-luc*) reporter activity upon stimulation with *E. coli* (**f**) or Toll^{ΔLR} overexpression (**g**). Luciferase expression in cells treated with dsRNA targeting *Imd*, *Dif/Dorsal* and *Deaf1* were normalized to *GFP* control. Error bars indicate the standard deviation of 6 replicates. ** p = 0.0015; *** p < 0.0001, using Student's t test. Data shown are representative of 3 independent experiments.

We then asked whether Deaf1 is not only required for reporter gene activity but also for the expression of endogenous Toll target genes. Using a stable cell line expressing Toll^{ΔLR} under the control of a metallothionein promoter, we monitored *Drs* mRNA expression after treatment with dsRNA and induction with Cu²⁺. As shown in figure 2e, depletion of *Dif/Dorsal*, MyD88 or Deaf1 using RNAi resulted in reduction of *Drs* mRNA to background levels. These results demonstrate that Deaf1 is required for Toll-dependent expression of endogenous *Drs* in SL2 cells.

To investigate whether Deaf1 depletion would affect the induction of other antimicrobial peptide genes, we monitored the expression of another anti-fungal gene, *mtk*. As *mtk* is activated by both Imd and Toll signalling we monitored the induction of a *mtk* promoter luciferase reporter (*mtk-luc*) upon stimulation with *E. coli* or Toll^{ΔLR} upon depletion of Deaf1. As shown in figure 2f and g, Deaf1 is required for the induction of the *mtk* reporter in response to both stimuli. These results are in agreement with the observations of Reed et al. [22], who showed that Deaf1 could bind to the *mtk* promoter in vitro. We also observed that depletion of Imd reduced reporter activity in response to both stimuli. S2 cells display a basal level of Imd pathway activity, suggesting that Imd knockdown also impacts *mtk-luc* reporter activity in the absence of *E. coli* stimulation (A.R. and N.P. unpubl. observations).

Deaf1 Required for Immune Target Gene Expression and Survival during Fungal Infection in vivo

According to previous studies, Deaf1 is expressed ubiquitously throughout development [38, 39]. In a classical genetic screen, 2 loss-of-function alleles of *Deaf1* were identified that are early embryonic lethal with segmentation defects [40]. These early developmental phenotypes prevent the analysis of Deaf1 function during innate immune responses in adult flies. To assess whether Deaf1 is required for innate immune responses in vivo, we used the UAS/GAL4 system to express RNAi-hairpins directed against the third exon of *Deaf1* in *Drosophila* adults. Selected transgenic insertions (UAS-Deaf RNAi) were crossed with different GAL4 driver lines to express the hairpin. Strong constitutive expression by *daughterless-Gal4* or *actin-Gal4* led to lethality in pupal stages, supporting the requirement of Deaf1 during development [40]. To assess Deaf1 function in innate immune responses, we crossed Deaf1-RNAi flies with *Cg-Gal4*, which provides expression in immune tissues, namely hemocytes and the fat body. *Cg-Gal4/+; Deaf1-RNAi/+*

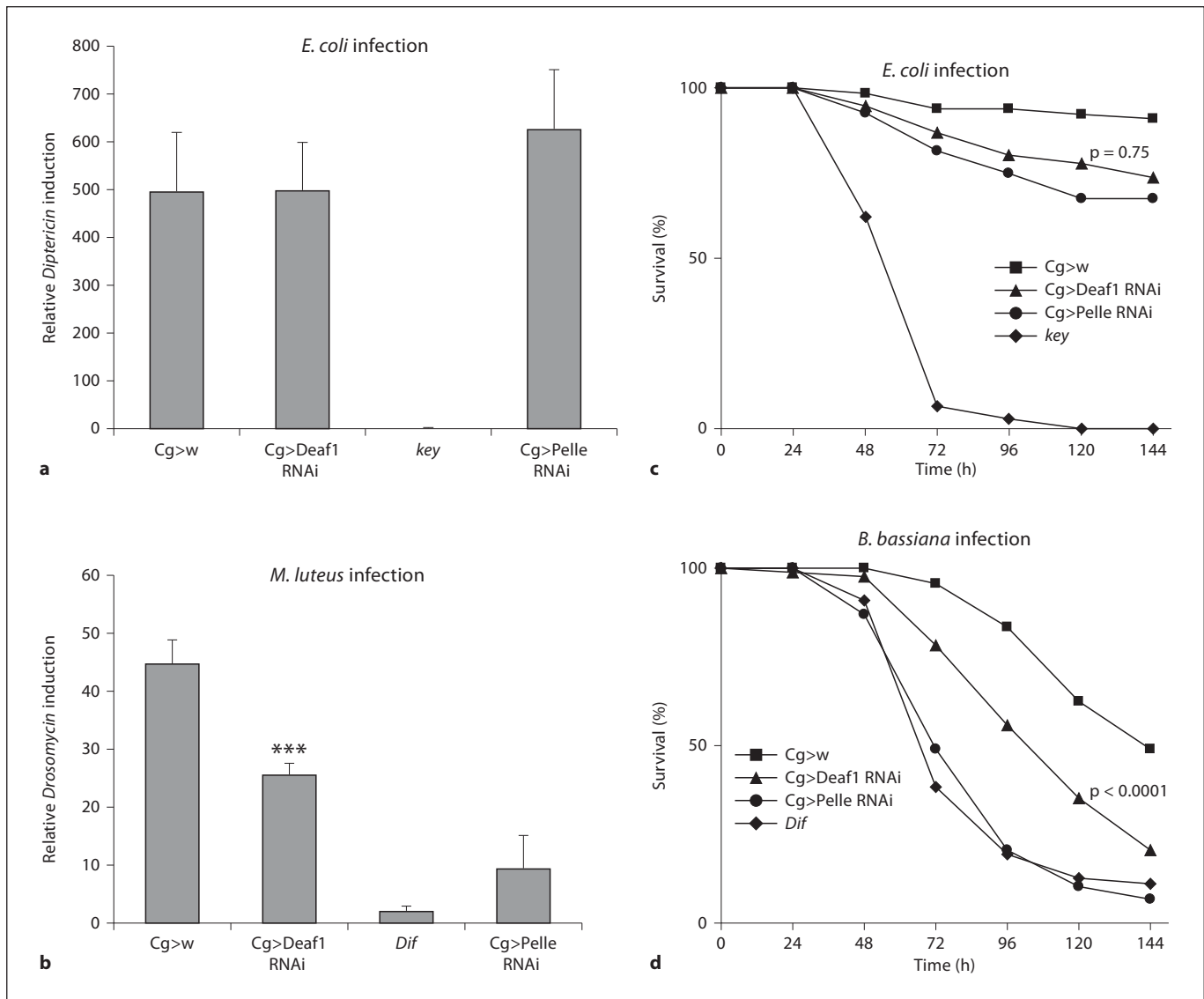


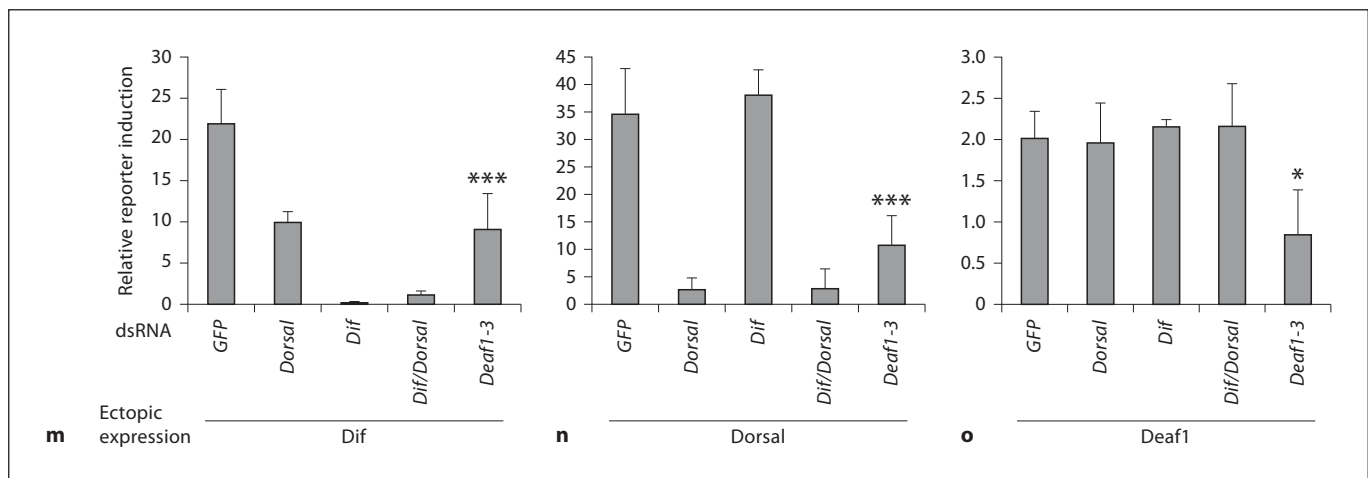
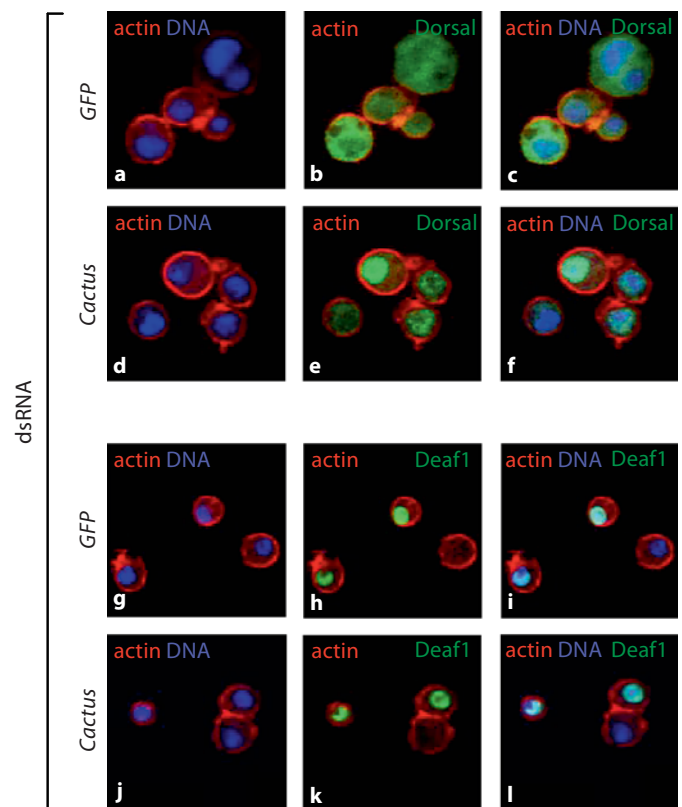
Fig. 3. In vivo function of Deaf1 in immune gene expression and survival upon infection. **a, b** Adult flies were infected with *E. coli* or *M. luteus* independently; *Dpt* expression was measured by qRT-PCR 6 hours post infection with *E. coli* (**a**), and *Drs* expression 24 h after infection with *M. luteus* (**b**). As negative control we used *w¹¹¹⁸*; *Cg-GAL4/+* flies (control). *kenny* (*key*) and *Dif* flies are mutant for the Imd pathway component IKK γ and the Toll pathway component *Dif*, respectively. *Cg>Deaf1-RNAi* or *Cg>Pelle-RNAi* flies carry 1 copy of a short hairpin RNA construct and the *Cg-Gal4* driver. All samples were normalized according to *Rp49* mRNA lev-

els. Transcript expression levels are shown relative to unstimulated wild-type flies. Error bars correspond to the standard deviation of 4 biological replicates from a representative experiment. *** $p < 0.0001$, using Student's *t* test. **c, d** Survival of *Cg>Deaf1 RNAi* flies is compromised upon fungal infection but not *E. coli* infection. **c** Percentage survival of adult flies upon infection with *E. coli*. **d** Percentage survival of adult flies infected with the fungal pathogen *B. bassiana*. Sample genotypes are as indicated above. Data set is representative of 2 independent experiments, with 120 animals per experiment. *p* values were determined using log-rank test.

(*Cg>Deaf1-RNAi*) animals were viable, fertile and did not show visible phenotypes. We further confirmed by qRT-PCR that *Deaf1* mRNA levels were reduced by approximately 80% in targeted tissues (online suppl. fig. 1).

To monitor the effect of Deaf1-RNAi on Toll and Imd pathway activity, we infected adult flies with either *E. coli* or *M. luteus* and quantified mRNA levels of the Imd target *Dpt* or the Toll target *Drs*. We used *w¹¹¹⁸*; *Cg-GAL4/+* (*Cg>w*) as the wild type reference. In contrast to flies

Fig. 4. Epistasis analysis of Deaf1 within the Toll pathway. **a–l** Immunofluorescence staining in SL2 cells. Endogenous Dorsal and V5-tagged Deaf1 were detected in SL2 cells depleted for GFP or cactus. Actin was stained with phalloidin-FITC (red) and DNA with Hoechst (blue). Dorsal protein was detected using the mouse α -dorsal antibody and goat α -mouse IgG TRITC (green). Deaf1 protein was detected using rabbit α -V5 antibody and goat α -rabbit TRITC antibodies (green). **m–o** Epistatic analysis using *Drs-luc* reporter assay. Subsequent to RNAi treatment as indicated, cells were transfected with *Drs-luc*, *Renilla* co-reporter and Dif, Dorsal or Deaf1 expression plasmids. Shown is the induction of the *Drs-luc* reporter upon stimulation, relative to luciferase expression in unstimulated cells treated with GFP dsRNA. Error bars represent standard deviations from 4 replicates. * $p = 0.0049$; *** $p < 0.0001$, using Student's *t* test. The data is representative of 2 independent experiments.



mutant for the Imd pathway component *kenny* (*key*), *Cg>Deaf1-RNAi* animals showed wild-type levels of *Dpt* induction after *E. coli* infection (fig. 3a). To test the requirement of Deaf1 in the Toll pathway we examined *Drs* expression, upon *M. luteus* infection, in *Cg>Deaf1-RNAi* animals versus Dif mutants or RNAi against the Toll pathway component Pelle. *Drs* expression was significantly reduced in *Cg>Deaf1-RNAi* animals (fig. 3b), indi-

cating a role of Deaf1 in the Toll mediated immune response. The fact that *Dpt* expression is wild type in flies depleted for Deaf1 suggests that the reduction in *Drs* level expression is not due to a defect in immune tissue function.

As Toll and Imd pathways are required for resistance to infections, we next asked whether Deaf1 plays a role in survival upon immune challenge. Thus, we infected flies

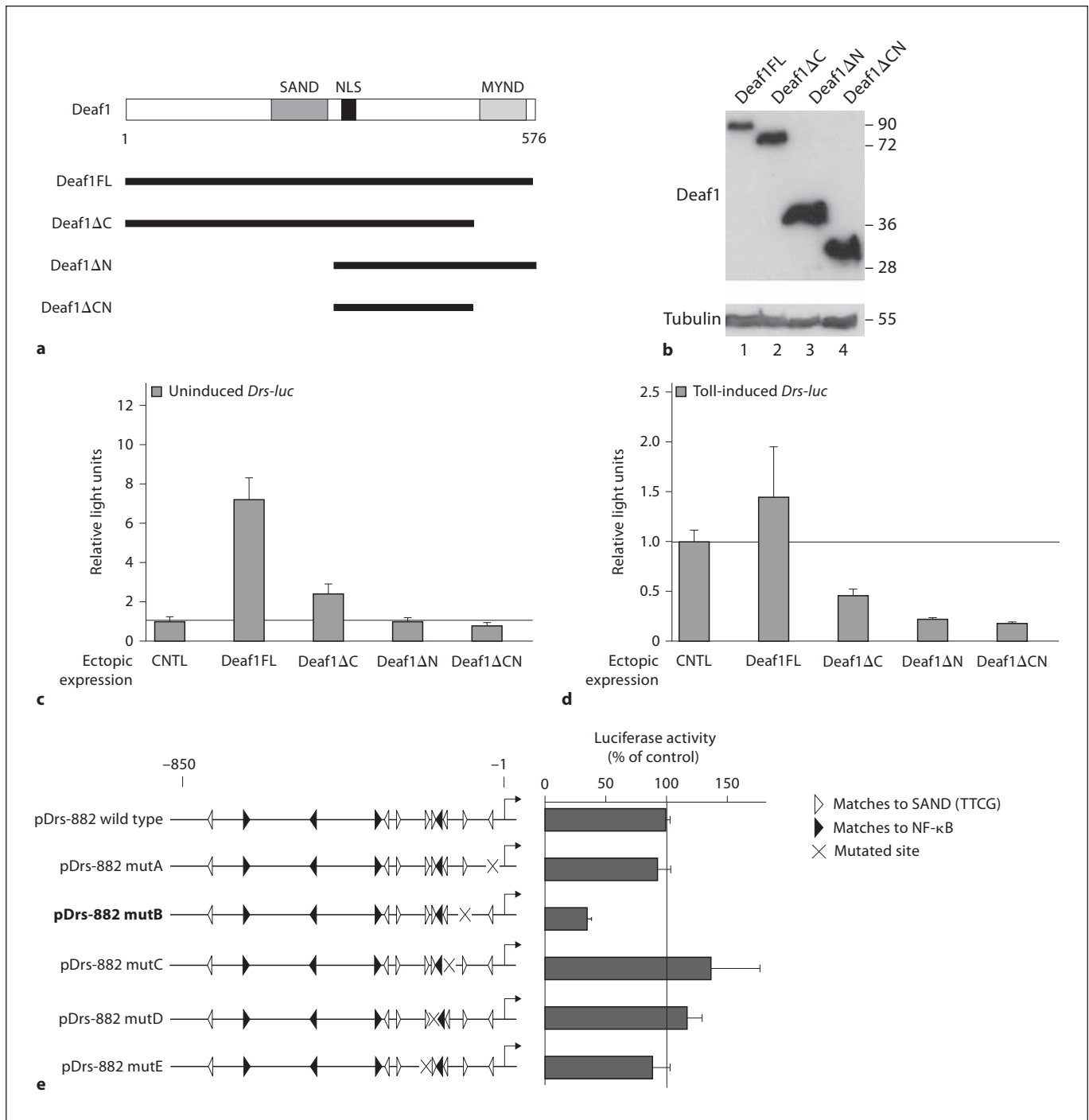


Fig. 5. Structure/function analysis of Deaf1. **a** Domain architecture of full length Deaf1 and cloned deletion constructs. The Deaf1 full-length protein contains 2 highly conserved domains, the N-terminal SAND domain and the C-terminal zinc-finger MYND motif. The deletion construct Deaf1ΔC lacks amino acids 501–576, Deaf1ΔN lacks residues 1–300 and Deaf1ΔCN contains only residues 301–500. **b** Western blot of SL2 cells transfected with Deaf1 deletion constructs. Proteins were detected using anti-His antibody. β-tubulin served as the loading control. **c, d** Functional

analysis of Deaf1 deletion mutants in a *Drs-luc* assay in the presence (**d**) and absence (**c**) of Toll^{ΔLRR} over-expression. SL2 cells were transfected with full-length or Deaf1 mutants with or without Toll^{ΔLRR}. Samples were normalized to GFP samples. Error bars represent standard deviations from 4 replicates. The experiment was independently performed twice. **e** Identification of a potential Deaf1 binding site in the *Drs* promoter. A luciferase reporter containing a *Drs* promoter sequence that ranged from +50 to -882 (pDrs-882) relative to the transcriptional start site was

with the entomopathogenic fungus *B. bassiana* or *E. coli*. *Cg>Deaf1 RNAi* animals displayed wild-type survival rates upon *E. coli* infection, showing that Imd mediated immune responses are independent of Deaf1 function (fig. 3c). However, RNAi knock-down of Deaf1 in adults significantly increased their sensitivity to fungal infections (fig. 3d). The survival phenotype in flies depleted for Deaf1 is not as strong as *Dif* mutants and *Cg>Pelle-RNAi* flies, reflecting the residual expression of *Drs* (fig. 3b). Altogether these experiments indicate that Deaf1 is a transcriptional modulator of immune target gene expression in adult *Drosophila*.

Cell-Based Epistasis Analysis Maps Deaf1 Function Downstream or at Level of Dif and Dorsal for Drosomycin Expression in SL2 Cells

As Deaf1 seems to be implicated in the response to fungal infection we next wished to map its action within the Toll pathway. Deaf1 belongs to a highly conserved protein family with members from insects to humans (online suppl. fig. 2) and was originally isolated in a *Drosophila* genetic screen for proteins binding to *deformed* autoregulatory enhancer regions [39].

Since Deaf1 has been proposed to be a sequence-specific transcription factor, we first asked whether any of the known core components of the Toll pathway are transcriptional targets of Deaf1. Quantitative RT-PCR experiments showed that the expression of known Toll pathway components was not significantly altered by Deaf1 depletion (data not shown). Being a transcriptional regulator we also hypothesized that Deaf1 acts at the downstream end of the Toll module and therefore examined whether Deaf1 translocates from the cytoplasm to the nucleus in a way similar to NF- κ B proteins after Toll activation. We monitored Deaf1 sub-cellular localization in cells treated with dsRNAs targeting *GFP* as a control or *cactus* to induce translocation of Dorsal to the nucleus. Immunofluorescence showed that in the absence of Toll signalling, Dorsal is primarily localized in the cytoplasm (fig. 4a–c) and is present in the nucleus of cells depleted

for *cactus* (fig. 4d–f). In contrast, Deaf1 proteins were localized to the nucleus in both activated (fig. 4j–l) and non-activated SL2 cells (fig. 4g–i).

Since Deaf1 solely resides in the cell nucleus, we next wanted to define its position in relation to the NF- κ B proteins Dif and Dorsal. We activated the Toll pathway at the most downstream position, at the level of Dif, Dorsal or Deaf1. We ectopically expressed these proteins in cells and measured *Drs-luc* reporter activity after RNAi against GFP (control), Dif, Dorsal, Dif/Dorsal and Deaf1. The induction of the Toll pathway by Dif-expression was impaired whilst depleting Dif, Dorsal, Dif/Dorsal or Deaf1 (fig. 4m). Similarly, Dorsal-dependent activity of *Drs-luc* was abolished by Dorsal-, Dif/Dorsal- and Deaf1-RNAi (fig. 4n). Surprisingly, Toll activity induced by *Deaf1* expression remained unchanged after any combination of Dif and Dorsal depletion (fig. 4o). This result implies that Deaf1 acts downstream or at the level of Dif and Dorsal in Toll mediated *Drs* expression. Taken together, our epistasis analysis indicates that Deaf1 localizes to the cell nucleus and functions downstream or at the level of Dif and Dorsal within the Toll pathway.

Deaf1 DNA Binding Domain Is Crucial for Drosomycin Regulation

To test whether Deaf1 DNA binding is essential for its function, we constructed deletion mutants that remove specific domains of the protein. The Deaf1 Δ N construct lacks the N-terminal SAND-domain (amino acids 1–299), whereas Deaf1 Δ C, a C-terminal deletion of amino acids 501–576 lacks the MYND-type zinc fingers. We further generated a deletion mutant that had both C- and N-terminal deletions and consisted of only amino acids 300–500 (Deaf1 Δ CN; fig. 5a). We confirmed that all constructs could be expressed in cells by Western blot analysis (fig. 5b).

To identify domains that are required for Deaf1 protein function, we examined the Deaf1 mutant proteins for their effect on Toll signalling using the *Drs-luc* reporter assay in both activated and inactive signalling states. In absence of Toll signalling, expression of full-length Deaf1 was sufficient to induce the *Drs* reporter 8-fold (fig. 5c). The induction was significantly lower when we expressed Deaf1 Δ C, indicating that the MYND domain is important for Deaf1 function. Expression of Deaf1 Δ N or Deaf1 Δ CN deletion mutants did not lead to any induction of the reporter (fig. 5c). Furthermore when the Toll pathway was active, deletion mutants, lacking the SAND or the MYND domain, led to strong dominant negative phenotypes (fig. 5d). In summary, these observations suggest

used to mutate TTCG Deaf1 binding sites (white triangles). NF- κ B sites are indicated by black triangles. Five TTCG motifs proximal to the transcriptional start were individually mutated by site-directed mutagenesis (indicated by black crosses). Constructs were expressed in SL2 cells transfected with Toll-EGFR chimera and *Renilla* co-reporter. Luciferase activity was measured 16 h after induction with hEGF. Error bars represent standard deviations from 4 replicates. Data are representative of 2 independent experiments.

that the DNA binding domain is crucial for the function of the Deaf1 protein in regulating *Drs* expression.

Deaf1 Function Depends on the Presence of a SAND Binding Motif in the Drosomycin Promoter

Previous studies have shown that Deaf1, similar to other SAND domain-containing proteins, binds to TTCG motifs [22, 39, 41]. To define the location of Deaf1 binding sites in the *Drs* reporter, we made deletion constructs and examined their activity in transient transfection experiments. As shown in online supplementary figure 3, we identified a 932-bp fragment (pDrs-882, ranging from +50 to -882) containing minimal promoter elements which are required for strong reporter expression. This fragment contained 4 NF- κ B binding sequences and several TTCG elements that clustered approximately 400 bp upstream of the transcriptional start site. To test whether these sites were essential for Deaf1 dependent *Drs* expression, we mutated the 5 most proximal TTCG sites in an immune-inducible pDrs-882 reporter construct (see also the extended Methods online). All mutated reporter constructs were tested for their expression after activating Toll signalling. As shown in figure 5e, mutation of the second TTCG site reduced the activity of the *Drs* promoter significantly. This correlates with the observation made by Reed et al. [22] and shows that putative Deaf1-binding sites in the *Drs* enhancer are required for *Drs* expression.

Discussion

The activation of transcriptional programs during development and later during homeostasis often relies on temporal and spatially restricted responses that lead to particular physiological outcomes. A key target gene induced during immune responses against bacterial and fungal infections is *Drs*, an antifungal peptide whose expression is controlled by the Toll signalling pathway. Large-scale RNAi approaches have been shown to be a powerful approach to systematically identify components of signalling pathways [19, 20, 42]. In this study, we took a systematic approach to identify novel transcriptional regulators of Toll signalling in immune responses by screening 1,033 putative transcriptional regulators encoded in the *Drosophila* genome. The phenotypic survey identified components of the general transcriptional machinery, in particular the TFIID and TFIIA isoforms Trf2 and TFIIA-S and TFIIA-L. Interestingly, although most components of the RNA polymerase II complex are

unique in the *Drosophila* genome, TFIIA and TFIID proteins occur in many isoforms which in varying combinations are thought to contribute in tissue- and time-specific gene regulation [43, 44]. Other components are essential for cell survival and were removed from the final list of candidates.

We identified Deaf1 that acts as a regulator of *Drosophila* immune response. Deaf1 belongs to a conserved family of proteins with homologs present in metazoans ranging from insects to humans. Our data show that RNAi-mediated knock-down of *Deaf1* transcripts in SL2 cells and the adult fat body results in strong misregulation of the Toll target gene *Drs*. To confirm the Deaf1 knock-down phenotype we demonstrated that it can be reproduced by 3 independent dsRNAs and by rescuing the loss-of-function phenotype using an RNAi-insensitive cDNA. Moreover, *Deaf1* has not been found in any other RNAi screen to date, suggesting that it is not a general transcriptional regulator.

One hypothesis for the mechanism of action of Deaf1 could be in the alteration of chromatin structure of immune regulated genes since MYND domain-containing proteins have been shown to interact with chromatin remodelling complexes [45]. As we found the known chromatin remodellers Brahma and Dalao as negative regulators in our screen, we tested if Deaf1 might function by reversing the inhibitory effect of Brahma and Dalao on Toll target genes. Epistatic experiments with Brahma and Dalao indicated that they do not act in the same pathway as Deaf1 (D.K. unpubl. observations). Previous work has shown that the mammalian Deaf1 homolog can act as a transcriptional co-activator or co-repressor depending on cellular context [46]. According to the immune phenotype we observed in *Drosophila* and the consequence of removing Deaf1 binding sites in the *Drs* promoter, we hypothesize that Deaf1 acts as a transcriptional co-activator in the context of *Drosophila* immune responses. However, we cannot exclude that Deaf1 might act as a co-repressor during development [40].

Parallel to our findings in SL2 cells, we also found Deaf1 to be necessary for the induction of *Drs* in *Drosophila* adult tissues. Flies with reduced Deaf1 levels express less *Drs* than control flies, and were significantly more sensitive to systemic infections with the entomopathogenic fungus *B. bassiana*. In accordance with the finding that Deaf1 acts as a positive regulator for Toll activity in vivo, over-expression of Deaf1 using the Cg-Gal4 driver resulted in the formation of melanotic pseudo tumours (online suppl. fig. 4). This phenotype was previously reported for Toll pathway mutants *cactus* (loss-of-

function) or constitutive active Toll alleles (Tl^D , Tl^{10B}) [8]. In addition Reed et al. observed a strong synergy between Deaf1 and Dif, a slight synergy between Deaf1 and Dorsal, but no synergy with Relish. Together with the observation that several Toll pathway targets contain consensus Deaf1 binding sites, they hypothesise that Deaf1 might function in the Toll signalling pathway [22]. We find that adult flies depleted for Deaf1 are sensitive to systemic infection with *B. bassiana* but not *E. coli*. This implies that Deaf1 is required primarily in Toll dependent immune responses in vivo.

Our epistasis analysis revealed that Deaf1 acts downstream or at the level of the NF- κ B molecules Dif and Dorsal. We therefore carried out a functional analysis of Deaf1 binding elements in the *Drs* promoter. Our results imply that Deaf1 binding sites are required for *Drs* induction. In accordance with our results, Reed et al. found Deaf1 to physically interact with immune gene enhancers in vitro [22]. In particular they showed that Deaf1 binds to the *mtk* and *Drs* promoters and that mutations in the Deaf1 binding sites of these promoters lead to reduced gene expression. It was not clear, however, whether Deaf1 is functionally required for expression of target genes. Using a loss of function approach we show that Deaf1 is required for *Drs* expression in SL2 cells and in vivo, and confirm the functional requirement of Deaf1 in *mtk* reporter expression. In addition our loss of function analysis showed that Deaf1 is required for *mtk-luc* expression both in response to *E. coli* stimulation and activated Toll signalling in SL2 cells. Reporter induction was

not dependent on Dif and Dorsal, suggesting that Deaf1 may be recruited or is bound to target gene promoters independently of the NF- κ B transcription factors. However, further studies are required to elucidate the mechanism of action of Deaf1 in relation to target gene activation.

In this study we show that Deaf1, the *Drosophila* member of a conserved family of DNA binding proteins, is required for the expression of *Drosophila* innate immune effector genes in SL2 cells and in vivo. Deaf1 protein localizes strictly to the nucleus and functions downstream or at the level of the NF- κ B molecules Dif and Dorsal. As other similar SAND domain containing proteins were associated with innate immune function in vertebrates [47], it might be possible that this family of transcription factors forms a novel functional class of immune regulatory genes.

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