ARTICLES

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Neuroimmune regulation of antimicrobial peptide expression by a noncanonical TGF-β signaling pathway in *Caenorhabditis elegans* epidermis

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After being infected by the fungus *Drechmeria coniospora*, *Caenorhabditis elegans* produces antimicrobial peptides in its epidermis, some regulated by a signaling cascade involving a p38 mitogen-activated protein kinase. Here we show that infection-induced expression of peptides of the Caenacin family occurred independently of the p38 pathway. The *caenacin* (*cnc*) genes enhanced survival after fungal infection, and neuronal expression of the transforming growth factor-β homolog DBL-1 promoted *cnc-2* expression in the epidermis in a dose-dependent paracrine way. Our results lead to a model in which antifungal defenses are coordinately regulated by a cell-autonomous p38 cascade and a distinct cytokine-like transforming growth factor-β signal from the nervous system, each of which controls distinct sets of antimicrobial peptide–encoding genes in the epidermis.

The free-living nematode *Caenorhabditis elegans* has emerged in the past decade as a powerful genetic model for the analysis of host-pathogen interactions[1–4]. It has been used successfully to identify virulence factors of diverse microbes and is contributing to the growing knowledge of the evolution and conservation of innate immune mechanisms. *C. elegans* has developed sophisticated defenses, including both behavioral (aversive) responses to pathogens[5,6] and inducible mechanisms of innate immunity[7]. Different pathogens trigger specific changes in gene expression. Several signaling cascades, including those involving the p38 and Erk mitogen-activated protein kinases (MAPKs), transforming growth factor-β (TGF-β) homologs and insulin-like peptides modulate the resistance of *C. elegans* to infection[8,9]. In addition, the single nematode Toll-like receptor is involved in host-pathogen interactions by as-yet-undefined 'downstream' effectors[10–12]. Furthermore, it has been shown that the response to unfolded protein, regulated by the apoptotic receptor CED-1, is involved in the immune response of *C. elegans* to bacterial infection[13].

The signaling mediators involved in innate immunity were all initially identified on the basis of their involvement in unrelated developmental or physiological processes. The molecular mechanisms that permit a single molecule to exert distinct functions in immune and nonimmune processes remain to be understood. Similarly, it is an open issue as to exactly how these pathways are triggered by infection; the *C. elegans* genome lacks genes encoding many of the receptors known to be important for pathogen recognition in other species[8,9].

The interaction between *C. elegans* and its natural fungal pathogen *Drechmeria coniospora* provides a model for delineating the genetic and molecular aspects of antifungal innate immune defenses[14–16]. Conidia of *D. coniospora* attach to the nematode cuticle by means of adhesive knobs and send out hyphal processes that pierce the cuticle. After penetrating the epidermis, hyphae then develop and grow inside the host, eventually killing it[17]. Genome-wide transcriptome analysis has shown that *C. elegans* responds to *D. coniospora* infection by rapidly upregulating several families of genes encoding putative antimicrobial peptides (AMPs) and proteins. A subgroup of the *nlp* family and the structurally related *cnc* genes are among the mostly highly induced. Most *nlp* and *cnc* genes that are induced by fungal infection are in two separate clusters on the 'left arm' of chromosome V. Overexpression of the six genes of the *nlp-29* cluster enhances pathogen resistance *in vivo*, which emphasizes their importance for defense[16]. Genes of the *nlp-29* cluster have considerably upregulated expression not only after infection but also after wounding. The induction of their expression after infection and physical injury is dependent on a conserved p38 signaling pathway that acts in a cell-autonomous way in the epidermis, 'downstream' of TIR-1, a TIR-domain adaptor protein and ortholog of the human protein SARM[15,16].

In this study, we investigate whether *cnc* genes also contribute to antifungal defense and whether they are subject to the same regulation as the genes of the *nlp-29* cluster. We identify a function for the *cnc* genes *in vivo* but, unexpectedly, we find that these two classes of phylogenetically related AMP-encoding genes are regulated in very distinct ways. Whereas the genes of the *nlp-29* cluster were controlled by a cell-autonomous p38 pathway, after infection, neuron-derived DBL-1 (a homolog of TGF-β) activated a noncanonical signal-transduction pathway in the epidermis that governs expression of the *cnc* genes.

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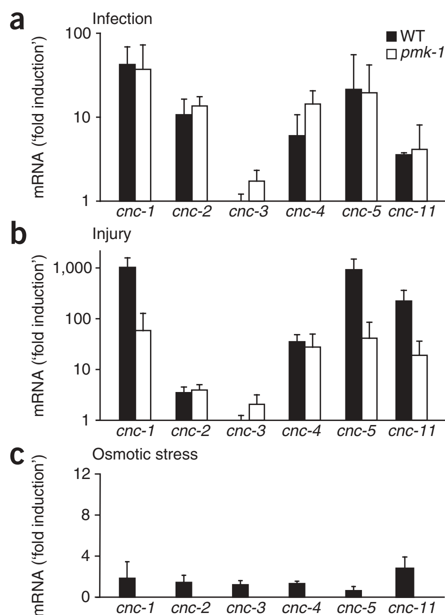

Figure 1 Expression of genes in the *cnc-2* cluster. Quantitative RT-PCR analysis of transcripts encoded by various genes (horizontal axes) in wild-type worms (WT) and *pmk-1*-mutant worms (*pmk-1*) after 24 h of infection by *D. coniospora* (**a**), 2 h after needle wounding (**b**) or after 6 h of osmotic stress in liquid (**c**), compared with the relative control and normalized relative to *act-1* expression. Data generated in each experiment with wild-type worms in **a** are in **Supplementary Figure 13a** online. Data represent at least three experiments (average and s.d.).

RESULTS

Regulation of *cnc* genes after infection and injury

The genes *cnc-1* through *cnc-5*, together with *cnc-11*, are present in a genomic cluster on the 'left arm' of chromosome V (**Supplementary Fig. 1a** online). We call this region the '*cnc-2* cluster' here. Microarray studies have indicated that many of these *cnc* genes are induced by infection with *D. coniospora*[14,16]. By quantitative RT-PCR analyses, we found considerable induction of expression after infection for five of the six genes of the *cnc-2* cluster, the exception being *cnc-3* (**Fig. 1a**). We also noted higher expression of the same five genes after wounding (**Fig. 1b**).

The expression of some *nlp* genes is also much higher when *C. elegans* is put in conditions of high osmolarity[16]. We detected little change in expression (less than twofold) of *cnc-1* through *cnc–5*, however, and a modest (two- to threefold) upregulation of *cnc-11* after exposure to a higher concentration of salt (**Fig. 1c**). In the same samples, we measured an average induction of seventeen-, eight- and fourfold for *nlp-29* after infection, wounding and osmotic stress, respectively[16]. Thus, the genes of the *cnc-2* cluster, which are structurally very similar[14,16], are controlled in a complex way after activation by different stimuli. Among these, *cnc-2* stands out, as it was induced more by infection than by injury.

The upregulation of genes of the *nlp-29* cluster triggered by infection with *D. coniospora* and injury is almost entirely dependent on a p38 signaling pathway; upregulation of *nlp* is severely compromised in mutant worms that do not express the p38 homolog PMK-1 (ref. 16). In contrast, induction of genes of the *cnc-2* cluster was essentially unchanged in *pmk-1*-mutant worms after infection (**Fig. 1a**). Injury-induced upregulation of *cnc-1*, *cnc-5* and *cnc-11*, the three genes that responded most strongly to wounding, was, however, consistently an order of magnitude lower in *pmk-1*-mutant worms than in wild-type worms (**Fig. 1b**). These results collectively indicate that in terms of regulation of genes of the *cnc-2* cluster, the full response of *C. elegans* to wounding requires *pmk-1* but the response to fungal infection is entirely p38 independent. Thus, at least one additional pathway acts together with the p38 cascade to activate antifungal defenses and control the expression of genes encoding AMPs in *C. elegans*.

The *cnc* genes promote survival after fungal infection

Whereas overexpression of *nlp-31* does not change the susceptibility of *C. elegans* to *D. coniospora*[14], overexpression of the six genes of the *nlp-29* cluster increases the resistance to infection[16]. Therefore, to address the question of whether the structurally related *cnc* genes could contribute *in vivo* to the capacity of *C. elegans* to resist infection, we generated transgenic worms carrying supernumerary copies of the *cnc-2* cluster (**Supplementary Fig. 1b**). We first determined by quantitative RT-PCR that there was more constitutive and inducible expression of genes in the *cnc-2* cluster in the transgenic worms (**Fig. 2a**). The transgenic worms showed minor but significantly improved survival compared with that of nontransgenic sibling worms after infection with *D. coniospora* (**Fig. 2b**). The worms carrying extra copies of the *cnc-2* cluster, however, had the same susceptibility as their nontransgenic siblings had to two bacterial pathogens, *Serratia marcescens* and *Pseudomonas aeruginosa* (**Supplementary Fig. 2** online; $P > 0.5$). These results indicate that genes of the *cnc-2* cluster can contribute *in vivo* to greater resistance to fungal infection. Although *in vitro* tests of their activity have not been done, these *in vivo* data, together with their structure, do suggest that some or all of these genes encode authentic AMPs.

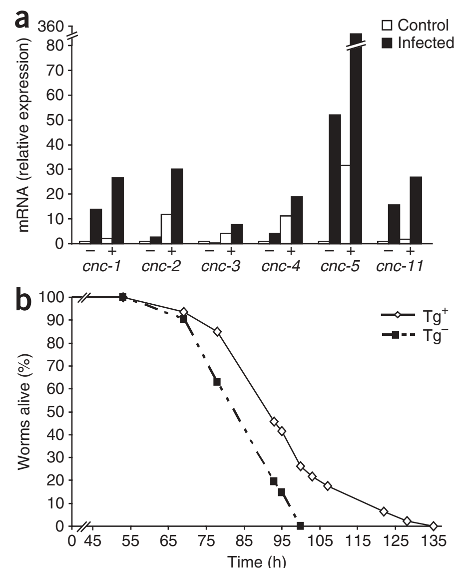

Figure 2 Overexpression of the *cnc-2* cluster is associated with greater resistance to infection. (**a**) Quantitative RT-PCR analysis of the expression of the six genes of the *cnc-2* cluster in transgenic worms carrying a DNA fragment encoding the cluster (+) and in nontransgenic worms (–), without (Control) or after (Infected) *D. coniospora* infection, presented relative to results obtained with uninfected nontransgenic worms, set as 1. Values are in **Supplementary Figure 13b**,**c**. Data are representative of at least three independent experiments. (**b**) Survival of transgenic worms (Tg^+) and their nontransgenic siblings (Tg^-) after infection with *D. coniospora*. $P < 0.0015$, transgenic versus nontransgenic (one-side log-rank test). Data are representative of four independent experiments.

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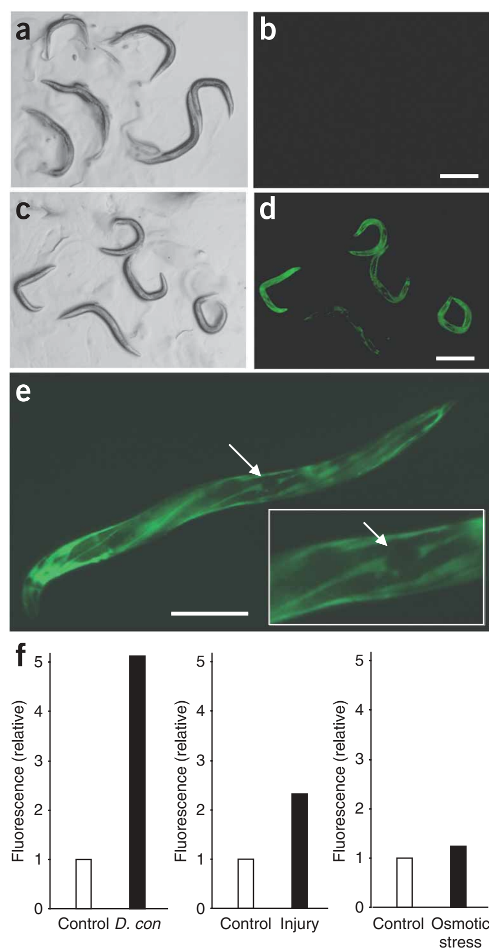

Figure 3 D. *coniospora* infection specifically induces expression of the *cnc-2* reporter. (**a**–**d**) Expression of p*cnc-2*::GFP in uninfected transgenic worms (**a**,**b**) and in transgenic worms 24 h after infection with *D. coniospora* (**c**,**d**), viewed by light microscopy (**a**,**c**) and epifluorescence microscopy (**b**,**d**). These worms have a 'roller' phenotype because they carry the pRF4 plasmid encoding a dominant mutation of *rol-6* (which encodes cuticle collagen). Scale bars, 0.5 mm. (**e**) Fluorescent image of the epidermal expression of p*cnc-2*::GFP in a worm 24 h after infection (head, left). Inset, enlargement of the vulval region. Arrows indicate lack of expression of the p*cnc-2*::GFP reporter in vulval epidermal cells, in contrast to expression of the p*nlp-29::*GFP reporter[14]. Scale bar, 50 μm. (**f**) Biosort quantification of the normalized fluorescence of worms carrying the p*cnc-2*::mCherry transgene at 24 h after infection with *D. coniospora* (*D. con*; left), 2 h after needle wounding (middle) and after 6 h of osmotic stress in liquid (right). Data are representative of at least three independent experiments (number of worms, **Supplementary Table 1** online).

Induction of *cnc-2* reporters by *D. coniospora*

To investigate the putative pathway that acts together with the p38 cascade to activate antifungal defenses and control AMP expression in *C. elegans*, we chose to focus on the regulation of *cnc-2*, as it was not strongly induced by wounding and its expression was entirely independent of *pmk-1* (**Fig. 1**). To monitor *cnc-2* expression *in vivo*, we generated and analyzed several transgenic worm strains expressing either green fluorescent protein (p*cnc-2*::GFP) or the 'mCherry' fluorescent protein (p*cnc-2*::mCherry) under control of the *cnc-2* promoter. In the absence of infection, no fluorescence expression was detectable in worms of any developmental stage (**Fig. 3a,b**). Consistent with the results obtained by quantitative RT-PCR, there was a small increase in fluorescence after injury with the p*cnc-2*::mCherry reporter, whereas there was no induction in worms carrying the p*cnc-2*::GFP reporter, possibly because of a truncation in its 5′ sequence (**Supplementary Methods** online). Fluorescence appeared in both the p*cnc-2*::mCherry and p*cnc-2*::GFP reporter strains when worms were infected with *D. coniospora* at any and all stages (**Fig. 3**, **Supplementary Fig. 3** online and data not shown). The *cnc-2* reporter genes were expressed exclusively in the epidermis (**Fig. 3e** and data not shown). We found no induction of expression of either reporter gene after infection with the bacterial pathogens *S. marcescens* and *P. aeruginosa* or exposure to high salt (**Fig. 3f**, **Supplementary Figs. 3** and **4** online and data not shown). Thus, these two *cnc-2* reporters can be used as specific 'readouts' of one arm of the innate immune response to infection with *D. coniospora*.

Consistent with the results obtained by quantitative RT-PCR, upregulation of the *cnc-2* reporter occurred independently of *pmk-1* (**Fig. 4a**), and it was not dependent on *tir-1* (**Supplementary Fig. 5** online). Infection-induced expression of the *cnc-2* reporter was also independent of *tol-1* (**Fig. 4b**), the only other gene encoding a TIR domain–containing protein in *C. elegans*[10]. We therefore studied the potential involvement of other pathways known to participate in pathogen responses in *C. elegans*. The Erk *mpk-1* signaling pathway is required for the response to the bacterium *Microbacterium nematophilum*[18]. Inactivation of *mpk-1* by RNA-mediated interference (RNAi), however, did not alter induction of the expression of

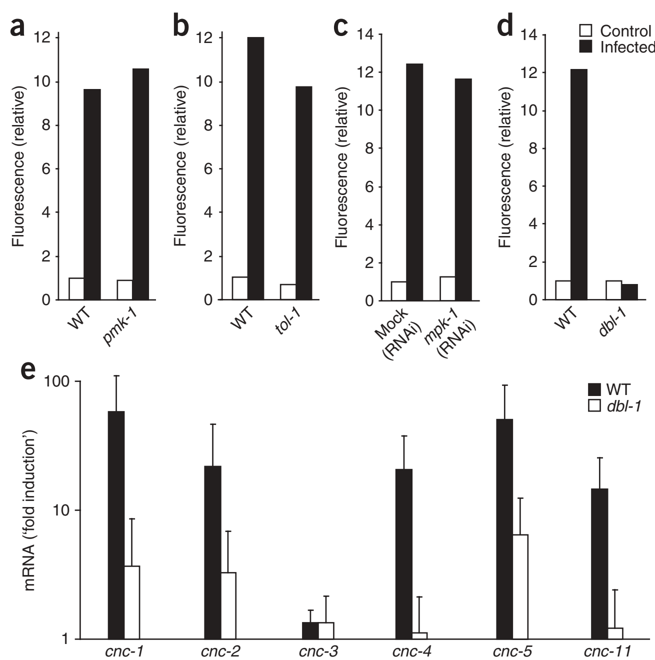

Figure 4 Upregulation of p*cnc-2*::GFP after fungal infection requires *dbl-1*. (**a**–**d**) Biosort quantification of the normalized fluorescence of wild-type worms and worms mutant for various genes (horizontal axes, **a**,**b**,**d**), or worms treated with an empty RNAi vector control (Mock (RNAi); **c**) or RNAi specific for *mkp-1* (*mkp-1* (RNAi); **c**), all carrying the *pcnc-2*::GFP reporter transgene, assessed without infection and 24 h after infection with *D. coniospora*. Data are representative of at least three experiments (number of worms, **Supplementary Table 1**). (**e**) Quantitative RT-PCR analysis of the expression of genes in the cnc-2 cluster in wild-type and *dbl-1*-mutant worms after 24 h of infection by D. coinospora, presented relative to act-1 expression. Data are representative of at least three experiments (average and s.d.).

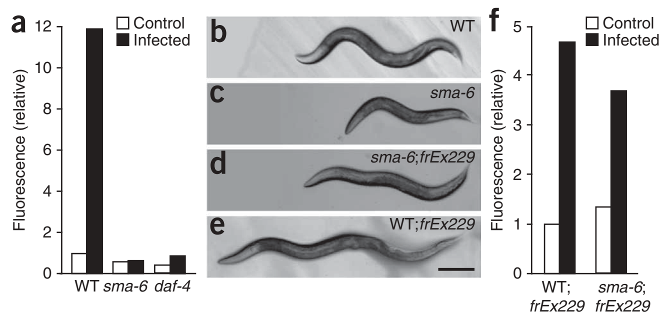

Figure 5 Upregulation of p*cnc-2*::GFP after fungal infection requires *sma-6* and *daf-4*. (**a**) Biosort quantification of the normalized fluorescence of wild-type worms and worms of the *sma-6*- and *daf-4*-mutant backgrounds, all carrying a p*cnc-2*::GFP transgene, without infection and 24 h after infection with *D. coniospora*. (**b**–**f**) Effect of epidermal expression of *sma-6* on body size and *cnc-2* reporter induction after fungal infection in *sma-6*-mutant worms. (**b**–**e**) Synchronized wild-type worm (**b**), *sma-6*-mutant worm (**c**), *sma-6*-mutant worm expressing *sma-6* specifically in the epidermis (**d**), and wild-type worm expressing *sma-6* specifically in the epidermis (**e**). The *frEx229* array (**d**,**e**) contains *sma-6* under control of an epidermal promoter, a *cnc-2* reporter and a DsRed marker of transgenesis (p*col-12*::SMA-6; p*cnc-2*::GFP; p*col-12*::DsRed). Scale bar, 100 μm. (**f**) Biosort quantification of the normalized fluorescence of wild-type and *sma-6*-mutant worms carrying the *fr Ex229* extrachromosomal array, assessed without infection and after 24 h of infection by *D. coniospora*. Data are representative of at least three independent experiments (number of worms, **Supplementary Table 1**).

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p*cnc-2*::GFP after fungal infection (**Fig. 4c**), which suggested that the Erk pathway is not involved in *cnc-2* regulation. One of the nematode TGF-β signaling pathways that involves the TGF-β-encoding gene *dbl-1* is required for the full resistance of *C. elegans* to the bacterial pathogens *S. marcescens* and *P. aeruginosa*[19,20]. We found that induction of expression of the *cnc-2* reporter normally seen after *D. coniospora* infection was much lower and sometimes was totally abolished in *dbl-1*-mutant worms (**Fig. 4d** and **Supplementary Figs. 6** and **7** online). We confirmed that result by quantitative RT-PCR and found that induction of the four other genes in the *cnc-2* cluster was also abolished or was very much lower in *dbl-1*-mutant worms than in wild-type worms (**Fig. 4e**). Thus, induction of the AMP-encoding genes of the *cnc-2* cluster triggered by fungal infection is dependent mainly on *dbl-1* activity. In contrast, expression of the AMP-encoding genes of the *nlp-29* cluster was unchanged in *dbl-1*-mutant worms (**Supplementary Fig. 8** online). Together these results suggest an important function for DBL-1 in the regulation of a specific aspect of inducible antifungal defenses.

To determine if *dbl-1* is directly involved in controlling fungal resistance, we compared the survival of wild-type and *dbl-1*-mutant worms after infection with *D. coniospora*. We noted much lower survival of *dbl-1* mutants (**Supplementary Fig. 9** online). The *dbl-1*-mutant worms, however, also have a shorter lifespan on *Escherichia coli* strain OP50, whether heat-killed or not[19]. This diminished viability in the absence of a pathogen precludes the definitive assignment of a function in antifungal resistance to DBL-1, but our results are not inconsistent with such a function.

DBL-1 acts on epidermal cells

The most prominent function of DBL-1 in the *C. elegans* hermaphrodite is in the regulation of body size[21,22]. Loss of TGF-β signaling results in small worms (the Sma phenotype), whereas excessive TGF-β signaling causes the worms to be longer than usual (the Lon phenotype)[22,23]. In its control of size, DBL-1 acts by means of the heterodimeric type I receptor–type II receptor formed by the transmembrane serine-threonine protein kinases SMA-6 and DAF-4 (ref. 24). Loss of function of either *sma-6* or *daf-4* results in small worms. We established that abrogation of either *sma-6* or *daf-4* was also sufficient to block the expression of *cnc-2* reporters after infection with *D. coniospora* (**Fig. 5a** and **Supplementary Figs. 6** and **7**). The type-II receptor DAF-4 can also form heterodimers with a second type I receptor, DAF-1 (ref. 24); however, *daf-1*-mutants showed normal induction of *cnc-2* reporter expression compared with that of wild-type worms after fungal infection (**Supplementary Fig. 6**).

In contrast to the broadly expressed DAF-4, SMA-6 is expressed mainly in the intestine and epidermis[24]. As *cnc-2* is expressed specifically in the epidermis, we sought to determine whether epidermal expression of *sma-6* was sufficient to restore the induction of *cnc-2* after infection in a *sma-6*-mutant worm. Expression of *sma-6* under control of the epidermis-specific *col-12* promoter partially restored body size (length) in worms of the *sma-6*-mutant background and produced a Lon phenotype in wild-type worms (**Fig. 5b–e** and **Table 1**). This suggests that SMA-6 can act in a dose-dependent and cell-autonomous way in the epidermis as a regulator of body size. In addition, after infection, p*cnc-2*::GFP expression in *sma-6*-mutant worms carrying the p*col-12*::SMA-6 transgene was as high as that in worms of the wild-type background (**Fig. 5f**). Expression of *sma-6* under control of the intestine-specific *mtl-2* promoter, however, did not restore the body size or the *cnc-2* phenotype of *sma-6*-mutant worm (**Table 1** and **Supplementary Fig. 10** online). These results collectively indicate that *sma-6* acts in the epidermis to regulate *cnc-2* expression and suggest that SMA-6 and DAF-4 act in a cell-autonomous way to transduce a DBL-1 signal associated with fungal infection.

Upregulation of *cnc-2* is independent of *sma-2* and *sma-4*

To regulate body length, the intracellular Smad signal-transducer homologs SMA-2, SMA-3 and SMA-4 act 'downstream' of the receptor SMA-6–DAF-4 (ref. 24). SMA-2 and SMA-3 are receptor-regulated Smad proteins, orthologous to mammalian Smad1 and Smad5, respectively, whereas the common-mediator Smad protein SMA-4 is the ortholog of mammalian Smad4 (ref. 24). As loss of function of any one of these elicits an identical phenotype, it has been

Table 1 Influence of TGF-β (DBL-1) signaling on body size

Genotype	Array	Transgene	Length (%)	*n*
WT	None	None	100	171
WT	*frEx229*	p*col-12*::SMA-6	112	130
WT	*frEx244*	p*rab-3*::DBL-1	121	59
WT	*ctIs40*	p*dbl-1*::DBL-1	122	129
sma-6	None	None	61	411
sma-6	*frEx229*	p*col-12*::SMA-6	76	168
sma-6	*frEx323*	p*mtl-2*::SMA-6	64	105
dbl-1	None	None	71	117
dbl-1	*frEx244*	p*rab-3*::DBL-1	85	97
sma-2	None	None	60	94
sma-2	*ctIs40*	p*dbl-1*::DBL-1	56	109
sma-3	None	None	56	82
sma-3	*ctIs40*	p*dbl-1*::DBL-1	61	192
sma-4	None	None	60	86
sma-4	*ctIs40*	p*dbl-1*::DBL-1	59	83
sma-4(RNAi)	None	None	72	153
sma-2;sma-4(RNAi)	None	None	61	121

Body size of worms mutant for the genes listed (far left) and/or carrying the arrays and transgenes listed, presented relative to that of wild-type worms with no arrays or transgenes. Data are representative of three independent experiments (*n* (far right) = number of worms).

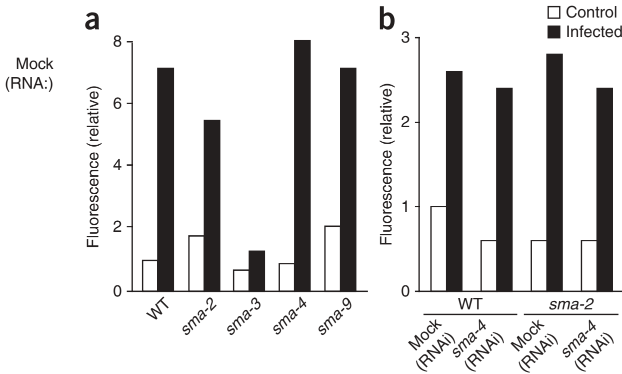

Figure 6 Upregulation of *cnc-2* after infection does not require *sma-2* or *sma-4*. (**a**) Biosort quantification of the normalized fluorescence of wild-type worms and worms of the *sma-2-*, *sma-3-*, *sma-4-* or *sma-9*-mutant background, all carrying a p*cnc-2*::GFP transgene, assessed without infection and 24 h after infection with *D. coniospora*. (**b**) Biosort quantification of the normalized fluorescence of wild-type worms and *sma-2*-mutant worms treated with an empty RNAi vector control (Mock (RNAi)) or *sma-4*-specific RNAi (*sma-4* (RNAi)), and carrying a p*cnc-2*::mCherry transgene, assessed without infection and 24 h after infection with *D. coniospora*. Data are representative of at least three independent experiments (number of worms, **Supplementary Table 1**).

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suggested that the functional Smad complex for this pathway is a SMA-2–SMA-3–SMA-4 heterotrimer. Notably, although a *sma-3* loss-of-function mutation blocked the induction of *cnc-2* reporters after fungal infection, we found that worms of the *sma-2-* or *sma-4*-mutant background did not have substantially lower reporter expression (**Fig. 6a** and **Supplementary Fig. 11** online). To determine whether this was due to redundancy in the activity of *sma-2* and *sma-4*, we analyzed the effect of disrupting *sma-4* activity by RNAi in a *sma-2*-mutant background. Consistent with published studies, worms in which both *sma-2* activity and *sma-4* activity was lower were no smaller than *sma-2*-mutant worms (**Table 1**). In addition, induction of expression of the *cnc-2* reporter gene was unchanged in *sma-2*-mutant worms treated with *sma-4*-specific RNAi relative to that in wild-type worms (**Fig. 6b**). These results suggest that *sma-2* and *sma-4* do not function either independently or redundantly to control the expression of *cnc-2* but that *sma-3* activity alone is required. Whereas mammalian receptor-regulated Smad proteins can form homodimers[25], to our knowledge, our results are the first evidence of a SMA-2- and SMA-4-independent function for SMA-3 in *C. elegans*.

In addition to SMA-2 and SMA-4, SMA-3 can interact with the transcription cofactor SMA-9 and with DAF-3, another Smad protein that functions as a transcriptional regulator and is required for the formation of the alternative dauer larval stage[24,26]. Neither SMA-9 nor DAF-3 was required, however, for *cnc-2* expression after infection (**Fig. 6a** and **Supplementary Fig. 12a** online). SMA-5, a homolog of the mammalian MAPK BMK1, is expressed in the worm intestine and epidermis and acts in parallel to the Sma signaling pathway to control body size[27]. Upregulation of the *cnc-2* reporter in response to fungal infection was also unchanged in *sma-5*-mutant worms (**Supplementary Fig. 12b**). Thus, not all of the genes necessary for the developmental function of the canonical DBL-1 signaling pathway are required for the regulation of *cnc-2*. This analysis therefore demonstrates the existence of a noncanonical DBL-1 signaling pathway important for innate immunity.

Dose-dependent regulation of *cnc-2* by DBL-1

To characterize further the mode of activation of the AMP-encoding gene *cnc-2* by DBL-1, we first determined whether the extent of upregulation of *cnc-2* after infection with *D. coniospora* correlated with the amount of *dbl-1* expression. By quantitative RT-PCR analyses, we found that *dbl-1* expression remained unchanged after infection, whereas *cnc-2* was highly upregulated (**Fig. 7a**). We then took advantage of an available integrated array (*ctIs40*)[22] associated with higher expression of *dbl-1*. On a wild-type background, worms carrying this array are longer than normal. Consistent with published results[28], we found that this larger size was dependent on *sma-2*, *sma-3* and *sma-4* (**Table 1**). We then transferred the *ctIs40* array into a strain containing two reporter genes, GFP under the control of the *nlp-30* promoter and mCherry under the control of the *cnc-2* promoter. As expected, p*nlp-30*::GFP expression was essentially like that of wild-type worms before and after infection (**Fig. 7b**). Also, we did not find any change in the constitutive expression of the p*cnc-2*::mCherry reporter. We did, however, note much greater induction of the reporter gene after infection of *ctIs40* worms than after infection of wild-type worms (**Fig. 7b**).

When we transferred the *ctIs40* array onto a *sma-2-* or *sma-4*-mutant background, we found that although the worms were no longer long, they still showed greater induction of the *cnc-2* reporter than did wild-type worms (**Table 1** and **Supplementary Fig. 11**). Only the *sma-3*-mutant worm had a concomitantly smaller size and lower expression of the *cnc-2* reporter gene (**Table 1** and **Supplementary Fig. 11**). Thus, control of body size can be 'decoupled' from the regulation of *cnc-2* expression.

LON-2, a conserved member of the glypican family of heparan sulfate proteoglycans, negatively regulates DBL-1 signaling[29,30]. As expected, a loss-of-function mutation of *lon-2* led to larger body size and also to overexpression of the p*cnc-2*::mCherry reporter after infection relative to that of infected wild-type worms (**Fig. 7c**); loss of

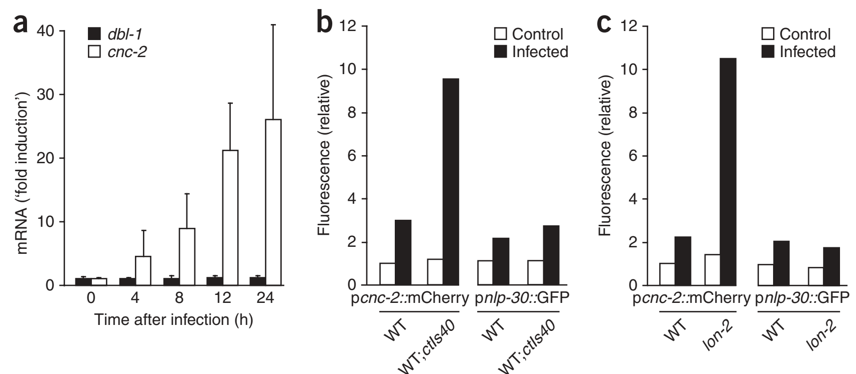

Figure 7 Influence of *dbl-1* expression on *cnc-2* upregulation. (**a**) Quantitative RT-PCR analysis of the expression of *cnc-2* and *dbl-1* in worms after infection with *D. coniospora*. Data represent at least three experiments (average and s.d.). (**b**,**c**) Biosort quantification of the normalized fluorescence of wild-type worms (**b**,**c**), worms of a *dbl-1*-overexpressing strain (WT;*ctIs40*; **b**) and worms of the *lon-2*-mutant strain (**c**), each carrying the p*cnc-2*::mCherry or p*nlp-30*::GFP transgene, assessed without infection and 24 h after infection with *D. coniospora*. Data are representative of at least three independent experiments (number of worms, **Supplementary Table 1**).

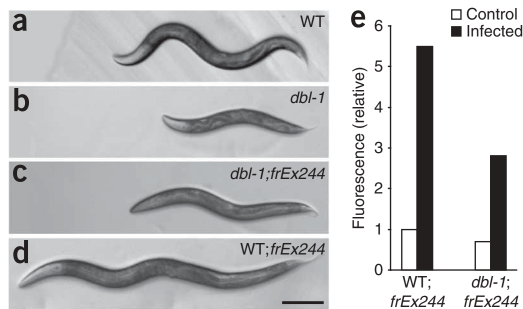

Figure 8 Neuronal expression of *dbl-1* is sufficient to restore body size (length) and upregulation of the *cnc-2* reporter after fungal infection in *dbl-1*-mutant worms. (**a**–**d**) Synchronized wild-type worm (**a**), *dbl-1*-mutant worm (**b**), *dbl-1*-mutant worm expressing *dbl-1* specifically in neurons (**c**), and wild-type worm expressing *dbl-1* specifically in neurons (**d**). The *frEx244* array (**c**,**d**) contains *dbl-1* under control of a neuronal promoter, a *cnc-2* reporter and a DsRed marker of transgenesis (p*rab-3*::DBL-1; p*cnc-2*::GFP; p*col-12*::DsRed). Scale bar, 100 μm. (**e**) Biosort quantification of the normalized fluorescence of wild-type and *dbl-1* mutant worms carrying the *frEx244* extrachromosomal array, assessed without infection and after 24 h of infection with *D. coniospora*. Data are representative of at least three independent experiments (number of worms, **Supplementary Table 1**).

lon-2 function, however, caused no alteration in expression of the p*nlp-30*::GFP reporter (**Fig. 7c**). These results collectively indicate that just as *dbl-1* functions as a dose-dependent positive regulator of body size[22], it also acts as a dose-dependent positive regulator of *cnc-2* expression after infection, acting through a signal-transduction pathway that involves *sma-3* but not *sma-2* or *sma-4*.

Neuronal *dbl-1* restores epidermal *cnc-2* induction

As *dbl-1* is expressed mainly in the nervous system[22,30], we sought to determine whether it acts in a non–cell-autonomous way to control *cnc-2* expression in the epidermis (**Fig. 8**). The specific expression of *dbl-1* in the nervous system under control of the 'pan-neuronal' *rab-3* promoter partially restored the body size of *dbl-1*-mutant worms and expression of p*cnc-2*::GFP after infection (**Fig. 8a**–**c**,**e** and **Table 1**). When we transferred the p*rab-3*::*dbl-1* transgene onto a wild-type background, it produced a long phenotype (**Fig. 8d** and **Table 1**) and was associated with higher expression of p*cnc-2*::GFP after infection (**Fig. 8e**). These results demonstrate that after fungal infection, neuronally produced DBL-1 can act in a paracrine way, through a noncanonical signaling pathway, to control expression in the epidermis of the AMP-encoding gene *cnc-2*.

DISCUSSION

Focused study of the regulation of a small number of AMPs in *Drosophila melanogaster* has provided fundamental insights into innate immune mechanisms[31]. One such peptide is Drosomycin, initially identified by biochemical approaches[32] as part of the antifungal response[33]. Delineation of the regulation of *Drosomycin* expression has shown that signaling in the infected host is initiated by a proteolytic cascade that leads indirectly to the activation of Toll. The consequent activation of a conserved intracellular signal-transduction pathway ultimately elicits the expression of *Drosomycin* as well as of many other genes, some of which also encode AMPs[31].

Here we focused on the regulation of the *C. elegans* AMP-encoding gene *cnc-2*. As with the better-characterized *nlp* AMP-encoding genes, *cnc-2* expression was not dependent on the single nematode Toll-like receptor encoded by *tol-1*. Infection-induced expression of the *nlp* genes[14–16] but not of *cnc-2* required the SARM ortholog *tir-1* and the associated conserved 'downstream' p38 pathway. Transcription of *cnc-2* was instead controlled in a paracrine way by the *C. elegans* TGF-β ortholog DBL-1.

We noted many parallels between the regulation of *Drosomycin* in adult flies and of *cnc-2* in worms. The former is controlled by an extracellular ligand, Spätzle, which has a cysteine-knot structure that is also found in many cytokines[34]. Spätzle expression is not greatly altered in immune-challenged flies[35–37]. Instead, the preexisting inactive precursor protein is processed by an infection-activated protease, Spätzle-processing enzyme[38]. In the developing embryo, Spätzle is cleaved by a different protease, Easter, and, after binding to Toll, influences dorso-ventral patterning[31]. DBL-1 is also a cysteine-knot protein that is important during development. We found that, as it does in its developmental function, in its regulation of *cnc-2* after infection, *dbl-1* acts in a dose-dependent way. Constitutive expression of *cnc-2* was not higher, however, in either in *ctIs40*-containing or *lon-2*-mutant strains, both of which have more active DBL-1. And, notably, infection did not increase *dbl-1* expression. This suggests that DBL-1 activity is controlled by post-translational modification. It seems possible that in a similar way to Spätzle, preexisting DBL-1 could be cleaved and activated by a 'sentinel' protease after immune challenge. Furthermore, as we noted different requirements for the intracellular Smad proteins for immune and developmental functions, it is possible that like the maturation of Spätzle, the maturation of DBL-1 in developmental and immune contexts requires two distinct proteases. The identification of these putative proteases and understanding of the molecular basis of the ability of the SMA-6–DAF-4 DBL-1 receptor to elicit distinct signaling cascades remain challenges for future studies.

At least four other pathways involved in *C. elegans* innate immunity are also involved in worm development (the *tol-1* pathway, the *tir-1*–*pmk-1* pathway, the *mpk-1* pathway and the *daf-2* pathway)[8,9]. This phenomenon emphasizes the intimate relationship between innate immune mechanisms and other aspects of organismal physiology. It also raises questions about the ways such pathways can evolve to counter the threat of an ever-changing microbial biota.

Part of the answer is that signaling cascades are used in a modular way. For example, in yeast MAPK signaling, adaptor proteins allow functional compartmentalization[39]. In *C. elegans*, whereas *tir-1*, the MAPK kinase kinase–encoding gene *nsy-1* and MAPK kinase–encoding gene *sek-1* are all required for correct neuronal development[40], this function does not depend on *pmk-1*, which is, however, necessary for immune regulation[15,16,41]. Presumably, *sek-1* can act through an alternative 'downstream' kinase during development. Such a separation of function is facilitated in multicellular organisms by tissue-specific expression of cofactors. For example, cells of the developing nervous system presumably express a different array of genes than do cells in the adult epidermis. It is notable that we found *sma-2*- and *sma-4*-independent immune functions occurring simultaneously with *sma-2*- and *sma-4*-dependent growth processes. Whether this difference depends on the activity of different transcription factors 'downstream' of *sma-3* remains to be established, but detailed investigation of regulatory motifs in the promoters of the *cnc* genes is warranted. This model of discrete pathways is, however, undoubtedly an oversimplification because although the *dbl-1*- and *sma*-6-mutant alleles we used were 'molecular nulls', expected to be associated with a complete loss of function, the observed phenotypes are not fully penetrant; there was induction of expression of the p*cnc-2*::mCherry reporter gene in a small proportion of mutant worms. The process of intracellular signal transduction initiated by TGF-β 'superfamily' ligands, from membrane-bound receptors to the nucleus, is conserved from worms to humans[25]. Thus, study of a function for the

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receptor-regulated Smad protein SMA-3 that is independent of the common-mediator Smad proteins may be of direct relevance to the comprehension of the previously poorly understood function of homomeric receptor-regulated Smad complexes in vertebrate TGF-β signaling.

Our results have also identified a function for a nervous system–derived ligand acting in a non–cell-autonomous way to regulate gene expression in another tissue and thereby contributing to pathogen resistance. This is not the first time that such a neuro-immune link has been proposed, as the well-characterized *daf-2* signaling pathway in *C. elegans* is controlled by insulin-like peptides, including INS-7, secreted by neurons[42,43]. In addition, activity of the *daf-2* pathway in the nematode intestine is modulated by the neurotransmitter serotonin[44]. Also, an indirect link between neuropeptide signaling and innate immunity has been described for *C. elegans*[45]. In such cases, the neuropeptide-secreting neuron is in direct contact with the pseudocoelomic cavity, which facilitates signal 'communication' with distant target tissues. The exact identity of all the neurons expressing *dbl-1* has not been established, and although it has not been formally demonstrated that DBL-1 is secreted into the pseudocoelom, this is widely assumed to be the case[22,30]. Whereas the insulin signaling pathway is influenced by a broad range of external stimuli, including temperature, heavy metals and starvation, as well as pathogens[46,47], for the time being, the regulation of *cnc-2* by *dbl-1* seems to represent principally an antifungal defense pathway.

Our results have shown that two groups of genes that share a relatively recent evolutionary origin have come under the control of very different signaling pathways. Future studies should examine the functional importance of this 'two-pronged' response to infection. Already, however, we can evoke different hypotheses to explain this complexity. First, hosts and pathogens are involved in a coevolutionary 'arms race' with repeated cycles of the emergence of new parasite virulence mechanisms and host countermeasures. For example, *P. aeruginosa* is able to suppress the immune response of both *D. melanogaster* and *C. elegans* during the early stages of infection by limiting the expression of defense genes[47,48], and infection of flies with zygomycetes leads to the downregulation of many genes involved in innate immunity, including those involved in pathogen recognition[49]. In such a situation, the expression of distinct sets of AMP-encoding genes through independent signaling pathways might be a strategy to limit a pathogen's capacity to suppress the host's immune response. Second, it is also possible that the combined activation after infection of a p38 cascade and a TGF-β pathway increases the worm's capacity to produce large quantities of AMPs very rapidly. This would be especially important if the NLP and CNC AMPs act synergistically. Finally, the control of *nlp* AMP-encoding genes by a p38 cascade after wounding and infection might represent the more ancestral response, rapid but relatively nonspecific, complemented by a more specific TGF-β-regulated expression of a second battery of AMP-encoding genes. Screens are needed to identify genes involved in triggering activation of the *dbl-1* pathway. Their identification should provide insight into these issues.

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METHODS

Nematode strains. All strains were maintained on nematode growth medium and were fed *E. coli* strain OP50. The wild-type reference strain is N2 Bristol. The strain *wt;ctIs40* (ref. 22) was provided by R.W. Padgett, and strains and *pmk-1(km25)*, *dbl-1(nk3)*, *daf-1(m40)*, *daf-3(e1376)*, *daf-4(m63)*, *sma-2(e502)*, *sma-3(e491)*, *sma-4(e729)*, *sma-5(n678)*, *sma-6(wk7)*, *sma-9(wk55)*, and *lon-2(e678)* were provided by the Caenorhabditis Genetics Center. All transgenic strains, including those containing p*cnc-2*::GFP, p*cnc-2*::mCherry, p*nlp-30*::GFP, p*col-12*::SMA-6, p*mtl-2*::SMA-6 or p*rab-3*::DBL-1 constructs, are described in the **Supplementary Methods**.

RNA preparation and quantitative RT -PCR. RNA preparation and quantitative RT-PCR were done as described[15] (primers, **Supplementary Methods**). Results were normalized to those of *act-1* and were analyzed by the cycling threshold method. Control and experimental conditions were tested in the same 'run'. Each sample was normalized to its own *act-1* control to take into account age-specific changes in gene expression.

RNAi. All RNAi feeding experiments were done essentially as described[50]. RNAi bacterial strains targeting an exon sequence common to all *tir-1* isoforms, *mpk-1* and *sma-4* were obtained directly from the Ahringer RNAi library and were used after insert verification (sequences available from WormBase).

Infection, wounding and osmotic stress. Infection, epidermal wounding and induction of osmotic stress were done as described[15].

Killing assays. A total of 50–70 worms at the L4 stage were infected at 15 °C with *D. coniospora* and surviving worms were counted every day as described[16] except that the nematode growth media plates were seeded with heat-killed OP50 bacteria. Killing was assayed at 15 °C. Assays with *S. marcescens* strain Db11 or *P. aeruginosa* strain PA14 used 70–100 worms at the L4 stage and were done at 25 °C as described[19,43]. A one-sided log-rank test and Prism software (Graphpad) were used for statistical analyses.

Analyses with the Biosort worm sorter. Upregulation of the expression of p*cnc-2*::GFP, p*cnc-2*::mCherry and p*nlp-30*::GFP reporter genes was quantified with the COPAS (Complex Object Parametric Analyzer and Sorter) Biosort system (Union Biometrica). Generally, a minimum of 100 synchronized worms were analyzed for length (assessed as time of flight), optical density (assessed as extinction), green fluorescence (GFP) and red fluorescence (red fluorescent protein)[15]. The ratio of green fluorescence or red fluorescence to time of flight was then calculated to normalize the fluorescence for variations in the size and health of individual worms. Mean values for the ratios were calculated and values for the different samples in a single experiment were normalized so that the control worms (wild-type) had a ratio of 1. As discussed before[15], because of factors such as conidial infectivity and the nature of the distribution of fluorescence values in a population, s.d. is not an informative parameter and is not presented for results obtained with the Biosort. Direct numerical comparisons can be made between age-matched populations in single experiments, and qualitative comparisons can be made between experiments done on different days.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS
O.Z. and J.J.E. designed the experiments, interpreted the data and wrote the manuscript, and O.Z. did the experimental studies.

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