The fatty acid synthase *fasn-1* acts upstream of WNK and Ste20/GCK-VI kinases to modulate antimicrobial peptide expression in *C. elegans* epidermis

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An important part of the innate immune response of the nematode *C. elegans* to fungal infection is the rapid induction of antimicrobial peptide gene expression. One of these genes, *nlp-29*, is expressed at a low level in adults under normal conditions. Its expression is upregulated in the epidermis by infection with *Drechmeria coniospora*, but also by physical injury and by osmotic stress. For infection and wounding, the induction is dependent on a p38 MAP kinase cascade, but for osmotic stress, this pathway is not required. To characterize further the pathways that control the expression of *nlp-29*, we carried out a genetic screen for negative regulatory genes. We isolated a number of Peni (peptide expression no infection) mutants and cloned one. It corresponds to *fasn-1*, the nematode ortholog of vertebrate fatty acid synthase. We show here that a pathway involving fatty acid synthesis and the evolutionary conserved *wnk-1* and *gck-3*/Ste20/GCK-VI kinases modulates *nlp-29* expression in the *C. elegans* epidermis, independently of p38 MAPK signaling. The control of the antimicrobial peptide gene *nlp-29* thus links different physiological processes, including fatty acid metabolism, osmoregulation, maintenance of epidermal integrity and the innate immune response to infection.

Introduction

The nematode *Caenorhabditis elegans* can come into contact with pathogens either at the surface of its intestinal epithelium following their ingestion, or when bacteria or fungi adhere to its cuticle.[1,2] Examples of the latter include Yersinia spp. and *Xenorhabdus nematophilum*, two Gram-negative species that can form a biofilm on head of worms,[3,4] the Gram-positive bacterium *Microbacterium nematophilum*, which adheres to a specific region in the tail,[5] and *Drechmeria coniospora*, a fungus with spores that attach to the worm and then penetrate the cuticle and epidermis.[6] Infection by *D. coniospora* provokes the rapid induction of genes encoding antimicrobial peptides (AMPs), including members of the *nlp* family.[7,8] When its epidermis is damaged, *C. elegans* also expresses AMPs of the *nlp* family, such as *nlp-29*, as part of a wound healing response, which concomitantly involves cellular repair.[9] The expression of *nlp-29* following injury or infection depends on a PKC-p38 MAPK pathway, which acts cell automonously in the epidermis.[10]

Mutants in some cuticular or epidermal proteins, including *dpy-9* and *osm-11*, also exhibit increased expression of *nlp-29*.[8] These two mutants have elevated levels of *gpdh-1*, which encodes the rate-limiting enzyme for the biosynthesis of the osmoprotectant glycerol and are resistant to osmotic stress.[11,12]

Surprisingly, it has recently been reported that this resistance can be decoupled from *gpdh-1* expression. Thus, when the nematode Wnk or Ste20/GCK kinase genes (*wnk-1* and *gck-3*, respectively) are inactivated in a *dpy-9* or *osm-11* background, osmotic stress resistance returns to normal, while *gpdh-1* expression remains high.[13] Further, both *gpdh-1* and *nlp-29* expression are upregulated upon exposure to high salt.[8,11] While the upregulation of *nlp-29* under these conditions is independent of the PKC-p38 MAPK pathway, abrogation of the function of the p38 MAPK *pmk-1* in an *osm-11* mutant blocks the elevated *nlp-29* expression but does not affect acute osmotic resistance.[8] Finally, death-associated protein kinase (*dapk-1*) mutants display both epidermal abnormalities and a high constitutive expression of *nlp-29*. The high level of *nlp-29* expression is independent of the morphological defects, but is dependent upon the p38 MAPK pathway.[14]

The *nlp-29* AMP gene is thus subject to a complex regulation. It links different physiological processes, osmoregulation, osmoresistance, epidermal integrity and the innate immune responses to infection and injury. In the present study, we used a genetic approach to try to tease apart these processes, and provide evidence that a pathway involving fatty acid synthesis and the evolutionary conserved *wnk-1*/WNK and *gck-3*/Ste20/GCK-VI kinases modulates AMP gene expression in the *C. elegans* epidermis.
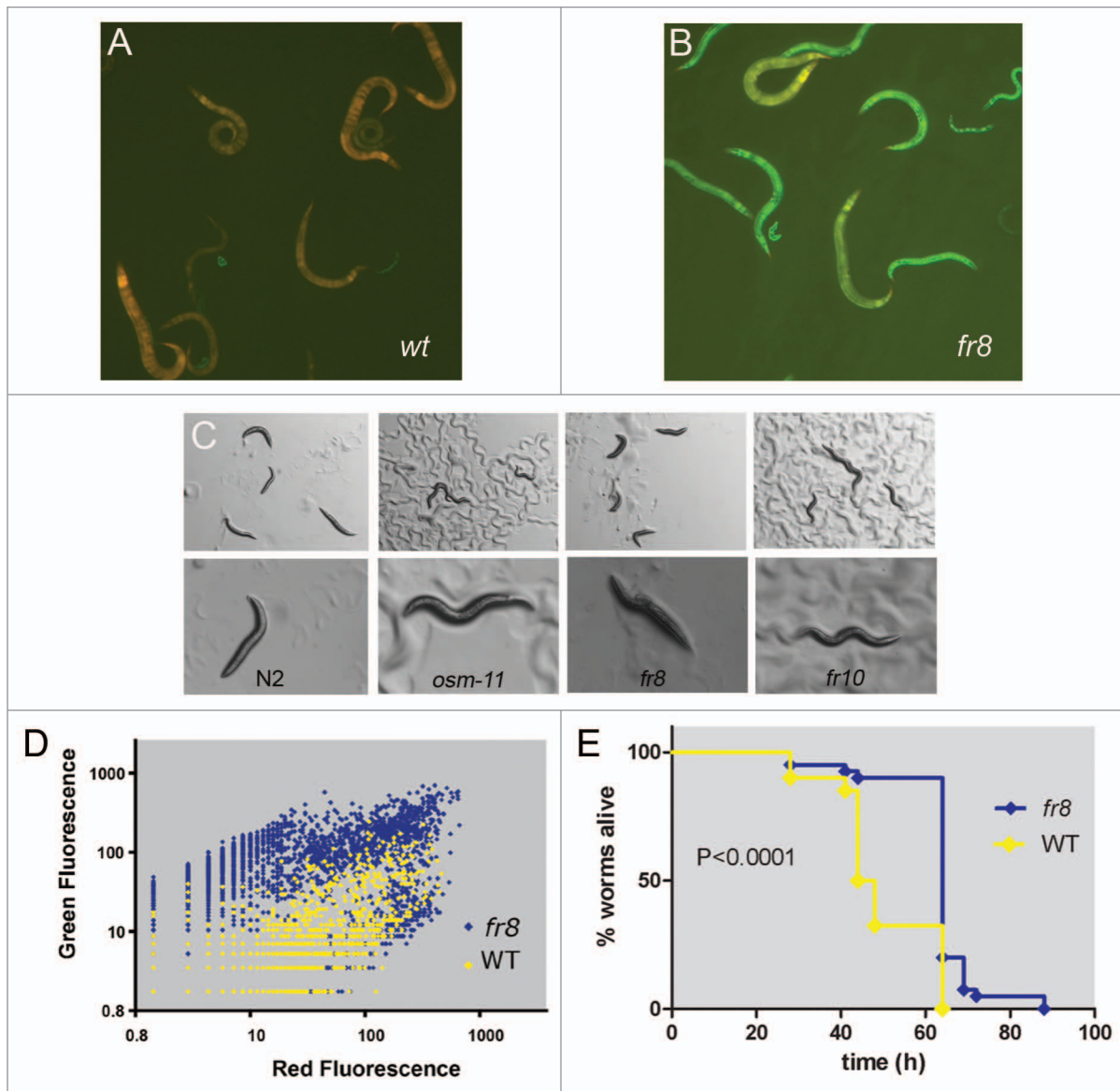
Figure 1. Characterization of Peni mutants. Uninfected transgenic worms carrying a p*nlp-29*::GFP reporter do not express high levels of GFP in the wild-type background (A) while in homozygous *fr8* worms there is a strong constitutive expression (B). The transgenic worms also carry a p*col-12*::dsRed reporter gene, which is expressed from the L2 stage onwards. Green and red fluorescence are visualized simultaneously. (C) The acute osmotic stress resistance (>15 minutes on NGM agar plates containing 500 mM NaCl) of wild type N2 worms is compared to *osm-11(n1604)*, *fr8* and *fr10* mutants (left to right). Upper and lower panel shows low and high magnification of the worms. (D) Quantification of fluorescence in the wild-type (yellow) and *fr8* (blue) mutant background of a mixed stage population with the COPAS Biosort. Each dot represents an individual worm (n = 2536 and 3349 for WT and *fr8*, respectively). Red and green fluorescence are shown on an arbitrary logarithmic scale. (E) Survival of *fr8* versus WT control worms (both containing the integrated *frIs7* reporter transgene) after infection with *D. coniospora* at 25°C. Data are representative of three independent experiments [n > 100; p < 0.0001, log-rank (Mantel-Cox) test].

Results

A screen for aberrant regulation of AMP expression. The majority of the *nlp* AMP genes, including *nlp-29*, are expressed at a low level under standard culture conditions.[8] Upon infection, their expression is upregulated, and this induction can be visualized in vivo using transgenic worms carrying suitable reporter constructs. With the aim of identifying negative regulators of the signaling pathway controlling AMP gene expression, we used a strain of *C. elegans* carrying an integrated p*nlp-29*::GFP reporter gene[9] to screen for mutants that showed abnormally high constitutive GFP expression. From a pilot screen of 10,000 mutagenized haploid genomes, seven Peni (peptide expression no infection) mutant alleles, *fr7-fr13*, were obtained (**Fig. 1A and B**, results not shown).

As mentioned above, as well as being induced by infection, p*nlp-29*::GFP can also be upregulated by hypertonic stress, such as upon exposure to concentrations of NaCl above 150 mM, and also in some mutant strains with defects in osmo-regulation, including *osm-11*.[8] We therefore tested whether any of our newly

isolated mutants were severely defective in osmo-regulation. Upon exposure to 500 mM NaCl, wild-type worms shrink due to water loss and are rapidly immobilized, whereas osmotic stress resistant (Osr) mutants like *osm-11* show no body fluid loss and move normally.[12] Two of the Peni mutants, *fr10* and *fr13*, displayed an Osr phenotype; *fr13* also displayed a Dpy phenotype. The remaining mutants behaved and looked wild-type (**Fig. 1C** and results not shown). Osmotic stress is normally associated with an increased expression of the rate-limiting enzyme for glycerol production, *gpdh-1*, and this can be followed using a GFP reporter under the control of the *gpdh-1* promoter. Certain Osr mutants, including *osm-11*, show a high constitutive expression of a p*gpdh-1*::GFP reporter, even in conditions of normal osmolarity. When this reporter gene was transferred into the different Peni mutants, a markedly increased fluorescence was observed in the strains homozygous for *fr10* and *fr13*, but not for *fr7, fr8, fr9* or *fr12* (results not shown). Taken together, these results suggest that *fr10* and *fr13* have a severe defect in osmo-regulation, which leads to increased p*nlp-29*::GFP expression; they were not characterized further.

The *fr8* mutant displays other phenotypes. To investigate whether the effect on *nlp-29* regulation seen in the Peni mutants extended to a second class of antimicrobial peptide genes, we looked at the expression of a Caenacin (*cnc*) gene. We chose the gene *cnc-2* as it is well characterized and its expression is only mildly influenced by changes in osmolarity.[15] We therefore transferred by mating a p*cnc-2*::GFP reporter transgene into the different mutant backgrounds. An increased constitutive expression of p*cnc-2*::GFP was only seen in the *fr8* mutant background (**Suppl. Fig. 1**, and results not shown); *fr8* was therefore chosen for detailed analysis.

Although roughly 40% of eggs from *fr8* homozygous mutants failed to hatch (**Suppl. Fig. 2A**), there were no obvious developmental defects in the hatching larvae and adults exhibited no gross morphological defects. The arrest in embryonic development was correlated with an abnormal permeability of the eggshell for the lipophilic dye Nile red (**Suppl. Fig. 2B and C**). As this is used as a criterion for solute permeability, and can reflect a problem in the eggshell or cell membrane, this suggests that although the *fr8* mutant does not have a pronounced osmo-regulatory defect as an adult, the structural integrity in a fraction of eggs is compromised.

We then characterized the age-dependent change in p*nlp-29*::GFP expression in wild-type and mutant animals, using the COPAS Biosort. This quantitative analysis demonstrated a clear difference in green fluorescence between the two strains, which was most evident from L2 to young adult stages, when it was 10–40-fold higher in the mutants. In older mutant worms, the green fluorescence declined (**Fig. 1D, Suppl. Fig. 3A and B**). The reporter strains used also carry a gene for the red fluorescent protein dsRed, under the control of a constitutive epidermal promoter (*pcol-12*::dsRed). In the *fr8* background, in L2 and L3 larvae there was an increase in the value of red fluorescence measured in the mutant worms (<5-fold compared to wild-type), but this difference disappeared in older worms, indicating that the observed increases in p*nlp-29*::GFP and p*cnc-2*::GFP expression

were likely not due to a general mis-regulation of transgene expression (results not shown). Despite the constitutively high level of expression of p*nlp-29*::GFP, infection of *fr8* mutant worms provoked a clear induction of reporter gene expression (**Suppl. Fig. 4**), showing that the signal transduction cascade triggered by infection was intact. Importantly, the *fr8* mutant worms also showed a significantly higher resistance to infection with *D. coniospora* compared to wildtype (**Fig. 1E**).

***fr8* is a mutant allele of the fatty acid synthase gene *fasn-1*.** To identify the molecular lesion in the *fr8* mutant, we first performed conventional SNP mapping, and placed *fr8* in a genomic region of 0.2 cM on the right arm of chromosome I. Microinjection and transformation rescue were used to narrow down the location of *fr8* to one gene, *fasn-1*. *fasn-1* encodes a putative fatty acid synthase, orthologous to human FASN. Sequencing revealed a single nucleotide change in *fr8* (G723A) (**Fig. 2A**), predicted to change a methionine to an isoleucine (M241I) in a highly conserved region of the protein (**Suppl. Fig. 5**). *fasn-1*(RNAi) provoked increased expression of both p*nlp-29*::GFP and p*cnc-2*::GFP reporter genes (**Suppl. Fig. 6** and results not shown), further confirming the identity of the gene. As previously reported,[16-18] we found that *fasn-1* is an essential gene; abrogation of its function by RNAi led to embryonic and larval lethality. In addition, blocking *fasn-1* function by RNAi in a transgenic strain carrying a p*gpdh-1*::GFP reporter gene, provoked a high level of GFP fluorescence. While consistent with prior studies,[11] the disparity between the results of RNAi and the observed phenotype of the *fasn-1* mutant indicates that *fasn-1(fr8)* is a mild loss of function allele.

The gene F32H2.6, which is 12 kb downstream of *fasn-1*, encodes a protein with high sequence similarity to the N-terminal region of *fasn-1* (65% identity over 167 amino acids). The similarity extends to the nucleic acid level, with more than 75% identity over 500 nucleotides for the predicted transcripts, and stretches of up to 27 contiguous identical nucleotides. RNAi of *F32H2.6* provokes the same pleiotropic phenotypes as *fasn-1*(RNAi), e.g., constitutive p*gpdh-1*::GFP expression, and embryonic and larval arrest.[17] RNAi of *F32H2.6* also provoked a marked increase in p*nlp-29*::GFP expression, i.e., a Peni phenotype (**Suppl. Fig. 7**). On the other hand, we found that an available deletion allele of *F32H2.6*, *tm3581*, predicted to be a molecular null, did not display a Peni phenotype. In all likelihood, therefore, the Peni phenotype observed with *F32H2.6*(*RNAi*) is a consequence of the concomitant interference of *fasn-1* expression.

***fasn-1* functions cell-autonomously in the epidermis of *C. elegans*.** To investigate which tissues and cells express *fasn-1*, we generated transgenic animals expressing a transcriptional reporter construct including more than 2 kb of upstream sequence and with the start of the second exon of *fasn-1* fused to GFP (**Fig. 2A**). Expression was visible in the intestine, spermatheca, many head and tail neurons, in the main epidermal syncytium hyp7 and the seam cells (**Fig. 2B–D**). Specific expression of *fasn-1* in the epidermis, driven by the *col-12* promoter completely rescued the Peni phenotype of *fasn-1(fr8)*, whereas expression of *fasn-1* in the intestine and neurons, under the control of the *mtl-2* and *rab-3* promoters, respectively, gave no rescue (**Fig. 2E–G**). Thus,
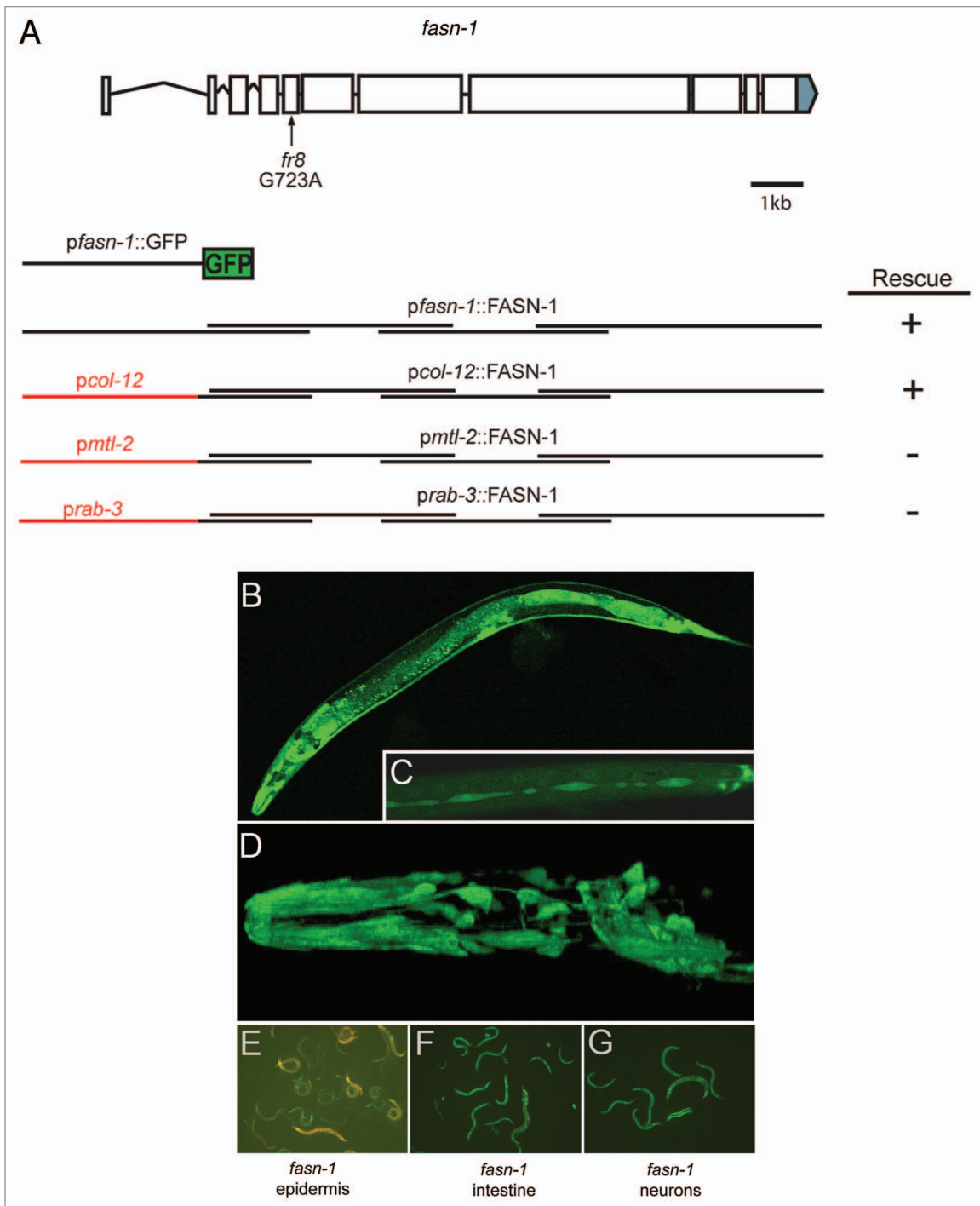
Figure 2. *fr8* is a mutation in the fatty acid synthase gene *fasn-1*. (A) Structure of the *fasn-1* genomic locus. The location of the *fr8* mutation is indicated with an arrow. Exons are shown as boxes, introns are represented as lines, the grey region shows the 3'UTR of *fasn-1*. Several constructs are shown below the gene structure, from top to bottom, respectively: the promoter region used for the GFP reporter construct (p*fasn-1*::GFP), the overlapping rescuing fragments, the rescuing fragments under the control of the tissue specific promoters of *col-12*, *mtl-2* and *rab-3*. The length of these promoters is not to scale. (B–D) Confocal fluorescence images of p*fasn-1*::GFP transgenic worms illustrating expression in multiple neurons in the head and tail region and socket cells (B and D), intestine, spermatheca and epidermis (B) and seam cells (C). (E–G) Expression of *fasn-1* in the epidermis is sufficient to rescue the Peni phenotype. Micrographs of *fasn-1(fr8)* worms carrying the *frIs7* transgene with a second transgene driving expression of *fasn-1* under the control of the epidermal *col-12* promoter (E), of the intestinal *mtl-2* promoter (F) and of neuronal *rab-3* promoter (G).

fasn-1 can act cell-autonomously in the epidermis to influence *nlp-29* expression.

***fasn-1(fr8)* has a subtly altered fatty acid composition.** FASN-1 is a key enzyme involved in the de novo synthesis of fatty acids, elongating malonyl-CoA in a step wise fashion to generate unsaturated fatty acids (FA), mainly C16:0 (palmitic acid). We therefore wished to address the question of whether the fat content in *fasn-1(fr8)* mutants was altered, and so used gas chromatography as a precise assay. We looked specifically at the stages between L2 and young adult stage, when differences in p*nlp-29*::GFP expression are most marked between wild-type and mutant worms. When we compared wild-type and *fasn-1(fr8)* mutants animals, we observed little difference (**Fig. 3A**). We then compared the FA content in worms from a transgenic rescued strain with their age-matched non-transgenic *fasn-1(fr8)* siblings. There was a marginal, but not significant difference in C17D levels (p > 0.05, paired student's t test). This cyclo-FA comes mainly from the bacterial diet. Variations in C17D levels can reflect differences in the amount of bacteria inside the worms' intestines or in the efficiency of washing worms from plates. Compared to the *fasn-1(fr8)* worms, the rescued siblings did show significantly increased levels of C16:0 (palmitic acid), the main product of FASN-1, and its elongated form C18:0 (**Fig. 3A and B**), which is directly synthesized from C16:0 by the elongation enzyme ELO-2.[19] This presumably reflects the overexpression of *fasn-1* in the transgenic animals and the associated increase in fatty acid synthase activity.

Loss of acetyl-CoA carboxylase function also provokes a Peni phenotype. The key rate-limiting enzyme in endogenous synthesis of fatty acids is the acetyl-CoA carboxylase (ACC). This enzyme carboxylates acetyl-CoA to malonyl-CoA, providing the substrate for FASN-1. Knockdown of the unique ACC gene in *C. elegans*, *pod-2*, by RNAi resulted in embryonic and larval lethality, consistent with previous results,[16] and also increased expression of p*nlp-29*::GFP (**Suppl. Fig. 8**). This result suggests that abnormalities in fatty acid metabolism, in particular the de novo synthesis of saturated FAs, can lead to a Peni phenotype.

Loss of function of downstream components of the fatty acid synthesis pathway do not alter p*nlp-29*::GFP expression. In mammals, saturated FA, formed through the action of FASN, is transformed into mono-unsaturated FA by Δ-9-stearoyl-CoA desaturase (SCD). Monounsaturated FAs are the most abundant group of FAs found in phospholipids. These serve as the main component of the lipid membrane and are responsible for sustaining optimal membrane fluidity, as well as being mediators of signal transduction.[20] The SCDs are essential and ubiquitous in eukaryotes. In *C. elegans* there are 2 such enzymes, FAT-6
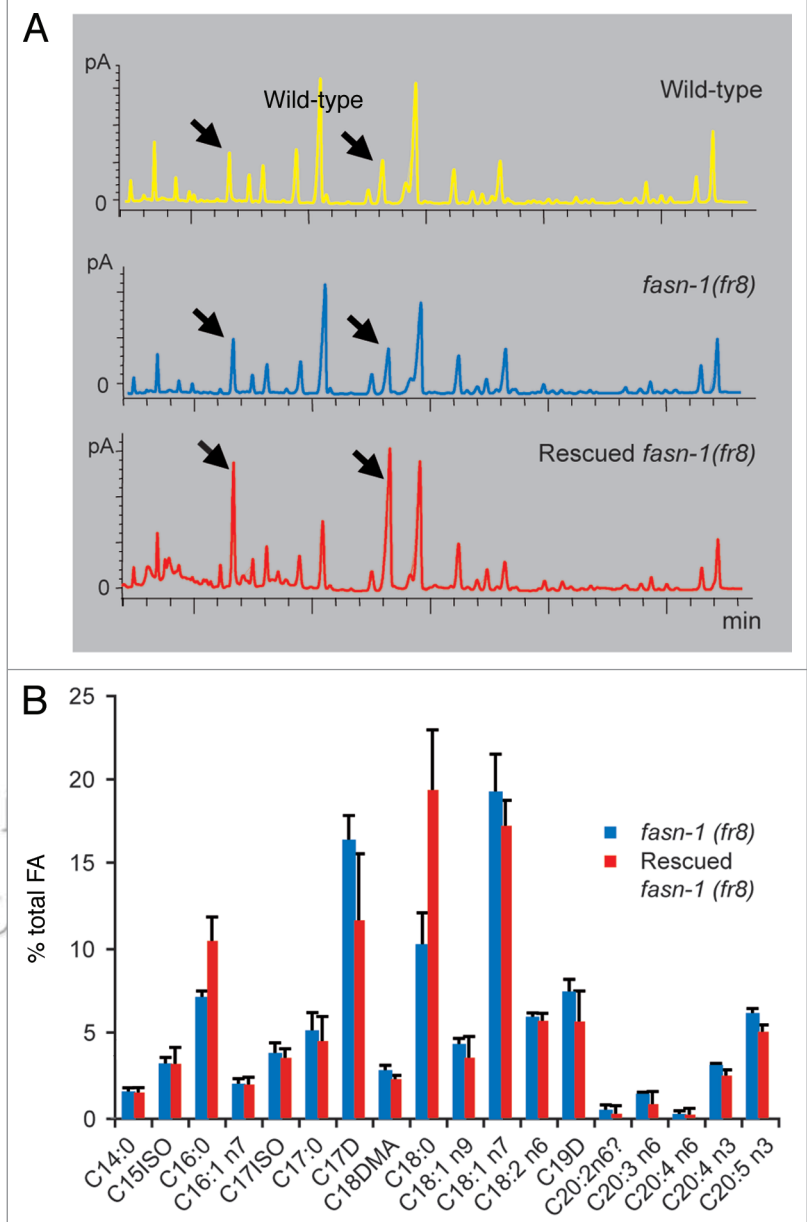

Figure 3. *fasn-1* mutants carrying a rescuing extrachromosomal array have an altered FA composition. (A) Gas chromatograms showing the FA composition in wild type worms, as well as rescued *fasn-1(fr8);frEx288* worms and their *fasn-1(fr8)* mutant siblings. Arrows point to the peaks corresponding to C16:0 and C18:0. The FA compositions in wild-type and *fasn-1* mutants are comparable suggesting that a decrease in FASN-1 activity does not preferentially affect a specific type of FA. Expression, and presumably overexpresion, of *fasn-1* from an extrachromosomal array resulted in an imbalance of FA composition, towards saturated C16 and C18. (B) The graph shows the average levels (with standard deviation) of individual FA species normalized to total FA from analysis of three biological replicates. The difference for C16:0 and C18:0 levels are significant (p = 0.05 and <0.05, respectively, paired Student's t test).

and 7 and a third enzyme, the palmitoyl-CoA desaturase FAT-5, which has high sequence similarity to the SCDs.[21] Inhibition of *fat-5, fat-6* or *fat-7* leads to altered FA composition,[22] causes changes in body fat, and reduces fertility and body size.[23] We tested whether these SCD genes are also involved in controlling
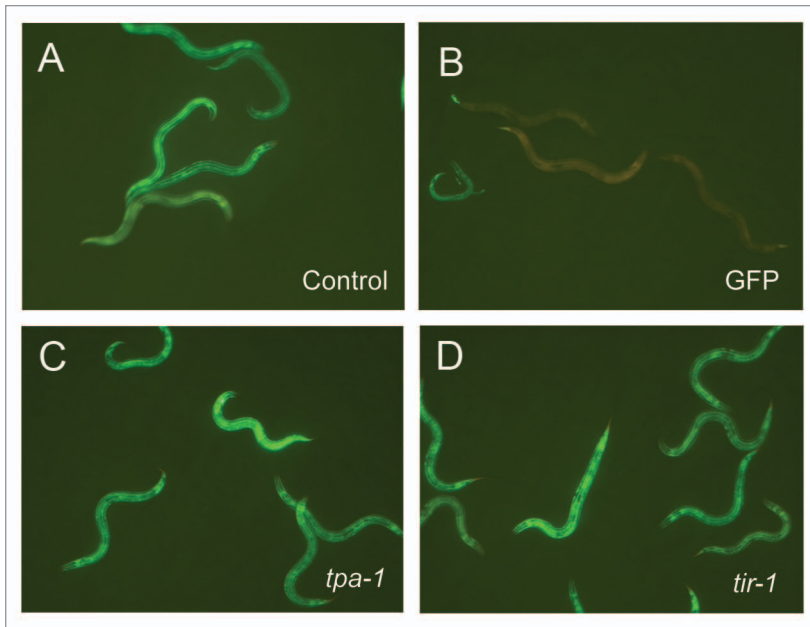
Figure 4. Regulation of p*nlp-29*::GFP by *fasn-1* is independent of the PKC-p38 MAPK pathway. RNAi of *tpa-1* (C) and *tir-1* (D) in *fasn-1(fr8)* background does not reduce the constitutive expression of p*nlp-29*::GFP seen in control worms raised on bacteria containing an empty RNAi vector (A), unlike GFP RNAi (B).
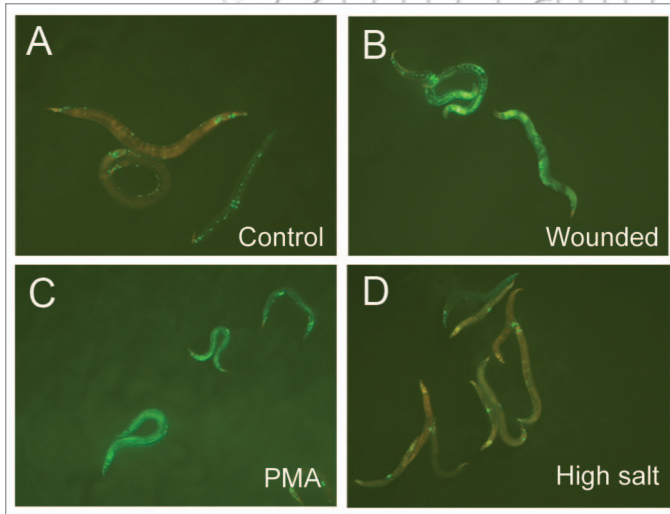

Figure 5. Extra copies of *fasn-1* block the osmotic shock-induced expression of p*nlp-29*::GFP. (A–D) Compared to untreated controls (A), wt;*frIs7;frEx288* worms exhibit a high level of p*nlp-29*::GFP expression after injury (B) or treatment with PMA (C), but not after osmotic stress (D).

involved in the biosynthesis of certain monomethyl branched-chain FA.[17,19] RNAi of the *elo* genes (*elo-1-7*) did not provoke an increased expression of p*nlp-29*::GFP expression (**Suppl. Fig. 9B**). Although this result could possibly be a result of inefficient RNAi, redundant gene function, or compensatory FA synthesis, it does suggest that only direct products of FASN-1 activity, and/or the FASN-1-specific contribution to the FA composition in a particular tissue are involved in the regulation of *nlp-29* expression.

***fasn-1* acts in parallel to, or downstream of, the p38 MAPK cascade.** Induction of *nlp* AMP gene expression in *C. elegans* following *D. coniospora* infection is known to depend on a PKC-p38 MAPK pathway.[10] To test whether *fasn-1* acts in this pathway, we tried to make a double mutant between *fasn-1(fr8)* and *pmk-1(km25)*. These attempts were unsuccessful, apparently due to a synthetic lethality between the two mutants during development. We therefore used RNAi to knock down expression of different target genes in the *fasn-1(fr8)* background.

When the activity of *tpa-1, tir-1, nsy-1, sek-1* or *pmk-1* was abrogated by RNAi, there was no suppression of the Peni phenotype in the *fasn-1(fr8)* mutant background (**Fig. 4A–D, Suppl. Fig. 10A**). Interestingly, a *tir-1;fasn-1* double mutant was viable and allowed us to confirm that loss of *tir-1* function did not diminish expression of p*nlp-29*::GFP in the *fasn-1(fr8)* background (**Suppl. Fig. 10B**). Taken together, these results suggest that *fasn-1* acts downstream or in parallel to the p38 MAPK pathway to control p*nlp-29::GFP* expression.

Supernumerary copies of *fasn-1* specifically block induction of p*nlp-29*::GFP expression after osmotic shock. We then transferred by mating the *fasn-1*-containing rescuing transgenic array into the wild-type background. When we assayed this strain's response to different stimuli, we observed the expected increase in p*nlp-29*::GFP expression after infection with *D. coniospora* and exposure to PMA (**Suppl. Fig. 11**). Unexpectedly, the induction of p*nlp-29*::GFP after osmotic shock was clearly blocked (**Fig. 5**), suggesting that *fasn-1* may in fact be involved in controlling the osmotic stress-mediated induction of *nlp* gene expression.

***fasn-1* acts upstream of *wnk-1*/WNK and *gck-3*/Ste20/GCK-VI kinases.** In *C. elegans*, the response to hypertonic shrinkage is believed to involve components of the cuticle and secreted proteins, such as DPY-10 and OSM-11, respectively. These proteins have been proposed to be part of a systemic mechanosensory apparatus that detects hypertonic shrinkage. Interestingly, *dpy-10* and *osm-11* mutants display a Peni phenotype, like *fasn-1(fr8)*.[8] The evolutionary conserved *wnk-1*/WNK and *gck-3*/Ste20/GCK-VI are required for the regulation of the systemic volume and survival after hypertonic shrinkage.[24] Knockdown of the gene function of *wnk-1* or *gck-3* by RNAi in *osm-11* or *dpy-10* mutants leads to the suppression of the Osr phenotype,

the expression of *nlp-29*. In contrast to the consequences of loss of *pod-2* function, no upregulation of p*nlp-29*::GFP was observed when *fat-5, fat-6* or *fat-7* expression was abrogated by RNAi (**Suppl. Fig. 9A**). This suggests that monounsaturated FAs may not be directly involved in the regulation of the expression of *nlp-29*.

The *elo* genes encode another group of FA modifying enzymes that elongate the carbon backbone of FAs. Some are also

but does not suppress glycerol accumulation or alter systemic volume.[13] When we abrogated *wnk-1* and *gck-3* expression by RNAi, *fasn-1(fr8)* worms showed a marked decrease in their level of p*nlp-29*::GFP expression. Additionally, *wnk-1(RNAi)* and *gck-3(RNAi)* reduced the expression of p*nlp-29*::GFP in wild-type worms exposed to conditions of high osmolarity (**Fig. 6**). These results suggest that *wnk-1* and *gck-3* act downstream of *fasn-1* and that part of the response of *C. elegans* to osmotic stress requires these two kinases.

wnk-1 and gck-3 regulate nlp-29 expression in parallel to the p38 MAPK pathway. Overexpression of *sek-1* leads to a constitutively high level of p*nlp-29*::GFP expression.[9] In contrast to *pmk-1(RNAi),* which as expected reduced p*nlp-29*::GFP expression, neither *wnk-1* nor *gck-3* RNAi provoked a change in the constitutively high reporter gene expression in the *sek-1* overexpressing strain. This suggests that *wnk-1* and *gck-3* do not act downstream of *sek-1* and so may regulate *nlp-29* via a pathway which is parallel to the p38 MAPK pathway (**Fig.** 7).

Discussion

Our previous studies have shown that regulation of AMP genes such as *nlp-29* is an important part of *C. elegans'* anti-fungal defenses. Activation of the expression of *nlp-29* after infection or wounding involves a PKC-p38MAPK pathway. In the current study, we identified a mutation in *fasn-1* that causes upregulation of the AMP gene *nlp-29*. In *C. elegans*, polyunsaturated FAs (PUFAs) gamma-linolenic and stearidonic acid play a role in resistance against *Pseudomonas aeruginosa* infection.[25] No alteration was evident, however, for PUFA's in the *fasn-1* mutant. Indeed, loss of *fasn-1* activity did not differentially affect FA content in *C. elegans*, but rather decreased the levels of all FA proportionally. It should be mentioned, however, that because of technical constraints, the GC analysis of FA is necessarily performed on whole animal extracts. There could conceivably be a significant imbalance of FA composition, or a more dramatic drop in total FA level, in the epidermis. In plants, fatty acid synthesis has also been implicated in the control of AMP expression. This was suggested potentially to be the consequence of alterations in lipid signaling.[26] While we cannot exclude such a possibility for *fasn-1*, we favor an explanation based on changes in FAs altering properties of the cuticle or affecting epidermal cell membrane integrity. Support for such an idea comes from the fact that overexpression of *fasn-1*
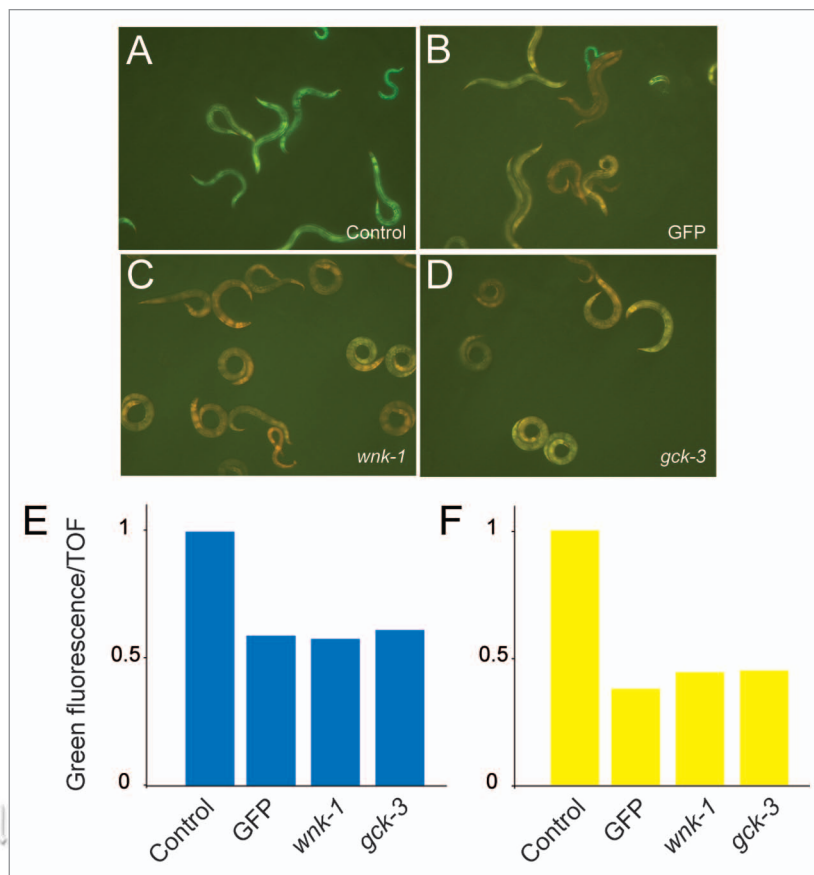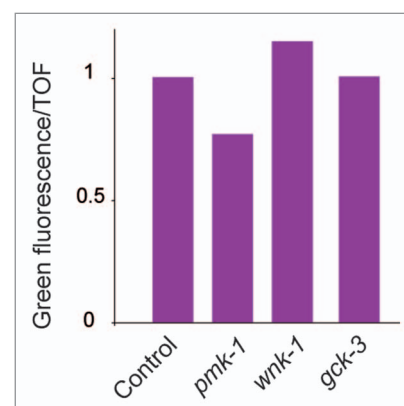

Figure 6. Abrogation of *gck-3* or *wnk-1* block the elevated level of p*nlp-29*::GFP expression seen in *fasn-1(fr8)*. Treatment of *fasn-1(fr8);frIs7* animals with RNAi for *gck-3* (C) and *wnk-1* (D) lead to the downregulation of p*nlp-29*::GFP expression similar to that seen upon worms treated with GFP RNAi (B), compared to an empty RNAi vector control (A). (E) Quantification of the effect of *gck-3* and *wnk-1* RNAi on p*nlp-29*::GFP expression in *fasn-1(fr8);frIs7* animals with the COPAS Biosort. The normalized average ratio of green fluorescence to time of flight (TOF) is shown. The analysis was restricted to worms with a TOF between 200 and 400. In each sample, data is from, from left to right, 100, 60, 87 and 67 worms. For reasons given elsewhere,[9] in this and the subsequent graphs, error bars are not shown. Data are representative of three independent experiments. (F) Treatment of *wt;frIs7* animals with RNAi for *gck-3* and *wnk-1* also lead to the downregulation of p*nlp-29*::GFP expression after 6 h of hyperosmotic stress, compared to the empty vector control. The number of worms used in each sample was, from left to right, 142, 122, 360 and 309 worms. Data are representative of two independent experiments.

Figure 7. *wnk-1* and *gck-3* act upstream or in parallel to the MAP2K *sek-1*. Overexpression of *sek-1* provokes constitutively high p*nlp-29*::GFP expression, which is reduced by *pmk-1(RNAi)* but not by *wnk-1* nor *gck-3* RNAi. The normalized average ratio of green fluorescence to TOF is shown. The analysis was restricted to worms with a TOF between 200 and 400. The number of worms used in each sample was, from left to right, 1381, 754, 371 and 367 worms. Data are representative of two independent experiments.
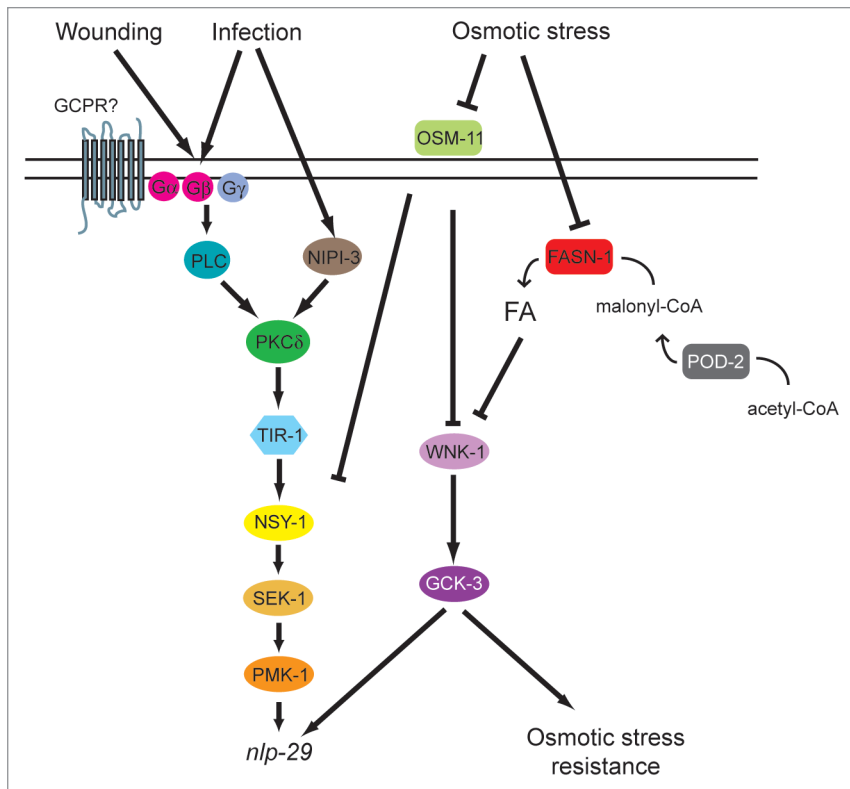
Figure 8. Model of the control of *nlp-29* expression. Signals perceived upon *D. coniospora* infection and injury are transduced by a PKC-p38 MAPK pathway to regulate the expression of *nlp-29*. OSM-11 acts as a negative regulator of this pathway, intervening at the level of *pmk-1*, or above. It also acts in a WNK-1- and GCK-3-dependent parallel pathway to negatively regulate *nlp-29* expression. The OSM-11/WNK-1/GCK-3 pathway additionally controls the organismal resistance to osmotic stress, and via a separate as yet undefined pathway, the expression of *gpdh-1*, and thereby glycerol levels (not shown here for the sake of simplicity). Both POD-2 and FASN-1 influence fatty acid (FA) levels, and negatively regulate *nlp-29* expression, in a WNK-1- and GCK-3-dependent manner. The exact manner in which FA levels alter *nlp-29* expression has yet to be determined. They may act indirectly by affecting the structure of the cuticle or epidermal cell membrane integrity.

diminishes the induction of *nlp-29* expression upon osmotic stress, and the observation that the control of *nlp-29* expression by *fasn-1* required *wnk-1* and *gck-3*, but was independent of the PKCδ/TIR-1/p38MAPK pathway. WNK-1 and GCK-3 act downstream of the putative systemic osmotic sensors DPY-10 and OSM-11.[13] Interestingly, the increased expression of p*nlp-29::GFP* in an *osm-11* mutant is dependent upon the p38 MAPK PMK-1.[8] This suggests that multiple pathways regulate the response to hypertonic stress, and that FA levels affect one of the branches that lead to increased *nlp-29* expression (**Fig. 8**).

One open question is why AMP expression should be part of the physiological response to hyperosmolarity, especially since it does not appear to correlate with resistance to osmotic stress.[8] Notably, expression of *gpdh-1*, and by extension glycerol, can be decoupled from osmotic stress resistance,[13] despite the clear role of glycerol as an osmoprotectant, indicating that this process is complex. It will be interesting to see what degree osmotic stress resistance pathways are intertwined with innate immune pathways in other organisms.

Material and Methods

Strains and culture condition. Worms were grown and maintained on nematode growth medium (NGM) and cultured with the *E. coli* strain OP50, as described (Stiernagle, 2006). The integrated transgene *kbIs5* [p*gpdh-1*::GFP, *rol-6*] *I* was a kind gift from Todd Lamitina. *tir-1(tm3036)* and *F32H2.6(tm3581)* were received from S. Mitani and the Japanese National Bioresource Project. The *osm-11(n1604), pod- 2(ye60), dpy-5(e61)* and *pmk-1(km25),* mutants were obtained from the Caenorhabditis Genetics Center (CGC).

EMS mutagenesis and screening. The current screen was conducted in parallel to one previously described.[9] Individual uninfected F2 worms that showed a high constitutive expression of p*nlp-29*::GFP were selected. Those showing severe morphological defects were discarded, and seven strains that showed a strong and penetrant phenotype were retained for further analysis and outcrossed three times.

Mapping and cloning of *fasn-1*. Standard genetic[27] and single nucleotide polymorphism (SNP) mapping procedures were used to map *fasn-1*.[28] The first round of mapping placed *fr8* on LGI between +2.87 and +3.58 cM. Subsequently the double mutant *fr8; dpy-5* was created and utilized for further SNP mapping. This procedure narrowed down the position of *fr8* to a 187 kb region containing 44 genes. To narrow down further the genomic location of *fr8*, different sets of seven overlapping fosmids were microinjected into *fr8* mutants. The concentration of the fosmid for microinjection was generally 30 ng/μl with 70 ng/μl of the coinjection marker pB*unc-53*::GFP.[29] Rescue of *fr8* was obtained with fosmids WRM0612bD08 and WRM062bH01, but not WRM0638dH10, narrowing down the candidates to one annotated gene, *fasn-1*. Sequencing of cDNA from *fasn-1* from the *fr8* strain revealed a G to A transition at a position 723 3' of the initiation codon (flanking sequences with *fasn-1* G/A substitution in bold: …CTT GAG ATT GGG CAT **A**CT CAC AGA TAA GGG A…).

Reporter gene constructs and transgenic lines. The IG274 strain containing the *frIs7* transgene and the p*col-12*::*sek-1* transgene is described elsewhere.[9] The rescuing construct *frEx288* consisted of four overlapping PCR amplicons including a promoter region that span 2.26 kb upstream region of the start ATG and the whole genomic locus of *fasn-1*. Each PCR fragment overlapped its neighbor by at least 900 bp. The primers for the PCR products were: A: JEP1283-JEP1105, B: JEP1104-JEP1143, C: JEP1142-JEP1115, D: JEP1114-JEP1121. The PCR products were microinjected at an estimated concentration of less than 5 ng/μl, together with the co-injection marker pB*unc-53*::GFP at 100 ng/μl. Tissue specific *fasn-1* rescue constructs were obtained

with the same method, concatenating by fusion PCR[30] tissue-specific promoters (from the *col-12*, *mtl-2* and *rab-3* genes) to amplicons derived from the *fasn*-1 coding region, starting with the second exon, which possess an in-frame ATG, and microinjecting these individually with the overlapping PCR products from the genomic region of *fasn-1*. The constructs were all injected at the concentration of 3 ng/µl with the coinjection marker pB*unc-53*::GFP at a concentration of 100 ng/µl. Transcriptional *fasn-1* green fluorescent protein (GFP) reporters were also generated by fusion PCR. The p*fasn-1*::GFP was produced by fusion PCR amplification with the functional promoter region of *fasn-1* fused with GFP, the concentration of the construct was 100 ng/µl. The strain IG938 [*fasn-1(fr8)* I; *frEx333* (F32H2.5, p*nlp-29*::GFP, p*col-12*::dsRed)] which was used for gas chromatography analysis was obtained by microinjection of p*nlp-29*::GFP at 60 ng/µl, p*col-12*::dsRed at 50 ng/µl and the four overlapping PCR amplicons able to rescue *fasn-1* at 3 ng/µl. The sequences of the primers used for all the different constructs are available upon request.

RNAi. All RNAi feeding experiments were performed as described,[31] using clones from the Ahringer library, except for *gck-3*. A *gck-3* fragment was amplified with the primers JEP1544-JEP1545 (sequence of the primers available upon request) with *Pst* I restriction sites. The *Pst* I digested *gck-3* fragment was ligated into L4440-PstI dephosporylated RNAi vector. All RNAi clones were sequence verified before use. In general, when exposure of L1 larvae to one or more RNAi bacterial clones in a given experimental series caused noticeable developmental defects, or larval lethality, the experiment was started with worms cultured on OP50 until the L4 stage.

Infection, wounding and osmotic stress. Infection, and wounding were performed as described.[9] The osmotic stress

resistance test with the different Peni mutants was performed as described.[32]

Biosorter. The quantification of fluorescent reporter gene (GFP or DsRed) expression was performed with the COPAS Biosort (Union Biometrica), essentially as described.[7,9,15] Generally, animals were analyzed for length (time of flight), optical density (extinction), green and red fluorescence.

Gas chromatography analysis. A stage matched population of the strain IG938 was washed off the plates with M9, and non-transgenic and transgenic siblings were separated with the COPAS biosorter on the basis of their red fluorescence. The two separated populations were frozen at -80°C, after aspiration of excess liquid. Lipid extraction was performed as described.[33] GC was performed as described.[17]

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/LeeVIRU1-3-Sup.pdf

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