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Unusual Regulation of a STAT Protein by an SLC6 Family Transporter in *C. elegans* Epidermal Innate Immunity

Katja Dierking,[1,2,3,4] Jolanta Polanowska,[1,2,3] Shizue Omi,[1,2,3] Ilka Engelmann,[1,2,3] Marta Gut,[5] Frederique Lembo,[6] Jonathan J. Ewbank,[1,2,3,*] and Nathalie Pujol[1,2,3]
[1]Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Case 906, 13288 Marseille, France
[2]INSERM, U631, 13288 Marseille, France
[3]CNRS, UMR6102, 13288 Marseille, France
[4]Department of Evolutionary Ecology and Genetics, University of Kiel, 24118 Kiel, Germany
[5]CNG, 91057 Evry, France
[6]CRCM, 13273 Marseille, France
*Correspondence: ewbank@ciml.univ-mrs.fr
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SUMMARY

The cuticle and epidermis of *Caenorhabditis elegans* provide the first line of defense against invading pathogens. Upon invasion by the fungal pathogen *Drechmeria coniospora*, *C. elegans* responds by upregulating the expression of antimicrobial peptides (AMPs) in the epidermis via activation of at least two pathways, a neuroendocrine TGF-β pathway and a p38 MAPK pathway. Here, we identify the sodium-neurotransmitter symporter SNF-12, a member of the solute carrier family (SLC6), as being essential for both these immune signaling pathways. We also identify the STAT transcription factor-like protein STA-2 as a direct physical interactor of SNF-12 and show that the two proteins function together to regulate AMP gene expression in the epidermis. Both SNF-12 and STA-2 act cell autonomously and specifically in the epidermis to govern the transcriptional response to fungal infection. These findings reveal an unorthodox mode of regulation for a STAT factor and highlight the molecular plasticity of innate immune signaling.

INTRODUCTION

Innate immunity is evolutionarily ancient and employs mechanisms that are highly conserved throughout the animal kingdom (Irazoqui et al., 2010; Lemaitre and Hoffmann, 2007). *Caenorhabditis elegans* has emerged as a powerful invertebrate model to study the origin and function of the innate immune system (Kawli and Tan, 2008; Kim et al., 2002; Means et al., 2009). Among other defense mechanisms, *C. elegans* responds to infection by activating the expression of proteins and peptides that can directly act against a pathogen. Several *C. elegans* immune pathways regulating the expression of these effector molecules have been identified (reviewed in Irazoqui et al., 2010; Partridge et al., 2010), including a Toll/IL-1 receptor (TIR) domain adaptor protein (TIR-1) that functions upstream of a highly conserved p38 mitogen-activated protein kinase (MAPK) pathway (Kim et al., 2002; Liberati et al., 2004). The activation of these immune signaling cascades depends on the pathogen and its mode of infection (Ren et al., 2009; Shivers et al., 2008; Wong et al., 2007; Ziegler et al., 2009). While most bacterial pathogens colonize the intestine of *C. elegans*, and affect principally the expression of defense genes in the gut, a few known pathogenic bacteria and fungi infect *C. elegans* by adhering to its cuticle. These include the nematode-specific fungus *Drechmeria coniospora*, a natural pathogen of *C. elegans*.

The cuticle and epidermis of *C. elegans* represent a physical barrier and provide a first line of defense against invading pathogens (Ewbank, 2002). The infectious conidia of *D. coniospora* attach to the nematode cuticle, germinate, and puncture the cuticle, and then hyphae invade the underlying epidermis to colonize the worm, eventually killing it (Dijksterhuis et al., 1990). *C. elegans* responds to this infection by activating the expression of several families of genes encoding antimicrobial peptides (AMPs) in the epidermis (Couillault et al., 2004; Pujol et al., 2008b). Among them, a cluster of *nlp* genes and a cluster of *cnc* genes have been demonstrated to enhance resistance to *D. coniospora* in vivo (Pujol et al., 2008b; Zugasti and Ewbank, 2009). We have focused on one representative of the *nlp* gene cluster, *nlp-29*, whose expression is robustly induced in the *C. elegans* epidermis following infection, but also upon physical injury and under conditions of osmotic stress (Pujol et al., 2008a, 2008b). Furthermore, a defect in the barrier integrity of the cuticle or epidermis can lead to the upregulation of *nlp-29* expression (Pujol et al., 2008a; Tong et al., 2009).

The generation of a strain carrying a reporter transgene with the gene encoding green fluorescent protein under the control of the *nlp-29* promoter (p*nlp-29*::GFP) has allowed us to begin to identify the molecular signaling mechanisms that underlie the regulation of *nlp-29* expression. We found that the TIR-1/p38 MAPK cascade regulates the expression of *nlp-29* in the epidermis after both fungal infection and wounding, but not osmotic stress (Couillault et al., 2004; Pujol et al., 2008a, 2008b). The activity of the pathway is normally held in check by the nematode ortholog of death-associated protein kinase (*dapk-1*) (Tong et al., 2009). In a direct genetic screen, we

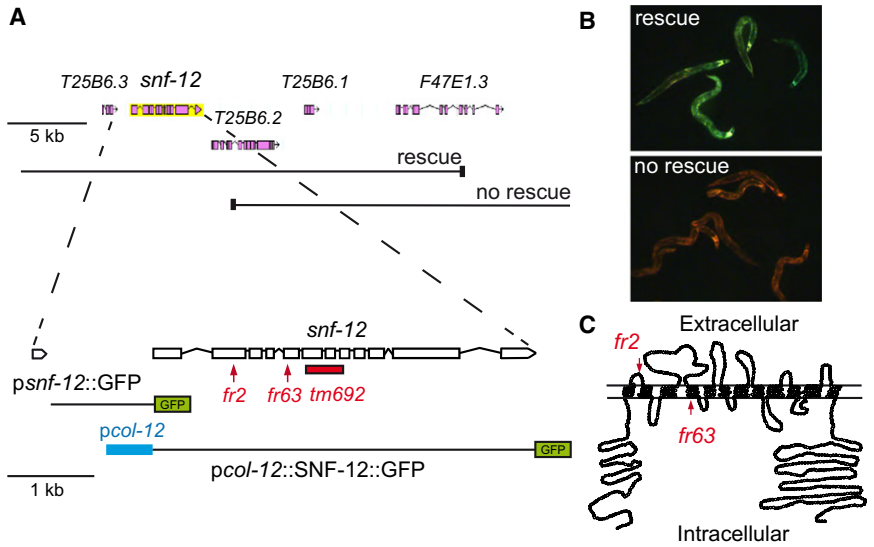

Figure 1. *nipi-2* Corresponds to *snf-12*

(A) Physical map adapted from the WormBase genome browser (WS200), showing the position of *snf-12* relative to nearby genes. Among the fosmids in this region, WRM0610aE08 rescued, whereas WRM068bG12 did not (see B). Exon-intron structure of *snf-12*, adapted from WormBase (WS200), with the positions of the *fr2* and *fr63* mutations and the *tm692* deletion indicated. Also shown is the structure of the *snf-12* promoter fusion and the p*col-12*::SNF-12::GFP fusion protein constructs.

(B) Worms carrying a gain-of-function mutation in the cuticle collagen gene *rol-6*(*su1006*) constitutively express *nlp-29*; this is completely abolished by the *snf-12*(*fr2*) mutation. Injection of the fosmid WRM0610aE08 together with *rol-6*(*su1006*) rescues the constitutive expression of *nlp-29* in *fr2* worms carrying an integrated p*nlp-29*::GFP reporter, whereas the fosmid WRM068bG12 shows no effect.

(C) Predicted topology of the SNF-12 protein, with its unusually long N- and C-terminal domains. The arrows indicate the glycine at position 193 affected in the *fr2* mutant and the premature stop codon in the *fr63* mutant.

isolated mutants that are defective for the upregulation of *nlp-29* following *D. coniospora* infection (Pujol et al., 2008a). Two of these *nipi* (no induction of peptide after *D. coniospora* infection) mutants have been identified: *nipi-3* encodes a kinase related to the human Tribbles homolog 1 and is specifically required for the induction of *nlp-29* expression after infection, but not wounding. It acts upstream of the TIR-1/p38 MAPK cascade. The identification of another *nipi* mutant as *tpa-1*, encoding protein kinase C δ (PKCδ), led to the delineation of a G protein/phospholipase C/PKCδ signaling cascade required for the upregulation of *nlp-29* following both infection and injury. This pathway is independent of *nipi-3* but also acts upstream of the TIR-1/p38 MAPK pathway. Together, this indicates that distinct signaling pathways converge onto the TIR-1/p38 MAPK cascade to govern part of the response of *C. elegans* to infection and wounding (Pujol et al., 2008a; Ziegler et al., 2009). The expression of the caenacin gene *cnc-2*, on the other hand, is p38 MAPK independent, is only induced by infection, and requires a noncanonical TGF-β signaling cascade (Zugasti and Ewbank, 2009). Currently, there are clear lacunae in our understanding of the pathways involved in regulating the innate immune response in the epidermis. Neither the signals involved in triggering the response nor the infection-specific transcription factors have been identified. It is interesting to note that *C. elegans* does not possess an obvious homolog of the NFκB transcription factor (Pujol et al., 2001), which is central to immune gene regulation in most animal species (Lemaitre and Hoffmann, 2007).

Here, we report the cloning and functional characterization of the *nipi-2* mutant that is defective in the induction of *nlp-29* expression following wounding and infection (Pujol et al., 2008a). We found that NIPI-2 corresponds to SNF-12, a member of the sodium-dependent neurotransmitter symporter family (SNF). We show that *snf-12* is expressed in the epidermis, where it functions cell autonomously to control *nlp-29* expression upon fungal infection. Epistasis analysis revealed that *snf-12* is also required for the transduction of signals associated with defects in the integrity of the epidermal barrier and that it interacts with the G protein/PKCδ/p38 MAPK cascade. Additionally, it is required for the expression of *cnc-2*. We also identified the STAT transcription factor-like protein STA-2 as a direct physical interactor of SNF-12, and showed that the two proteins function together to regulate the expression of both *nlp* and *cnc* AMP genes in the *C. elegans* epidermis.

RESULTS

SNF-12, a Member of the Sodium-Dependent Neurotransmitter Symporter Family, Regulates AMP Expression

From the pilot-scale genetic screen that allowed the isolation of the *tpa-1(fr1, fr3)* and *nipi-3(fr4)* mutants (Pujol et al., 2008a; Ziegler et al., 2009), we isolated two other mutant alleles, *fr2* and *fr5*, that strongly suppressed the induction of p*nlp-29*::GFP following infection and wounding. Characterization of *fr5* led to the identification of a new allele of the p38 MAPK gene *pmk-1*, a known component of the pathway regulating *nlp-29* expression (Pujol et al., 2008a). In *pmk-1*(*fr5*) a G-to-A transition abolishes the splice donor site at the end of the third exon. On the other hand, *nipi-2(fr2)* was not allelic to any previously characterized innate immunity gene. Hermaphrodite *nipi-2(fr2)* mutants did not exhibit any obvious phenotypes with regards their development, growth, fertility, movement, or morphology (data not shown).

We cloned *nipi-2* and found that it corresponds to the previously annotated gene *snf-12* (Figures 1A and 1B). It encodes a member of the highly conserved sodium-dependent neurotransmitter symporter family (see Figure S1A available online). The SNF family in mammals is also referred to as the sodium- and chloride-dependent neurotransmitter transporter family, or

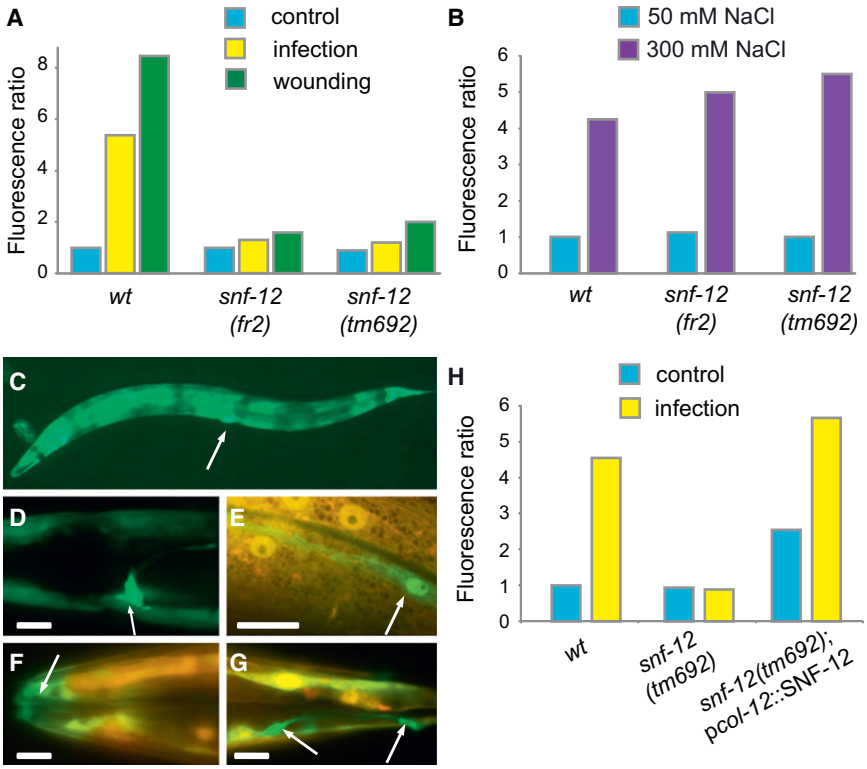

Figure 2. *snf-12* Is Required for the Response to Infection in the Epidermis

(A) Biosort quantification of the normalized fluorescence ratio in *wt*, *snf-12(fr2)*, and *snf-12(tm692)* worms carrying an integrated p*nlp-29*::GFP reporter following infection and wounding. For this and subsequent figures, see the Supplemental Experimental Procedures for details of the data processing and the number of worms analyzed.

(B) *snf-12* mutants do not block the induction of *nlp-29* expression upon osmotic stress. Biosort quantification of the normalized fluorescence ratio in *wt*, *snf-12(fr2)*, and *snf-12(tm692)* worms carrying an integrated p*nlp-29*::GFP reporter following osmotic stress.

(C–G) Fluorescence images of adult worms carrying a p*snf-12*::GFP transgene and a p*col-12*::DsRed2 reporter visualized with a green filter (C and D) or a filter allowing red and green fluorescence to be seen simultaneously (E–G). Expression of *snf-12* is seen throughout the epidermis (C), in vulval cells (arrow in C), in the excretory cell (arrow in D), in the seam cells (arrow in E), and in the amphid and phasmid socket cells (arrow in F and G, respectively). Scale bar, 10 μm.

(H) Biosort quantification of the normalized fluorescence ratio in *wt* and *snf-12(tm692)* worms carrying an integrated p*nlp-29*::GFP reporter and additionally a transgene that specifically drives the expression of SNF-12 in the epidermis (p*col-12*::SNF-12).

solute carrier 6 family (SLC6; Human Genome Organization Nomenclature system). Like other members of the SLC6 family, SNF-12 is predicted to have 12 transmembrane domains and cytoplasmic C and N termini (Figure 1C).

The *fr2* allele corresponds to a single base mutation changing a glycine to an aspartic acid codon. This glycine at position 193 in the first predicted extracellular loop (between transmembrane domain 1 and 2, Figures 1A and 1C) is adjacent to a conserved residue that is considered to be one of the hallmarks of SLC6 transporters (Figure S1B) (Nelson, 1998). A second allele of *snf-12*, *fr63*, was obtained in an independent genetic screen (N.P., unpublished data) and is predicted to introduce a premature stop codon into the fourth transmembrane domain of the protein (Figures 1A and 1B). Another allele of *snf-12*, *tm692*, obtained from the Japanese Gene Knockout Consortium, corresponds to a 436 bp deletion and is likely to be null (Figure 1A). We found that the *snf-12(fr63)* and *snf-12(tm692)* mutants exhibited the same phenotypes as the *fr2* allele, i.e., the induction of p*nlp-29*::GFP expression is almost completely abolished upon infection and injury (Figure 2A and data not shown), so that *fr2* and *fr63* are likely to be null or near null alleles.

When *snf-12* mutant worms were subjected to an osmotic stress, we found that they exhibited a normal induction of p*nlp-29*::GFP (Figure 2B). This demonstrates that *snf-12(fr2)* mutants are not generally deficient in their capacity to upregulate p*nlp-29*::GFP. It also reinforces the notion that the signaling pathways that control *nlp-29* expression in response to infection/wounding and osmotic stress are genetically distinct (Lee et al., 2010; Pujol et al., 2008b). The expression of the enzyme glycerol 3-phosphate dehydrogenase GPDH-1 is also increased under high-salt conditions, but not by infection or wounding, and so provides a marker of a different aspect of the osmotic stress response. Transgenic worms carrying a p*gpdh-1*::GFP construct show an enhanced fluorescence upon exposure to increasing concentrations of salt (Lamitina et al., 2006). We found that loss of *snf-12* function did not alter the induction by high salt of a p*gpdh-1*::GFP reporter in either the epidermis or the intestine (Figures S2A–S2D). Together, these results suggest that all three *snf-12* alleles are null or near null and confirm the specific role of SNF-12 in the regulation of *nlp-29* expression following physical injury and during the antifungal response.

SNF-12 Functions Cell Autonomously in the Epidermis of *C. elegans*

To identify the cells in which *snf-12* is expressed, we generated transgenic animals expressing a GFP transcriptional reporter construct (Figure 1A). We observed strong expression in the epidermis of *C. elegans* throughout development. *snf-12* was also expressed in the seam cells, which are specialized epithelial cells that secrete the cuticle, as well as in the excretory cell, and the amphid and phasmid socket cells (Figures 2C–2G).

Since *nlp-29* is expressed specifically in the epidermis following *D. coniospora* infection, we investigated whether *snf-12* functions cell autonomously in the epidermis to regulate *nlp-29* expression. We therefore expressed *snf-12* under the control of the strong epidermis-specific *col-12* promoter (p*col-12*::SNF-12). Overexpression of *snf-12* in the epidermis resulted in an increased constitutive expression of p*nlp-29*::GFP, and rescued reporter gene induction in the *snf-12(tm692)* mutant upon infection (Figure 2H) and wounding (data not shown). Thus

snf-12 acts cell autonomously in the epidermis to control *nlp-29* expression.

SNF-12 Is an Atypical SLC6 Protein with a Specific Role in Regulating AMP Gene Expression

In mammals, based on amino acid sequence conservation, the sodium-dependent neurotransmitter transporters can be divided into four subfamilies (Figure S1B). Pairwise BLAST alignments suggested that SNF-12 was not more closely related to one SLC6 subfamily than another. When we generated a cladogram using the sequences of selected SLC6 members from mammals and insects, using only proteins for which the substrate has been established experimentally, together with all 13 *C. elegans* SLC6 proteins, we found that while the known *C. elegans* GABA, monoamine, and amino acid transporters fell into the expected positions, SNF-12 lies outside the clusters representing the classical SLC6 subfamilies (Figure S1B). If SNF-12 acts as a transporter, it is difficult to predict what its substrate might be from the very extensive list of candidate molecules.

To investigate if the role in the immune response of *C. elegans* to *D. coniospora* is specific to SNF-12 or applies more generally to members of the SLC6 family, we tested the *mod-5(n3314)* and the *snf-1(ok790)* mutants. They both showed a strong induction of *nlp-29* expression after infection and wounding (Figure S2E), indicating that regulation of *nlp-29* expression is not a general function of *C. elegans* SLC6 proteins.

Neurotransmitters Are Dispensable for the Induction of *nlp-29* Expression

The sequence similarity of SNF-12 to other *C. elegans* SLC6 proteins with neurotransmitter substrates, such as MOD-5 (serotonin) and DAT-1 (dopamine), and recent studies showing a role for the neuroendocrine axis in *C. elegans* antibacterial defenses (Kawli and Tan, 2008) led us to investigate if any neurotransmitter might be required for the infection-induced expression of *nlp-29*. While noradrenaline, adrenaline, and histamine appear not to be synthesized in *C. elegans* (Chase and Koelle, 2007), the genes encoding the enzymes responsible for the synthesis of the neurotransmitters acetylcholine, serotonin, GABA, dopamine, tyramine, and octopamine have all been identified. Viable *C. elegans* mutants deficient for each of these enzymes are available. We investigated the effect of the inactivation of these genes on the expression of *nlp-29*. We found that the constitutive expression of *nlp-29* and its induction after infection, wounding, and osmotic stress were unaltered in all these mutants (Table S1). This indicates that no single neurotransmitter plays an essential role in the induction of *nlp-29* expression.

SNF-12 Physically Interacts with a STAT-like Transcription Factor

SNF-12 has an atypical structure for a SLC6 protein, currently shared only with clear orthologs (CBR-SNF-12 and CRE-SNF-12) in the closely related nematode species *C. briggsae* and *C. remanei*. It has an N-terminal extension of 60–90 amino acids, compared to other nematode SNF family proteins, and an unusually long C terminus (Figure 1C). This latter region of 300 amino acids does not resemble any other known protein sequence and contains no obvious structural motifs. When we attempted to rescue the *snf-12(tm692)* mutant with a truncated version of the SNF-12 protein lacking the C-terminal domain, we obtained no rescue (data not shown). This suggests that the C terminus has a functionally important role. We therefore searched for direct physical interactors of the C-terminal part of SNF-12 using a yeast two-hybrid (Y2H) screen. We identified five potential interactors, the most highly represented being the protein F58E6.1 (Table S2). This protein is a member of the STAT family of transcription factors and is most similar to the vertebrate STAT-5B (Figure S3). There is one other STAT family transcription factor in *C. elegans*, STA-1, so we named F58E6.1 STA-2. We then performed coimmunoprecipitation (coIP) experiments to provide an independent corroboration of the Y2H result. When we coexpressed tagged versions of the C terminus of SNF-12 and STA-2 in 293T cells, we were able to coimmunoprecipitate the two proteins as a complex (Figure 3). These results support the idea that the two proteins can directly interact.

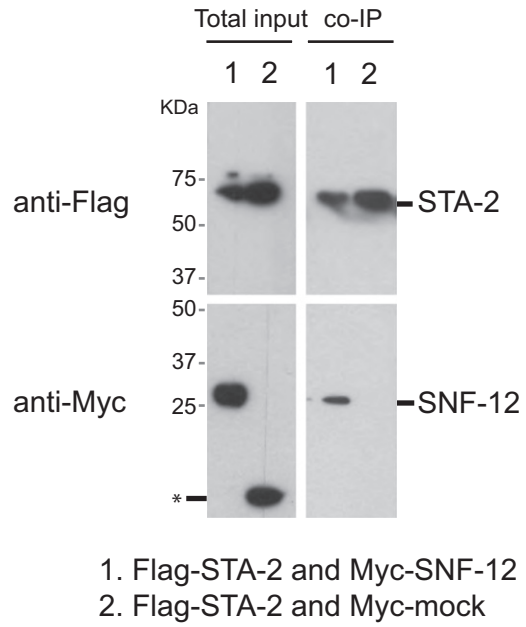

Figure 3. Coimmunoprecipitation of the SNF-12 C Terminus with STA-2

Flag-tagged STA-2 and Myc-tagged SNF-12 or a Myc-tagged unrelated peptide (mock) were coexpressed in 293T cells. Flag-STA-2 was immunoprecipitated by using anti-Flag-Sepharose beads. Western blot analysis was performed on cell lysate (total input) and coimmunoprecipitates (coIP) using anti-Flag (upper panel) and anti-Myc (lower panel) antibodies to detect SNF-12 and STA-2 or the mock peptide (labeled as asterisk), respectively.

STA-2 Plays a Specific Regulatory Role in the Epidermis

To investigate the possible role of STA-2 in the regulation of defense genes, we first tested the effect on the induction of p*nlp-29*::GFP of knocking down the expression of the corresponding gene by RNAi and in an available deletion mutant *sta-2(ok1860)*. We found that abrogation of *sta-2* function in both cases blocked the response to wounding and infection (Figure 4A, and data not shown). Loss of *sta-2* function did not affect the induction by high salt of p*nlp-29*::GFP (Figure 4B) or of

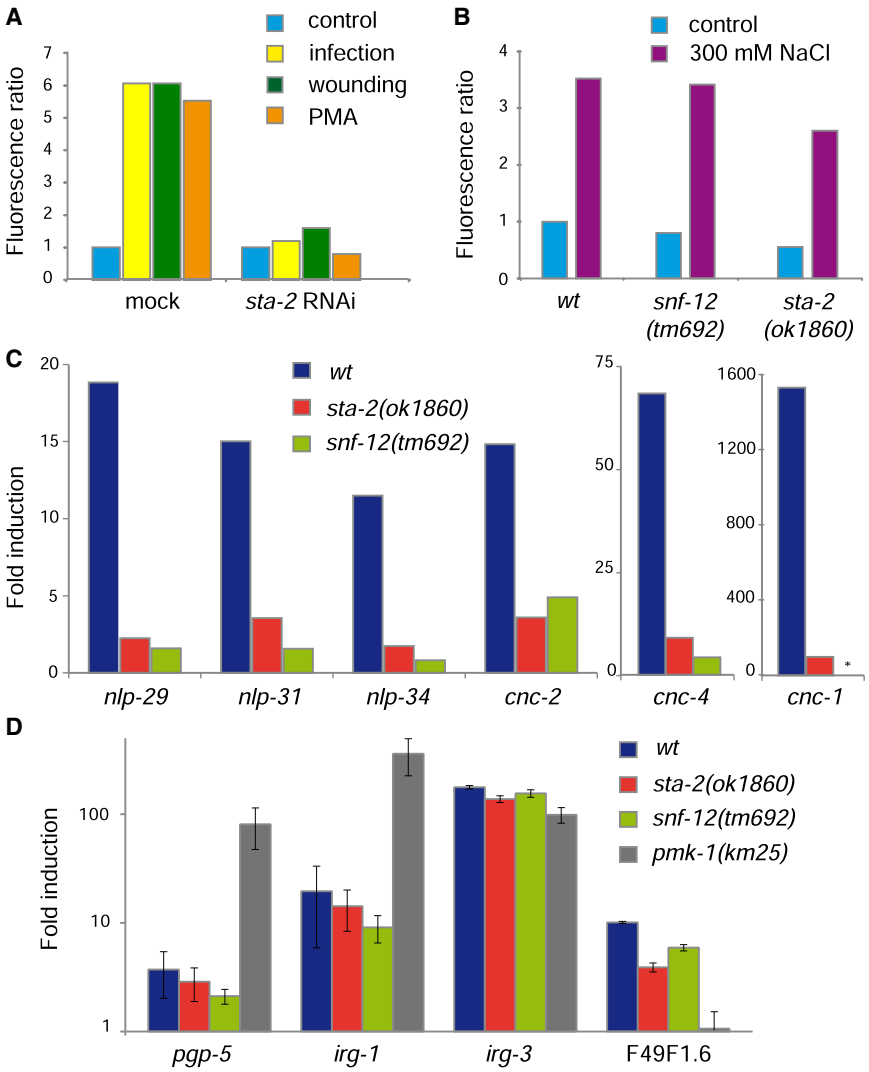

Figure 4. *sta-2* and *snf-12* Specifically Control AMP Gene Induction in the Epidermis upon Infection and Injury

(A and B) Biosort quantification of the normalized fluorescence ratio in worms of different genetic backgrounds carrying an integrated p*nlp-29*::GFP reporter. (A) *sta-2* RNAi-treated animals following *D. coniospora* infection, wounding, and exposure to PMA. Mock corresponds to worms fed on bacteria containing the control RNAi plasmid. (B) *wt*, *snf-12(tm692)*, and *sta-2(ok1860)* worms upon exposure to high salt. (C and D) Quantitative RT-PCR analysis of gene expression levels showing the infection-associated "fold induction" (infected/noninfected values).

(C) *D. coniospora* induced expression of representative *nlp* and *cnc* genes in *wt*, *snf-12(tm692)*, and *sta-2(ok1860)* worms 6 hr postinfection. The results are representative of three independent experiments.

(D) *P. aeruginosa* PA14 induced expression (average and SEM from three independent experiments) of *pgp-5*, *irg-1*, *irg-3*, and F49F1.6 in *wt*, *sta-2(ok1860)*, *snf-12(tm692)*, and *pmk-1(km25)* worms 4 hr postinfection. As previously observed, loss of *pmk-1* function increases the expression of *irg-1* (Estes et al., 2010); the same applies for *pgp-5*.

a p*gpdh-1*::GFP reporter in either the epidermis or the intestine (Figures S4A–S4D). In these respects, *sta-2* function mirrors that of *snf-12*.

To confirm the role of *sta-2* and *snf-12* in controlling the response upon fungal infection, we analyzed the expression of other genes that have been shown to be induced upon *D. coniospora* infection (Pujol et al., 2008b). We could confirm by qRT-PCR that in *sta-2* and *snf-12* mutants, the induction of the endogenous *nlp-29* gene was essentially abrogated, as it was for other genes of the same locus such as *nlp-31* and *nlp-34* (Figure 4C). A second group of AMPs, including *cnc-2*, is regulated in a distinct manner from *nlp-29* as they require signaling via a noncanonical TGF-β/DBL-1 pathway and are independent of the p38 MAPK/PMK-1 pathway (Zugasti and Ewbank, 2009). We found by qRT-PCR that loss of function of either *snf-12* or *sta-2* affected the expression of *cnc-1*, *cnc-2*, and *cnc-4*, both their constitutive level and that after infection (Figure 4C and Figure S4E). Thus *snf-12* and *sta-2* play a role in innate immune signaling and influence targets of both the p38 MAPK/PMK-1 and TGF-β/DBL-1 pathways.

To extend this investigation, as *sta-2* is expressed in the intestine (see below), we looked at the expression of a number of genes that are induced in the intestine of *C. elegans* upon infection with the bacterial pathogen *Pseudomonas aeruginosa*. We chose to measure the levels of targets reported to be both p38 dependent and independent (Estes et al., 2010; Kurz et al., 2007). In no case could we find a positive regulatory role for either *snf-12* or *sta-2* (Figure 4D). Further, when we knocked down by RNAi the expression of the only other STAT family transcription factor, *sta-1*, we observed no effect on either *nlp-29* or *cnc-2* reporter gene expression (data not shown). These results suggest that *snf-12* and *sta-2* have a specific function in the regulation of antifungal defenses in the epidermis.

STA-2 Functions Cell Autonomously in the Epidermis of *C. elegans*

In transgenic worms expressing a *sta-2* reporter gene, we observed strong expression in the epidermis of *C. elegans* throughout development. There was also an expression in the seam cells, the intestine, the phasmid socket cells, and the excretory duct cell, as well as cells in the vulva and the dorsal rectal cells (Figures 5A–5C, data not shown). When we expressed *sta-2* under the control of the epidermis-specific *col-12* promoter in the *sta-2(ok1860)* mutant, we restored the induction of *nlp-29* expression upon exposure to *D. coniospora* (Figures 5D and 5E). Thus, just like *snf-12*, *sta-2* acts cell autonomously in the epidermis to control *nlp-29* expression.

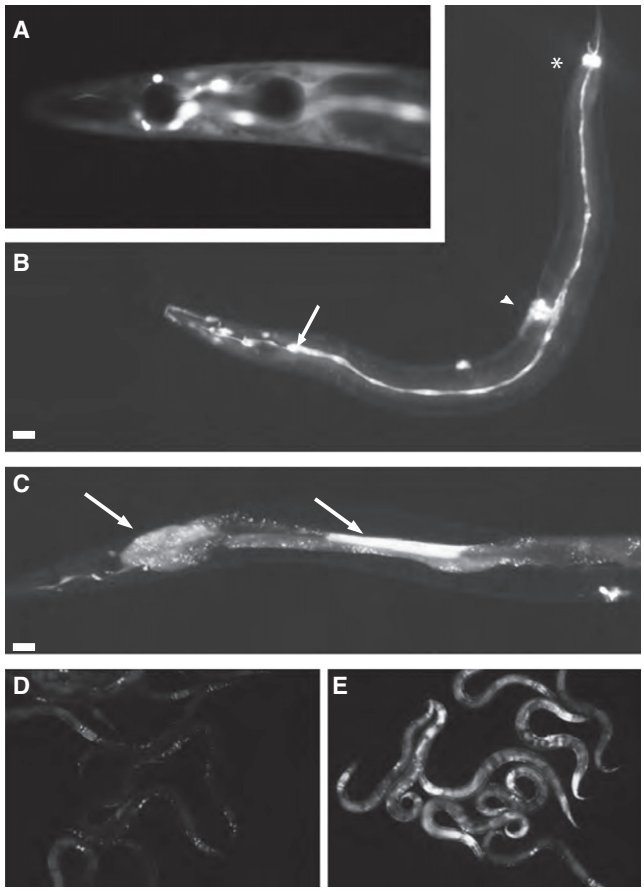

Figure 5. The *sta-2* Gene Is Expressed in the Epidermis, Where It Functions Cell Autonomously

(A–C) Fluorescence images of adult worms carrying a p*sta-2*::GFP transgene. Expression of *sta-2* is seen (A) throughout the epidermis, including in the head region shown here; (B) in the seam cells (arrow), vulval cells (arrowhead), and rectal cells (asterisk); as well as (C) in the intestine (arrows). Scale bar, 10 μm. (D and E) Fluorescence images of *sta-2* adult worms carrying an integrated p*nlp-29*::GFP reporter and a transgene that specifically drives the expression of STA-2 in the epidermis (p*col-12*::DsRed2::STA-2) with the coinjection marker p*unc-53*::GFP (Stringham et al., 2002). (D) Noninfected control, (E) after *D. coniospora* infection.

snf-12 and *sta-2* Genetically Act Downstream of a G Protein/PKCδ Pathway

Upregulation of *nlp-29* expression upon infection or physical injury in the *C. elegans* epidermis requires a signaling cascade involving the Gα protein GPA-12 acting upstream of the PKCδ TPA-1, which in turn lies upstream of a conserved p38 MAPK pathway (Figure 6A). The death-associated protein kinase DAPK-1 and the cuticle collagen family member DPY-9 both negatively regulate this p38 MAPK pathway. Thus in both *dapk-1* and *dpy-9* loss-of-function mutants, as well as in worms with a gain-of-function mutation in *gpa-12*, there is a constitutive activation of *nlp-29* which is dependent on the p38 MAPK *pmk-1* (Pujol et al., 2008b; Tong et al., 2009; Ziegler et al., 2009). The pathway is also activated in worms by exposure to the diacylglycerol mimetic phorbol 12-myristate 13-acetate (PMA) which stimulates TPA-1 (Ziegler et al., 2009). We found that *snf-12* and *sta-2* mutant blocked the increased *nlp-29* expression associated with the expression of activated GPA-12, loss of function of *dapk-1* or *dpy-9*, and exposure to PMA (Figures 4A and 6B–6D and data not shown), indicating that *snf-12* and *sta-2* function downstream of the PKCδ *tpa-1* in the regulation of *nlp-29* expression.

snf-12 and *sta-2* Act Partially Downstream of p38 MAPK

Overexpression of the MAP2K gene *sek-1* in the epidermis of *C. elegans*, with a p*col-12*::SEK-1 transgene, leads to an increased constitutive and infection-induced expression of *nlp-29* (Pujol et al., 2008a). This constitutive expression was reduced when the p*col-12*::SEK-1 transgene was transferred into a *snf-12* or a *sta-2* mutant background (Figure 6E). Nevertheless, it was still elevated when compared to *pmk-1* mutant worms carrying the p*col-12*::SEK-1 transgene, when the constitutive expression of *nlp-29* is essentially abolished (Pujol et al., 2008a). These results suggest that *snf-12* and *sta-2* act partially downstream or parallel to MAP2K *sek-1* to regulate the expression of *nlp-29*.

Biochemical studies have shown that even under normal culture conditions, p38/PMK-1 is phosphorylated by the MAP2K SEK-1. In a *sek-1* mutant, no phosphorylated PMK-1 can be detected (Kim et al., 2002). When we expressed *sek-1* specifically in the epidermis in a *sek-1* mutant, using the p*col-12*::SEK-1 transgene, we detected constitutive phosphorylation of PMK-1 (Figure 6F). Taken together with previous genetic evidence (Pujol et al., 2008a), this suggests that PMK-1 is a direct target of SEK-1 phosphorylation in the epidermis, as it is proposed to be in the intestine. In a *snf-12* or a *sta-2* mutant, in contrast to the *sek-1* mutant, there was no change in the constitutive phosphorylation of PMK-1. Together these results show that *snf-12* and *sta-2* do not act upstream of the p38 MAPK PMK-1. Moreover, biochemical assays in a heterologous system indicated that STA-2 is a potential substrate of activated PMK-1. While STA-2 expressed alone in 293T cells is constitutively phosphorylated by endogenous kinases, without the requirement for *C. elegans* PMK-1 and SEK-1, overexpression of PMK-1 together with its activator SEK-1 in these cells led to accumulation of an additional phosphorylated form of STA-2 (Figure 6G), suggesting that STA-2 can be modified by p38 MAPK PMK-1.

SNF-12 and STA-2 Are in Endocytic Vesicles

SLC6 family transporters traffic to and from the plasma membrane (Melikian, 2004). To determine the subcellular localization of SNF-12, we first generated transgenic worms expressing a C-terminal SNF-12::GFP fusion reporter, under the control of the *snf-12* promoter. As these animals had a fluorescent signal that was not strong enough to be analyzed, we then generated transgenic worms carrying the same fusion reporter construct but driven by a strong epidermis-specific promoter (p*col-12*). While under these conditions protein expression levels are higher than those of the endogenous protein, the chimeric protein p*col-12*::SNF-12::GFP was functional and was able to restore the expression and inducibility of p*nlp-29*::GFP in a *snf-12* mutant (Figure S5A). This strongly suggests that the localization seen with this reporter protein reflects that of the endogenous protein. Some SNF-12::GFP could be seen in

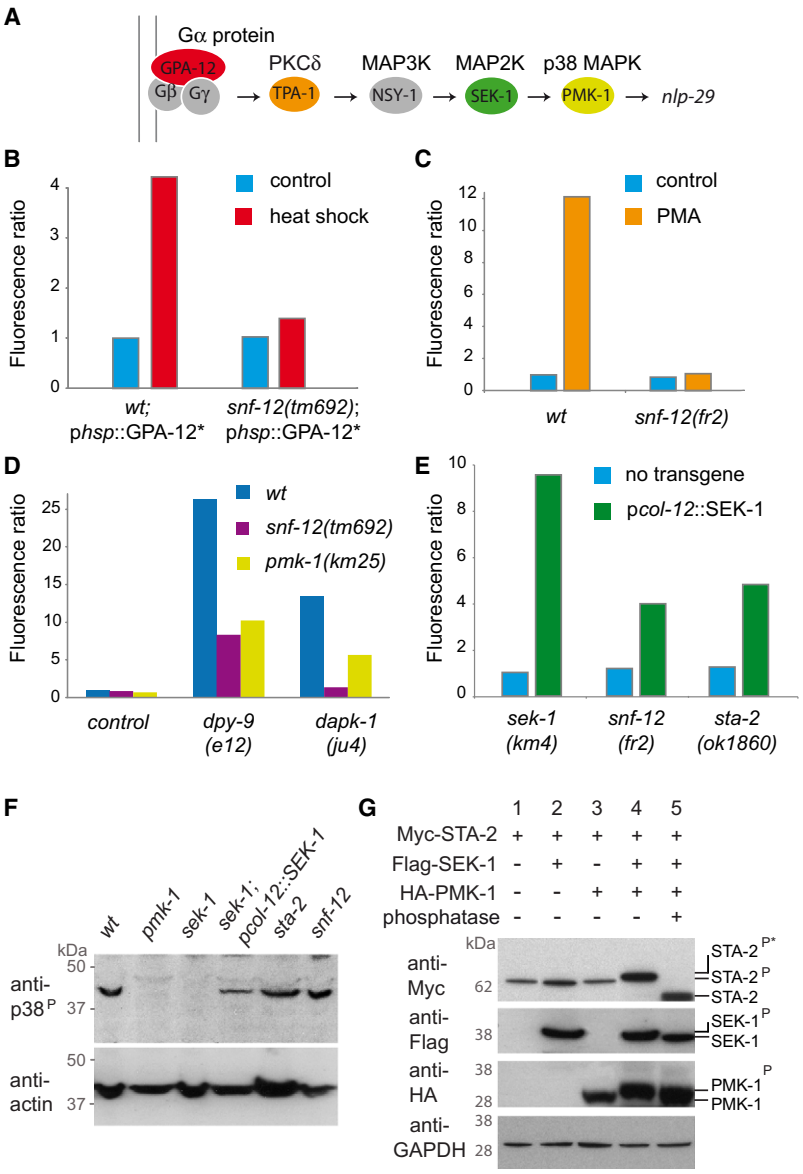

Figure 6. *snf-12* and *sta-2* Genetically Interact with the G Protein/PKCδ/p38 MAPK Cascade

(A) The G protein/PKCδ/p38 MAPK cascade regulates the expression of *nlp-29* after infection and wounding. Epistasis analyses were done between the *snf-12* mutant and the elements of the cascade shown in color. (B–E) Biosort quantification of the normalized fluorescence ratio in worms of different genetic backgrounds carrying an integrated p*nlp-29*::GFP reporter.

(B) *wt* and *snf-12(tm692)* mutant worms carrying an activated form of GPA-12 under the control of a heat-shock promoter (p*hsp*::GPA-12*).

(C) *wt* and *snf-12(fr2)* worms upon exposure to PMA.

(D) *wt*, *snf-12(tm692)*, *pmk-1(km25)*, *dpy-9(e12)*, *dpy-9(e12);snf-12(tm692)*, *dpy-9(e12)pmk-1(km25)*, *dapk-1(ju4)*, *dapk-1(ju4);snf-12(tm692)*, and *dapk-1(ju4);pmk-1(km25)*.

(E) *sek-1(km4)*, *snf-12(fr2)*, and *sta-2(ok1860)* mutant worms overexpressing *sek-1* in the epidermis (p*col-12*::SEK-1).

(F) Activated PMK-1 is present in *sta-2* and *snf-12* mutants. Immunoblot analysis of worm lysates from *wt*, *pmk-1(km25)*, *sek-1(km4)*, *sek-1(km4)*;Ex[p*col-12*::SEK-1], *sta-2(ok1860)*, and *snf-12(tm692)* worms. Upper panel, anti-phospho p38; lower panel, anti-actin.

(G) STA-2 is phosphorylated by activated PMK-1 in a heterologous system. Whole-cell extracts from 293T cells expressing Myc-STA-2 with HA-PMK-1 and/or Flag-SEK-1 were immunoblotted with antibodies directed against Myc (top), Flag (middle top), HA (middle bottom), and GAPDH, with (sample 5) or without (samples 1–4) phosphatase treatment. The phosphorylated forms of each protein (as judged by mobility shift) are indicated by "^{P}." For STA-2, three differentially migrating forms are observed, and two different phosphorylation states are indicated by STA-2^{P} and STA-2^{P*}.

the cytoplasm, but more was in a banded pattern, underneath the cuticle, typical for apical membrane proteins. It was also found in vesicles at the same level as DLG-1::RFP, a marker of apical junctions (McMahon et al., 2001) (Figure 7A and Figures S5B–S7D). These SNF-12::GFP-expressing vesicles were distinct from exocytotic vesicles that are characterized by the presence of markers such as the V-ATPase subunit VHA-5 (Liegeois et al., 2006) (Figures 7C–7E). We conclude that SNF-12 is predominantly at the apical surface in epidermal cells, in endosome-like vesicles.

In worms expressing a GFP::STA-2 fusion reporter in the epidermis (p*col-12*::GFP::STA-2), a fluorescent signal was clearly seen in the nucleus but also in bands underneath the cuticle and in some vesicles (Figure 7B and Figures S5E–S5G). Equivalent patterns were also obtained when STA-2 was tagged with DsRed2 or mCherry (Figure 7G and data not shown) and when the fusion protein was expressed under the control of the *sta-2* promoter (p*sta-2*::GFP::STA-2) (Figure S5K). As the different chimeric proteins were functional (Figures 5D and 5E and data not shown), the localization seen with these reporter proteins is likely to reflect the distribution of the endogenous protein. Using the mCherry::STA-2 construct, we found that the fluorescent signal colocalized with a clathrin heavy-chain CHC-1::GFP reporter protein (Figures 7F–7H), a specific marker of newly formed endocytic vesicles (Greener et al., 2001; Sato et al., 2009). Comparing the patterns seen for STA-2 and SNF-12 reporter proteins, there was an overlap at the apical membrane and most obviously, in strains expressing both DsRed2::STA-2 and SNF-12::GFP in the epidermis, in endocytic vesicles (Figures S5H–S5J).

To address the potential functional role of endosomes in the signaling pathways controlling *nlp-29* expression, we used RNAi to knock down the expression of dynamin (*dyn-1*) that is involved in the scission of newly formed clathrin-coated endocytic vesicle from the cell membrane and the small GTPase Rab5 (*rab-5*) that characterizes early endosomes derived from dynamin-dependent and independent endocytosis, using conditions in which RNAi did not provoke any lethality. While RNAi of *rab-5* reduced the level of induction of *nlp-29* provoked by both

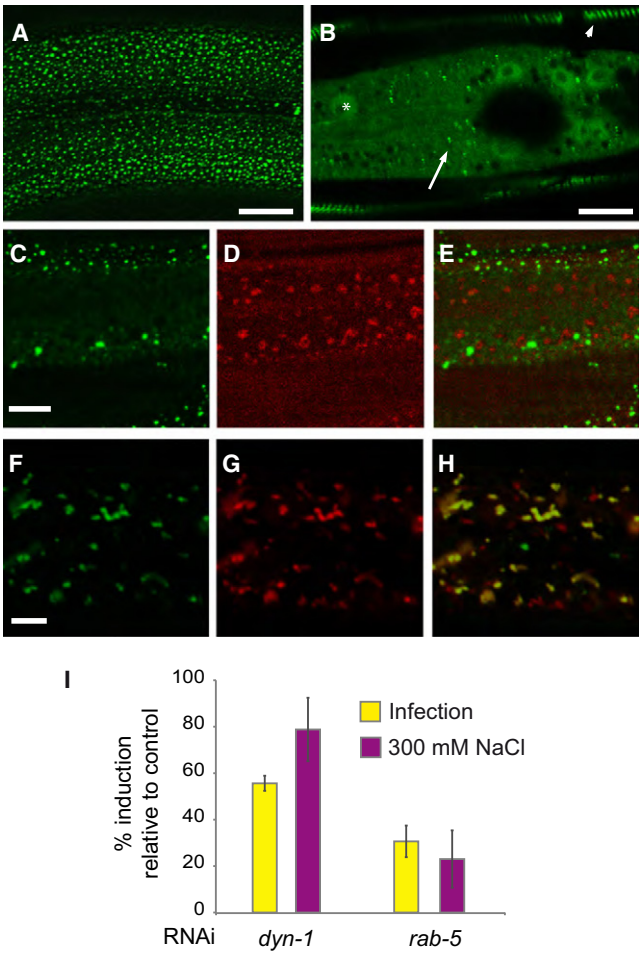

Figure 7. SNF-12 and STA-2 Are Found in Endosome-like Vesicles

(A–H) Confocal micrographs showing the distribution of different chimeric reporter proteins expressed in the epidermis.

(A) The expression of p*col-12*::SNF-12::GFP appears predominantly as dots at the apical side.

(B) p*col-12*::GFP::STA-2 is found in nuclei (asterisk), in vesicles (arrow), and in an annular pattern at the apical plasma membrane (arrowhead).

(C–E) p*col-12*::SNF-12::GFP does not colocalize with VHA-5::RFP, which is a marker of exocytotic vesicles (Liegeois et al., 2006).

(F–H) p*col-12*::mCherry::STA-2 colocalizes to a large degree with CHC-1::GFP, which is a marker of endosomes (Greener et al., 2001) (C and F, green channel; D and G, red channel; E and H, both channels). Scale bar, 10 μm (A and B) or 5 μm (C–H).

(I) Quantitative RT-PCR analysis of *nlp-29* gene expression showing the level of induction in worms treated with RNAi against *dyn-1* and *rab-5*, relative to control, after infection or osmotic stress (average and SEM from three independent experiments).

fungal infection and osmotic stress to a similar degree, *dyn-1* had a markedly stronger effect after infection compared to osmotic stress (Figure 7 and Figure S5L). This suggests that infection-specific signaling requires dynamin-dependent endocytic vesicles that are dispensable for the response to osmotic stress. Taken together, our results suggest that SNF-12 and STA-2 physically and functionally interact within *dyn-1*-dependent endocytic vesicles to regulate part of the innate immune response to fungal infection.

DISCUSSION

C. elegans responds to *D. coniospora* infection, physical injury, and osmotic stress by upregulating the expression of certain AMP genes in the epidermis via distinct molecular cascades (Pujol et al., 2008a, 2008b; Rohlfing et al., 2010). There are many common elements in the pathways that are involved in the response to infection and wounding, including a conserved PKCδ that activates a p38 MAPK cassette (Ziegler et al., 2009). AMP gene expression after osmotic stress, however, involves a distinct regulatory circuit (Lee et al., 2010). In this study, we show that *snf-12*, which encodes a previously uncharacterized member of the sodium-dependent neurotransmitter symporter family, is specifically required for the regulation of expression of the AMP genes after infection and injury. The protein SNF-12 cannot be assigned to one of the classical SNF subfamilies, and its potential ligand has yet to be defined. Our results with mutants deficient in neurotransmitter biosynthesis rule out this class of bioactive amines as ligands. But we cannot exclude the possibility that the main function of SNF-12 in this context is not as a transporter. Indeed, here we provide evidence that the role of SNF-12 in regulating *nlp-29* expression is mediated through physical interaction with the STAT transcription factor-like protein STA-2.

STA-2 is one of the two STAT-like proteins in *C. elegans* (Plowman et al., 1999). STAT proteins in other species, including *Drosophila* (Boutros et al., 2002) and mammals, are well-known for their role in innate defenses. In other species, the general model of their action has STAT proteins as latent, cytoplasmic transcription factors that require phosphorylation for their activation. They are phosphorylated by receptor-associated Janus kinases (JAKs), dimerize, and then translocate into the nucleus. There, they regulate the transcription of target genes. Although *C. elegans* does have some cytokines and cytokine receptors, it does not possess a clear JAK homolog, nor homologs of the best-characterized cytokines such as interleukins and interferons or the receptors that activate JAK (Bromberg, 2001). We showed that STA-2 is a potential target for p38 MAPK phosphorylation and can be found in the nucleus of epidermal cells that express AMP genes. Further studies will be needed to establish whether it is able to bind DNA directly and acts as a bone fide transcriptional activator.

STAT genes are also present in *Dictyostelium* without cytokines, cytokine receptors, or JAK partners, so the STAT family of transcription factors is likely to have arisen early in eukaryote evolution, regulated in a manner independent of cytokine-triggered phosphorylation. Indeed, the STAT protein in *Dictyostelium* is activated by a G protein-coupled receptor (Wang and Levy, 2006). Given that SNF-12 has no counterpart outside closely related nematode species, its direct interaction with a STAT protein would appear to be phylogenetically very limited. But this regulatory module is integrated within an ancient p38 MAPK pathway that is conserved from worms to man. This therefore contributes to illustrate the extraordinary evolutionary plasticity of innate immune signaling pathways.

Recent studies have also revealed adaptability in the molecular cascades that control defenses within different *C. elegans* tissues. For example, although PKCδ and the p38 MAPK cascade are required for innate immunity in both the epidermis

and intestine, the protein kinase D DKF-2 is essential in the intestine but dispensable in the epidermis (Ren et al., 2009; Ziegler et al., 2009). Here, we have shown that two further components, SNF-12 and STA-2, have a specific role in the epidermis, but apparently not in the intestine. They are required for the expression of genes of both the *nlp-29* and *cnc-2* loci. These are controlled, respectively, by p38 MAPK and TGF-β signaling cascades (Pujol et al., 2008a, 2008b; Zugasti and Ewbank, 2009). This could reflect a generic role for SNF-12 and STA-2 in epidermal cell function or development. But as in both *snf-12* and *sta-2* mutants there are no obvious morphological phenotypes, and since the osmotic response is intact in the epidermis, we favor instead a model in which *snf-12* and *sta-2* link the p38 MAPK and TGF-β pathways specifically during the innate immune response to fungal infection. When we assayed the effect of *snf-12* and *sta-2* on the resistance of *C. elegans* to infection with *D. coniospora*, we saw no difference between mutant and wild-type worms, despite a small decrease in longevity of the worms under control conditions (Figures S4F and S4G). Under these conditions, it is difficult to assign unambiguously a role in resistance to *snf-12* or *sta-2*. This is, however, not the only case of a lack of correlation between defense gene expression and resistance to infection (e.g., Couillault et al., 2004), which can arise as infection influences the expression of many genes. Indeed, the expression of certain putative defense genes is increased in a *pmk-1* mutant, despite the fact that it is hypersusceptible to infection (Troemel et al., 2006 and Figure 3D). A fuller understanding of these results must await a complete transcriptional profiling of the *snf-12* and *sta-2* mutants.

Our epistasis and biochemical analyses place SNF-12 and STA-2 downstream of the PKCδ TPA-1 and the p38 MAPK pathway. Both *snf-12* and *sta-2*, however, only partially block the increased expression of *nlp-29* provoked by *sek-1* overexpression, suggesting that they also act either upstream of *sek-1* or in a pathway parallel to *sek-1*. Their role in regulating PMK-1-independent genes, such as *cnc-2*, would be consistent with a role for *snf-12* and *sta-2* in a parallel pathway. Given their respective structures, one model would be that SNF-12 tethers inactive STA-2 and retains it out of the nucleus, like cytokine receptors in other animal species. Upon stimulation, STA-2 would be activated, released, and able to regulate nuclear gene expression. On the other hand, we found STA-2 in the cytoplasm and in the nucleus of epidermal cells, even in uninfected individuals. *C. elegans* STA-1, which is a DNA-binding protein, is also found in both the cytoplasm and the nucleus (Wang and Levy, 2006). Indeed, there are now many examples of STATs shuttling between the cytoplasm and nucleus in a constitutive manner (see, e.g., the review Sehgal, 2008). Clearly, the precise role of STA-2, its activation, and intracellular trafficking all merit further study.

SNF-12 potentially acts as a transporter for a bioactive amine. It is still an open question whether such a putative transport function is required for its role in regulating AMP gene expression. We demonstrated a role for endocytosis in regulating AMP expression. SNF-12 is localized to endocytic vesicles. It therefore might be in a position to "sample" what is at the epidermal surface, and this might be part of the mechanism that triggers an innate immune response. This could be especially important for an organism like *C. elegans*, as it lacks motile scavenger cells (Ewbank, 2002). There are precedents for vesicular sampling in host defense, such as with certain TLRs (Blasius and Beutler, 2010). It is also notable that endocytosis has recently been demonstrated to play an essential role in *Drosophila* in the regulation of AMP gene expression controlled by the Toll signaling pathway (Huang et al., 2010). Thus, endosomal membranes can function as important platforms for innate immune signaling. It is possible that they also play this role, via SNF-12 and STA-2 in *C. elegans*.

EXPERIMENTAL PROCEDURES

Nematode Strains

All strains were maintained on nematode growth media and fed with *E. coli* strain OP50, as described (Stiernagle, 2006). The strains *mod-5(n3314)*, *snf-1(ok790)*, *cha-1(y226)*, *cat-2(tm2261)*, *tph-1(mg280)*, *unc-25(e156)*, *tbh-1(n3247)*, *tdc-1(ok914)*, *cat-4(ok342)*, and *sta-2(ok1860)* were obtained from the CGC. *snf-12(tm692)* was kindly provided by S. Mitani and outcrossed three times. Strains containing an integrated transgene with VHA-5::RFP (ML1542) or with CHC-1::GFP (RT1378) were kindly provided by M. Labouesse and B. Grant, respectively. Strains containing the *frls7* and the p*col-12*::*sek-1* transgenes are described in Pujol et al. (2008a). All other transgenic strains are described in the Supplemental Experimental Procedures.

Mutant Isolation, Cloning, and Rescue

As described in Pujol et al. (2008a), *wt*; *frls7* worms were mutagenized with EMS using standard procedures (Wood, 1988). *nipi-2(fr2)* was mapped between *dpy-6* and *dpy-22* in the middle of chromosome X, through standard genetic and SNP mapping. Further analysis of 90 recombinants with a double mutant *dpy-6 nipi-2(fr2)* allowed us to place the mutation between T25B6.3 and F47E1.4 (WS200). Two fosmids, WRM0610aE08 and WRM068bG12 (Geneservice), prepared with standard arabinose induction (0.1%) were injected at 100 ng/μl with the coinjection marker *rol-6* (pRF4) into *nipi-2(fr2); ls7* mutant worms. Only the WRM0610aE08 fosmid rescued the Nipi phenotype in two independent lines, IG431 *snf-12(fr2); frls7; frEx105* and IG432 *snf-12(fr2); frls7; frEx106*. Sequencing the remaining three candidate transcripts, T25B6.3, T25B6.7, and T25B6.2, revealed a mutation in T25B6.7 (*snf-12*). Complementation tests with a deletion in *snf-12(tm692)* confirmed that *nipi-2* corresponds to *snf-12*.

Infection, Wounding, Exposure to High Salt, PMA, and GPA-12 Heat Shock

Infections with *D. coniospora* and wounding were carried out at 25°C as described (Powell and Ausubel, 2008). Briefly, animals were infected with *D. coniospora* at the L4 stage and incubated. After 18 hr, noninfected animals were used for wounding assays or exposure to high salt or PMA, or were kept as control. Exposure of worms to high salt (300 mM NaCl) and PMA (1 μg/ml) was done in liquid using 96-well plates with 50 mM NaCl solution as a control, and on plate for the RNAi experiments with *dyn-1* and *rab-5*. Synchronized L4s carrying the p*hsp*::GPA-12 transgene were heat shocked for 2 hr at 33°C, then transferred to 25°C and analyzed after 24 hr. Synchronized L4s carrying the p*gpdh-1*::GFP reporter were placed on NGM agar plates containing 50 or 300 mM NaCl and analyzed after 24 hr.

Yeast Two-Hybrid Screen

The SNF-12 C terminus, comprising the 325 last amino acid residues, was used as bait in a yeast-two hybrid screen using the ProQuest *C. elegans* cDNA library and the ProQuest two-hybrid system with Gateway technology (Invitrogen). Only the interactors that were positive for the three phenotypes tested (growth on -Leu, -Trp, -His, 3-amino-1,2,4-triazole, or -Ura or β-galactosidase) were further analyzed.

Coimmunoprecipitation

CoIP assays using M2-Flag-Sepharose beads (Sigma) were done as described in Lenfant et al. (2010) on protein extracts from 293T cells transfected with a Flag-STA-2 (full-length STA-2 ORF) and a Myc-SNF-12 (C terminus of SNF-12) or a Myc-mock (unrelated peptide) all in pDEST-cmv

vectors described in Xu et al. (2003). The same extracts were used with anti-Myc Sepharose beads, giving similar results.

Immunoblotting of *C. elegans* Lysates

Mixed staged animals were collected and washed five times in S-basal media. After centrifugation, the dry pellet was resuspended in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton, 1% sodium deoxycholate, 10% glycerol) supplemented with protease and phosphatase inhibitor cocktails (Pierce). Worms were then subjected to three freeze/thaw cycles and mechanical grinding with a Dounce pestle. After high-speed centrifugation to clarify the extracts, protein concentration was estimated with DC Protein Assay (BioRad). Of total protein, 15 μg was resolved on 4%–12% NuPage gels, transferred to PVDF membrane, and immunoblotted with anti phospho-p38 (9211, Cell Signaling) or anti-actin (C4, Millipore) antibodies.

Phosphorylation Assay

293T cells were transfected with Myc-STA-2 (full-length STA-2 ORF in pDEST-cmv-Myc [Xu et al., 2003]), with or without Flag-SEK and HA-PMK-1 kindly provided by K. Matsumoto (Shivers et al., 2010) using HD Fugen reagent (Roche). After 48 hr, cells were collected, washed, and resuspended in lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1mM EDTA, 0.2% NP-40) with protease inhibitor (Pierce) and complete phosphatase inhibitor (Pierce), with the exception of the samples subsequently treated with phosphatase. After high-speed centrifugation to clarify the extracts, protein concentration was estimated with DC Protein Assay (BioRad). Of total protein, 25 μg was treated with 0.4 u/μl lambda protein phosphatase (New England Biolabs) for 1 hr at 30°C. Samples were resolved on 15% Tris-glycine gel, transferred to PVDF membrane, and blotted with anti-Myc 9E10 (Sigma) anti-Flag M2 (Sigma), anti-HA11 (Covance), or anti-GAPDH 6C5 (Millipore) antibodies.

qRT-PCR and Biosort Analyses

Quantitative real-time PCR was performed as described with L4 worms infected for 4 hr at 25°C with *D. coniospora* (Pujol et al., 2008b) or PA14 (Powell and Ausubel, 2008). Primers sequences and description of the COPAS Biosort analyses are given in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.chom.2011.04.011.

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