The Functions of Grainy Head-Like Proteins in Animals and Fungi and the Evolution of Apical Extracellular Barriers

Adam Paré1*, Myungjin Kim1, Michelle T. Juarez1, Stuart Brody2, William McGinnis1*

1 Section of Cell & Developmental Biology, University of California San Diego, La Jolla, California, United States of America, 2 Section of Molecular Biology, University of California San Diego, La Jolla, California, United States of America

Abstract

The Grainy head (GRH) family of transcription factors are crucial for the development and repair of epidermal barriers in all animals in which they have been studied. This is a high-level functional conservation, as the known structural and enzymatic genes regulated by GRH proteins differ between species depending on the type of epidermal barrier being formed. Interestingly, members of the CP2 superfamily of transcription factors, which encompasses the GRH and LSF families in animals, are also found in fungi – organisms that lack epidermal tissues. To shed light on CP2 protein function in fungi, we characterized a Neurospora crassa mutant lacking the CP2 member we refer to as grainy head-like (grhl). We show that Neurospora GRH has a DNA-binding specificity similar to that of animal GRH proteins and dissimilar to that of animal LSF proteins. Neurospora grhl mutants are defective in conidial-spore dispersal due to an inability to remodel the cell wall, and we show that grhl mutants and the long-known conidial separation-2 (csp-2) mutants are allelic. We then characterized the transcriptomes of both Neurospora grhl mutants and Drosophila grh mutant embryos to look for similarities in the affected genes. Neurospora grhl appears to play a role in the development and remodeling of the cell wall, as well as in the activation of genes involved in defense and virulence. Drosophila GRH is required to activate the expression of many genes involved in cuticular/epidermal-barrier formation. We also present evidence that GRH plays a role in adult antimicrobial defense. These results, along with previous studies of animal GRH proteins, suggest the fascinating possibility that the apical extracellular barriers of some animals and fungi might share an evolutionary connection, and that the formation of physical barriers in the last common ancestor was under the control of a transcriptional code that included GRH-like proteins.

Introduction

Grainy head (GRH) transcription factors are crucial for many aspects of development. For instance, Drosophila GRH (also called Elf-1 or NTF-1) regulates development of the epidermis and head skeleton [1,2], wound healing [3–6], neuroblast proliferation [7,8], early embryonic patterning [9,10], and tracheal-tube morphology [11]. However, the functions of GRH family proteins with respect to epidermal-barrier formation and wound healing have received the most attention, as these functions appear to be widely conserved in animals.

Drosophila grh mutant embryos have slack and fragile cuticles, as well as “grainy” and discontinuous head skeletons [1,2,12]. Null mutations are lethal, as the embryos fail to develop past the embryonic/larval transition point due to their extremely fragile epidermal barriers. These phenotypes clearly point to defects in the formation of cutin-based cuticular structures in grh mutant embryos. These defects are likely due to lowered epidermal expression of a wide variety of genes, among them Dlk, which encodes dopa decarboxylase, an enzyme required to generate the reactive quinone molecules used to cross-link cutin fibers and proteins in the Drosophila cuticle [1,3]. Furthermore, grh embryos are permeable to exogenously applied dyes [6], and the removal of GRH from imaginal disc cells results in reduced expression of at least two cell-adhesion genes [13]. These findings suggest that the paracellular integrity of the epithelial barrier underlying the cuticle becomes compromised in Drosophila grh mutants. In addition to the developmental functions of GRH in Drosophila, it is also necessary for the proper expression of several cuticular-barrier genes that are activated during the regenerative process following epidermal wounding [3–5].

GRH family proteins are also important for epidermal-barrier formation in the distantly related invertebrate C. elegans. RNAi targeted against Ce-Grh-1 results in embryos with a fragile and puckered hypodermis – a similar phenotype to that seen in Drosophila [14]. Ce-Grh-1 binds the same palindromic consensus DNA sequences as Drosophila GRH, and the Dkc gene in C. elegans has GRH binding sites upstream of its promoter [14]. Strikingly, conservation of GRH family transcription factor function extends to vertebrates as well, despite vast differences in the structural components of epidermal barriers between and within protostomes and deuterostome animals. In Xenopus laevis, expression of a
Grhl2 and hair loss due to poor anchoring of hair shafts within follicles deficient mice display delayed coat growth, thickened paw skin, Grhl1 ability to exogenous dyes, and severe postnatal water loss, as well as defects in neural-tube and eyelid closure [16,19-24]. Grhl1-deficient mice display delayed coat growth, thickened paw skin, and hair loss due to poor anchoring of hair shafts within follicles [25]. Grhl2 appears to regulate neural-tube closure as well as E-cadherin expression [26]. Furthermore, all three mouse Grhl transcription factors have been shown to bind preferentially to the same consensus DNA sequences as Drosophila and C. elegans GRH proteins [27].

While the DNA-binding specificity of GRH family proteins has been conserved between protostome and deuterostome animals, the downstream effectors of GRH-like proteins in distantly related species do not appear to be homologous, but instead carry out analogous functions suited to the specific barrier being generated or regenerated after wounding. For instance, the epidermal defects in Grhl3-deficient mice correlate with reduced levels of transglutaminase 1 transcription (which has upstream GRH binding sites), as well as reduced transcription for many genes that are structural barrier components of differentiated corneocytes [19,21]. Transglutaminase 1 is an enzyme necessary for the cross-linking of keratin and other proteins in the mammalian epidermis, and it plays an analogous role to that of dopa decarboxylase in the Drosophila cuticle. In sum, there exists a high-level functional conservation of GRH proteins as regulators of epidermal integrity and wound healing in both protostome and deuterostome animals (which diverged approximately 700 million years ago), despite the significant structural differences in barrier composition across the animal kingdom. This functional conservation is reminiscent of other cases in which high-level transcription factor function has been conserved over great evolutionary time (e.g., Hox genes, Pox6/eyless, and Nkd2.5/tumnin in body-axis, eye, and heart specification, respectively) despite the drift of specific downstream effectors.

Since the function of GRH-like proteins in epidermal-barrier formation and wound healing appears well conserved in triploblastic animals, we were interested in determining what role GRH-like proteins might be playing in more distantly related organisms. GRH family proteins (along with the related LSF family proteins) belong to the CP2 superfamily of transcription factors, members of which are found in the opisthokont lineage, which includes Metazoa (Animals), Fungi, and several closely related sister-species [28]. Considering the fact that Fungi utilize a very different type of extracellular physical barrier (the cell wall) compared with animals, we thought that by studying the role of CP2 superfamily transcription factors in Fungi we might shed some light on the origins of transcriptional control of physical-barrier formation in the opisthokont ancestor. Towards this end, we have characterized the function of the CP2 superfamily gene in the ascomycete fungus Neurospora crassa using microarray and phenotypic analyses. We show that the loss of this Neurospora gene, which we call grainy head-like (grhl), leads to a developmental defect in cell wall remodeling during conidial development, which is associated with the down-regulation of numerous genes predicted to encode abundant components of the cell wall. We also carried out microarray and phenotypic analyses of Drosophila grh mutants, and we present evidence that, in addition to its crucial role in cuticular- and epidermal-barrier formation, GRH may also be involved in microbial defense during adulthood in Drosophila. Our results suggest an ancestral role for CP2 superfamily proteins as regulators of extracellular-barrier formation in opisthokont ancestors.

**Results**

**Sequence Analyses Suggest Fungal CP2 Proteins are More Functionally Similar to Animal GRH Proteins than to Animal LSF Proteins**

The CP2 superfamily is composed of the GRH and LSF families of transcription factors. A comprehensive review of the functions of LSF-like proteins is beyond the scope of this paper, but there appears to be little overlap between the biological roles of the GRH and LSF families in animals [29], and the two families have diverged greatly in their modes of DNA binding [30,31]. It is clear that the last common ancestor of Metazoa and Fungi possessed at least one CP2 superfamily protein, although phylogenetic analysis indicates that fungal CP2 superfamily proteins form a separate outgroup with respect to metazoan GRH and LSF family proteins [28]. With few exceptions, all sequenced metazoan genomes possess one or more copies of both GRH and LSF family proteins. Among the Fungi, only ascomycete and zygomycete genomes encode a CP2 superfamily protein (or multiple paralogs), while known basidimycyte genomes do not. Some ascomycetes (e.g., Saccharomyces cerevisiae) appear to have lost the CP2 superfamily. The unicellular sister-group organisms M. brevicollis (a choanoflagellate) and C. owczarzaki (a filasterean) both contain single CP2 superfamily proteins [32,33].

Although a recently published phylogenetic analysis using gap-free alignments of near full-length protein sequences showed that fungal CP2 superfamily proteins are roughly equally related to both the GRH and LSF family proteins [28], we decided to look more closely at the DNA-binding domain sequences of extant opisthokont CP2 superfamily proteins to identify specific residues that might be characteristic of GRH or LSF proteins. An alignment between the DNA-binding domains of two GRH family proteins ([D. melanogaster GRH and H. sapiens Grhl1], two LSF family proteins ([D. melanogaster Gem and H. sapiens LSF]), and a representative fungal CP2 superfamily protein (referred to as Neurospora Grainy head-like, or GRHL, for reasons described below) highlights the extensive sequence conservation throughout this domain (Figures 1A and B). It has been predicted that part of the region containing the DNA-binding domain of CP2 superfamily proteins adopts a similar tertiary structure to the DNA-binding domain of p53 [34], which has a well-characterized three-dimensional structure. Strikingly, the identity of eight amino acid residues at and around positions predicted to be crucial for DNA binding, based on mapping to the p53 structure (i.e., major- and minor-groove contacts, zinc-binding residues, and residues involved in dimerization) suggest that the DNA-binding properties of fungal CP2 proteins might be more similar to GRH than to LSF family proteins. For example, relative to positions 194–198 of the Neurospora GRHL DNA-binding domain (a region predicted to be involved in major-groove interaction) the same amino acid sequence GAERK is found in nearly all available metazoan GRH and fungal GRHL ortholog sequences, while the sequence GADRK is found in all available metazoan LSF sequences (Figure 1B). Similarly, in three other regions predicted to be important for DNA binding (positions 81–84, 142–147 and 150–
(153), we find that some *Neurospora* GRHL amino acid residues are identical to those found in nearly all fungal CP2 proteins and metazoan GRH proteins, but they differ from those found in LSF proteins (indicated with asterisks in Figure 1B). Based on these observations, we hypothesized that the DNA-binding characteristics of the *Neurospora* GRHL protein might be more similar to those of animal GRH family proteins than animal LSF family proteins. Another indication that fungal CP2 superfamily proteins might be more functionally similar to animal GRH proteins is that fungal CP2 superfamily proteins all lack SAM oligomerization domains – animal GRH proteins also lack SAM domains, but all known animal LSF family proteins possess SAM domains [28].

The *Neurospora* CP2 Protein GRHL and *Drosophila* GRH have Similar DNA-binding Specificities

To test whether a fungal CP2 protein binds DNA similarly to animal GRH family proteins, we decided to study the *Neurospora crassa* CP2 superfamily protein. We chose *Neurospora* as our model organism because it is a fairly typical representative of a filamentous ascomycete fungus, there exist a number of molecular tools to work with (including gene-knockout technologies), and it

**Figure 1.** *Neurospora* GRHL has a similar DNA binding specificity as *Drosophila* GRH. (A) The *Neurospora* GRHL protein shares sequence similarity with both *Drosophila* GRH and mammalian Grhl proteins, as well as with LSF family proteins [28]. The areas of highest similarity include the region containing the GRH DNA-binding domain, and a region near the C-terminus containing the GRH dimerization domain [30]. (B) A comparison of the DNA-binding domain of a representative fungal CP2 superfamily protein (*N. crassa* GRHL) with those of the *Drosophila* and human GRH family proteins (*D. melanogaster* GRH and *H. sapiens* Grhl1) and LSF family proteins (*D. melanogaster* GEM and *H. sapiens* LSF). Amino acid residues predicted to be important for DNA interactions based on comparisons with p53 transcription factors [34] are marked above the alignment as follows: “D” – dimerization; “Z” – zinc-binding; “m” – minor-groove interaction; and “M” – major-groove interaction. Residues that distinguish GRH family from LSF family proteins are indicated below the alignment with asterisks. The indicated amino acids in nearly all known fungal CP2 superfamily proteins are identical to the *Neurospora* residues. (C) Oligonucleotides used in the gel-shifts. Bases that include the LSF or GRH optimal consensus binding sites are indicated with asterisks. (D) Gel-shift assays testing *Drosophila* GRH and *Neurospora* GRHL binding to the oligonucleotides in (C). The bottom panels were exposed for 16.5 h, and the top panels were exposed for 75 h. Specific bands are indicated with black arrowheads, and weak specific bands are also highlighted with asterisks in the top panels. Nonspecific (NS) bands were also detected in the no-protein-template negative controls (data not shown), and they are indicated with white arrowheads in the top panels and with a bar in the bottom panels.

doi:10.1371/journal.pone.0036254.g001
has a fully sequenced genome. The Neurospora crassa genome possesses a single CP2 superfamily gene (designated as NCU06095), which has been called grainy-head homolog (grhl) [53], but which we will hereafter refer to as grainy head-like (grhl) or csp-2, for the reasons described in the preceding and following sections. Using RT-PCR and primers specific to the predicted start and stop sites, we cloned and sequenced the full-length grhl coding region and found the sequence and exon structure to be identical to that in the Broad Institute Neurospora database. No splice variants were detected, although we cannot rule out the possibility of 3′UTRs.

We synthesized full-length Neurospora GRHL protein in order to characterize its DNA-binding properties using gel-shift analyses. It has been shown that Drosophila and C. elegans GRH family proteins can both bind with high affinity as homodimers to the palindromic DNA sequence ACCGGTT from the Ddc promoter [14,30] and that the optimal consensus binding site for marine GRH family proteins contains the palindromic DNA sequence AACCCTTT [19,27]. Mammalian LSF has been shown to bind as a tetramer to DNA fragments containing the sequence CTGG-N6-CTGG; LSF does not bind to DNA fragments containing a GRH site from the Ubx promoter [31]. On the other hand, Drosophila GRHL can weakly bind to both full-length LSF sites and CTGG half-sites [31]. Therefore, we tested the ability of the Neurospora GRHL protein to bind DNA oligonucleotides containing one of the following sites: the endogenous GRH binding site (GRH-Ddc), a mutated GRH binding site (GRH-mut), or the consensus GRH binding site (GRH-con) (Figure 1C). We also tested the ability of the GRHL protein to bind DNA oligonucleotides containing one of the following sites: the endogenous LSF consensus site (LSF-con), an LSF half-site (LSF-1/2), or a mutated LSF site (LSF-mut) (Figure 1C). The binding of full-length Drosophila GRH protein to these oligonucleotides was tested as a comparison.

Drosophila GRH bound DNA sequences as previously reported [30,31], interacting strongly with the GRH-Ddc and GRH-con oligonucleotides, but not with the GRH-mut oligonucleotide (Figure 1D, right panels). Drosophila GRH also bound very weakly to both the LSF-con and LSF-1/2 oligonucleotides, but not to the mutated LSF-mut oligonucleotide (Figure 1D, top right panel). Neurospora GRHL bound with a similar specificity as Drosophila GRH, albeit with apparent lower affinity. GRHL bound strongly to the GRH-con oligonucleotide, weakly to the GRH-Ddc oligonucleotide, and very weakly to the LSF-con oligonucleotide (Figure 1D, left panels). Considering these results, along with the similarities in their DNA-binding domain sequences, we conclude that the last common ancestor of opisthokonts possessed a CP2 superfamily protein with a similar DNA-binding specificity to existing metazoan GRH family proteins.

Phenotypes of the Neurospora grhl Knockout Mutants

The fungus Neurospora crassa has a simple cellular organization and life cycle compared with most animals and plants (for an in-depth treatment on the subject, see [36]). The most visually obvious phase of the Neurospora life cycle is asexual proliferation – single spores (conidia) germinate on a food source and form a densely interwoven mat of thread-like mycelia, which spreads quickly to form a colony. Neurospora colonies exist as syncytial collections of “cells” which share a common extracellular barrier – the cell wall. While there are regularly spaced septa along the length of the mycelial and hyphal axes, these divisions are not complete, and the “cells” use vigorous cytoplasmic streaming to move nutrients and other molecules throughout the colony. After about a day (and every day after that, according to a circadian rhythm) aerial hyphae grow up and away from the food source and bud off chains of new conidia. These conidial chains become quite delicate as they mature, as the thick cross-walls between individual conidia are remodeled into thin, easily broken connectives – this allows mature conidia to readily detach and disperse to found new colonies.

Neurospora strains containing precise deletions of the entire grhl coding region were obtained from the Fungal Genetics Stock Center (FGSC) for both mating type (mat) backgrounds: FGSC13563 (mat A) and FGSC13564 (mat a). In addition, we created multiple independently derived grhl knockout strains using targeted homologous recombination to replace the grhl locus with a hygromycin cassette. The phenotypes of these mutant strains were indistinguishable from those of the deletion mutant stocks obtained from the FGSC, indicating that the phenotypes described below are indeed due to the loss of grhl function. PCR amplification of a region within the grhl locus verified that all strains were indeed lacking the grhl gene (Figure 2A). Furthermore, RT-PCR amplification of a region of the grhl mRNA yielded no product when RNA from grhl mutants was used as template, compared with robust detection of grhl transcripts using wild-type RNA as template (Figure 2B). Transcripts from the grhl gene were readily detectable by RT-PCR using wild-type RNA templates from either pure mycelial samples or samples of aerial hyphae and conidia (the latter yielding slightly stronger amplifications; data not shown), which suggests the GRHL transcription factor is expressed in most Neurospora cell types during asexual proliferation.

Mutant grhl strains are viable and can be propagated asexually as homokaryotic colonies (i.e., all nuclei in the colony are clonal) on minimal media. Both grhl mating-type strains can serve as males or females in sexual crosses to wild-type or grhl strains of the opposite mating type, indicating that grhl function is dispensable for sexual reproduction (data not shown). The grhl strains appear quite healthy and in many ways are indistinguishable from wild type, at least under laboratory conditions (Figures 2D–F).

The grhl mutant strains display a slightly altered circadian rhythm [35], develop orange pigmentation slightly more quickly than wild type (Figure 2C), and sometimes have paler mycelia than wild type (data not shown). However, the most striking phenotype of grhl mutants is a pronounced conidial-separation defect. In grhl strains, conidial chains fail to completely separate, even upon physical stress or immersion in liquid (Figures 2G–I). This phenotype is identical to that observed in the conidial separation mutants csp-1 and csp-2, whose phenotypes have been investigated in some detail. It was shown that the csp-1 mutant strains begin conidial development normally; however, the chitinous cross-walls between adjacent conidia do not become remodeled into thin connectives, precluding conidial separation [37]. This phenotype was correlated with a decrease in the autocatalytic activity of the Neurospora cell wall, which was hypothesized to be due to the loss of secreted enzymes such as chitinase [38].

While csp-1 and csp-2 have long been popular background strains for Neurospora researchers (they help prevent the cross-contamination of stocks), the nature of the mutant genes responsible for these phenotypes remained unknown for many years. Recently, it was shown that csp-1 (NCU02713) encodes a zinc-finger transcription factor on chromosome 1 [39]. However, nature of the gene underlying the csp-2 phenotype remained unclear, except that it mapped to chromosome 7 between the genes thi-3 and ace-8 [40] – precisely the region where grhl is located. Therefore, we believed that a lesion in the grhl gene might be responsible for the csp-2 phenotype.

We first carried out genetic complementation tests with the recessive alleles to test whether csp-2 and grhl are allelic. Different
Neurospora strains can fuse to form syncytial heterokaryonic colonies containing nuclei from both parental strains; these fused colonies are often able to grow in conditions that their parents cannot, as each type of nucleus will complement the requirements of the other. For instance, fused colonies from different nutritional-auxotroph parents can survive on minimal media, which can be taken advantage of to test for genetic complementation at another non-selectable locus. Using standard sexual-crossing procedures, csp-2 and grhl mutations were placed into different auxotrophic backgrounds (inos and his-3, respectively), and conidia from each strain were combined on minimal media. We found that all viable heterokaryotic fusions resulted in colonies that still displayed the conidial-separation phenotype, demonstrating that csp-2 and grhl mutant alleles fail to complement (see Materials and Methods for details). To assay for the basis of the non-complementation, we sequenced the grhl open reading frame of the csp-2[FS590] allele, and found a one base pair deletion in codon S509. This mutation would be predicted to result in a premature stop codon after 14 out-of-frame codons, leading to the removal of the proper C-terminal amino acids of the GRHL protein (Figure S1). Therefore, we conclude that grhl and csp-2 are allelic and that the conidial-separation phenotype observed in grhl strains is due to a reduction in the autocatalytic activity of the cell wall [38], which in turn precludes remodeling of the cross-walls between adjacent conidia [37].

Microarray Profiling of Neurospora grhl Knockout Mutants

To determine the genes directly and indirectly under the control of GRHL in Neurospora, we carried out microarray-based transcriptome profiling of three different sample types: 1) MYC – actively growing pure mycelial samples; 2) AHC – aerial hyphae and conidia from 48 h old colonies; and 3) ALL – all cell types from 48 h old colonies. We only describe the results from the AHC samples, as these are the cell types that displayed the conidial-separation phenotype (see the Materials and Methods section for the accession numbers of the MYC and ALL microarray datasets).

Of the 10,526 genes that were probed on the microarray, 167 were seen to be misregulated in the grhl AHC samples at a False Discovery Rate (FDR) threshold of less than 0.01 (meaning 1%, or about 2 of these genes, are expected to be false positives). This threshold roughly corresponds to a greater than twofold change in expression up or down relative to wild-type levels. Nearly equal numbers of genes were seen to be up- or down-regulated (84 and 83 genes, respectively), and verification of microarray fold-change directionality for ten genes using quantitative RT-PCR is shown in Figure S2. Up-regulated genes on the grhl AHC microarrays are shown in Figure S3, the largest classes of which include genes involved in nitrogen, sulfur, and selenium metabolism, as well as genes involved in membrane transport and cellular import. As we were interested in finding commonalities between the gene
products activated by GRH-like transcription factors in animals and fungi, we focused on the down-regulated genes on the ghl AHC microarrays.

Highly Enriched FunCat Categories of the Down-regulated Genes from the Neurospora grhl AHC Microarrays

In order to parse microarray results, researchers often use the Gene Ontology (GO) functional annotation system (www.geneontology.org) to look for highly enriched classes of genes. As a comprehensive GO annotation of the Neurospora crassa genome did not exist at the time of these analyses, we used an alternative classification system – The Functional Catalogue (FunCat) – for which there did exist a high-quality annotation for Neurospora genes [41]. For the 83 genes that were seen to be significantly down-regulated, there were highly significant enrichments in five FunCat categories (Table 1). Three of these categories are composed of genes involved in amino acid metabolism – specifically that of cysteine, phenylalanine, and tryptophan. A fourth category, “C-compound and carbohydrate transport”, is composed of membrane transport proteins. The fifth highly significant category found was “disease, virulence, and defense”, which is composed of genes predicted to be involved in fungal pathogenicity, defense against other organisms, and certain stress responses.

We could find no direct connections in the literature between cysteine metabolism and barrier formation in animals. However, most amino-acid-metabolism networks are interlinked, and three of these genes (NCU05499, NCU09183, and NCU01402) are also a part of the significantly enriched phenylalanine- and tryptophan-metabolism FunCat categories, for which there are some intriguing connections to barrier formation in animals. Melanization reactions in Drosophila are used to harden and cross-link cuticular structures, and are known (at least in the epidermis) to rely on GRH for activation [3]. The reactive quinone molecules used to carry out these processes are derivatives of dopamine, which is itself a derivative of the amino acids tyrosine and phenylalanine (for a review see [42]). It is possible that an ancestral role in phenylalanine regulation by GRH-like transcription factors could have been co-opted by cuticle-forming animals for use in cross-linking apical extracellular barriers. As for the last amino-acid-related FunCat category, “degradation of tryptophan”, there is some evidence that it is a general mechanism of all cells to degrade tryptophan in response to infection, which is used as a means to slow microbial growth through tryptophan deprivation [43]. If true, this function would link tryptophan degradation to the fifth FunCat category – “disease, virulence, and defense” – the presence of which we found especially intriguing, due to the numerous documented connections in animals between physical epidermal barriers and chemical defense against pathogens [44,45].

Down-regulated Genes from the Neurospora grhl AHC Microarrays

To investigate the down-regulated genes from the Neurospora grhl AHC samples in more detail, we undertook a manual classification of these genes based on database and literature searches. We were especially interested in finding studies carried out directly on the Neurospora crassa genes or on their close homologs in other fungal species. Of the 83 significantly down-regulated genes, 54 had known functions, or predicted functions based on homology to genes in other fungi (Figure 3).

Strikingly, the most strongly down-regulated gene on the entire microarray (other than ghl itself) was chinamase 1 (NCU04083) (Figure 3), the lack of which is likely to contribute to the conidial-separation phenotype observed in ghl1/cup-2 mutants. The chinamase 1 gene of Neurospora has two consensus GRHL DNA binding sites (AAACGGTT & CACCGGTT) within 875 bp of the ATG codon for the chinamase 1 gene. This suggests that the microarrays had identified at least some biologically relevant genes that are directly regulated by GRHL. There were at least six other GRHL-dependent down-regulated genes that encode proteins predicted (from research on other fungi) or known to be involved in normal “Cell Wall Structure” (Figure 3). All of these proteins contain predicted secretion signals, and four are experimentally verified components of the Neurospora cell wall: gel1 (NCU07253, which has an AACCGGTT sequence ~130 bp upstream of the transcription start), Mag1 (NCU05974, which has an AACCGGTT sequence ~400 bp upstream of the 5’ end of the open reading frame), non-anchored cell wall protein-5 (NCU00716, which has an AACCGGTT sequence ~1.8 kb upstream of the transcription start), and BISI domain-containing protein (NCU08907) [46]. Three of the down-regulated cell wall genes – Mag1 and NCU04431 (both belonging to glycoside hydrolase family 16) as well as gel1 (belonging to glycoside hydrolase family 72) – encode beta-1,3-glucanases. Beta-1,3-glucans are the major biopolymer constituent of the cell wall in filamentous fungi, and it has been shown in many fungal species that beta-1,3-glucanase enzymes are very abundant components of the cell wall, where they play an active role in cell wall biosynthesis and remodeling, as well as in processes such as biofilm formation [46–49]. For instance, mutations in enzymes from glycoside hydrolase family 72 cause cell wall defects in S. cerevisiae [48] and also affect morphogenesis and virulence in Aspergillus fumigatus [50]. We also found that seven of the 29 down-regulated genes that could not be assigned a function encode proteins with predicted secretion signals, and therefore might be components of the cell wall (Figure 3).

We classified 15 ghl-dependent down-regulated genes in the category “Virulence/Defense/Detoxification” (Figure 3). Defense/ Virulence have not been studied experimentally in Neurospora crassa, as its normal host-pathogen relationships are unknown, so the functions of the following genes are inferred from research in other fungi. Genes potentially involved in defense include the following: kynureninase (NCU09183, which has two AACCGGTT sites ~650 bp upstream of the open reading frame) and indoleamine 2,3-dioxygenase (NCU01402), which are involved in tryptophan catabolism and microbial growth control [43] (see above); NCU03495 encodes a putative anti-viral factor [51,52]; and exo-beta-1,3-glucanase (NCU04850) is possibly involved in the degradation of foreign polysaccharides. Other Neurospora grhl-dependent genes potentially involved in fungal virulence include the following: the metalloprotease MEPI (NCU07200), whose homolog in C. posadasii has been shown to be crucial for evasion of host-detection [53]; the p450 monooxygenase loc4 (NCU05376), whose homolog in a Fuscariun species has been shown to be directly involved in mycoxin synthesis [54]; cerato-platanin (NCU07787), which is potentially important for phytotoxin synthesis [55]; the integral membrane protein pith11 (NCU06328), whose homolog in another fungal species is important for appressorium formation [56]; and NCU03643, which encodes a cutinase transcription factor that is likely to control plant cuticle digestion during fungal infection [57]. Finally, several genes potentially involved in the detoxification of harmful chemicals and the stress response include the following: the p450 gene pisatin demethylease (NCU06327), whose ortholog is important in fungal pathogenesis for detoxifying host defensive chemicals [58]; the aldehyde dehydrogenase gene (NCU03415), which encodes a broadly acting
detoxification and metabolic enzyme; the genes catalase-3 (NCU00355) (another verified component of the cell wall [46]) and NAD(P) transhydrogenase (NCU01140), which encode enzymes known to be important for oxygen-radical detoxification; and the YBH1 flavohemoglobin gene (NCU10051), which may be involved in the stress response [59].

Taken together, these results suggest that Neurospora GRHL plays an important role in the regulation of genes that form and remodel the cell wall (at least in developing conidia). Additionally, a significant number of GRHL-dependent down-regulated genes in the aerial hyphae and conidia are involved in virulence, defense, and detoxification. It is should be noted that the cell wall and virulence/defense categories are not mutually exclusive, as seven of the 15 "defensive" gene products (e.g., cerato-platanin and MEPl) have secretion signals (Figure 3) and are likely to be deposited into the cell wall or released into the extracellular space.

Microarray Profiling of Late-stage Drosophila grh Embryos

As a comparison to the Neurospora microarray dataset, we also carried out microarray-based transcriptome profiling of Drosophila grhIM and control wild-type embryos collected during late-stage 16 and early-stage 17 of embryogenesis [60], when cuticle deposition is occurring. We used flies homozygous for the grhIM allele because it is the strongest grh allele available (with respect to its cuticle and head-skeleton phenotypes) and because homozygous embryos do not produce any detectable GRH protein (assayed using an antibody against the C-terminal half of the protein [6]). By sequencing the grhIM transcript, we identified the lesion responsible for the grhIM allele as a TAT to TAA stop-codon introduction in exon seven, in the N-terminal end of the DNA-binding domain and about half-way through the protein (Figure 1B, amino acid
Figure 3. Down-regulated genes from the *Neurospora grhl* Aerial Hyphae and Conidia microarray samples. A manual classification of the significantly down-regulated genes from the *Neurospora grhl* AHC microarrays. "Broad ID" entries correspond to the gene IDs found in the Broad Institute Neurospora crassa database. The two italicized entries in this column refer to probes that do not correspond to genes in the Broad database.
but which correspond to genes in the MIPS database. “Gene name or Description” and “Function” entries were based on the annotations found in the Broad and MIPS databases, as well literature and homology searches. Numbers in curly brackets indicate genes that belong to one of the five highly enriched FunCat categories: (1) metabolism of the cysteine - aromatic group, (2) metabolism of phenylalanine, (3) C-compound and carbohydrate transport, (4) degradation of tryptophan, and (5) disease, virulence and defense. Entries with asterisks encode experimentally verified components of the *Neurospora* cell wall [46]. “Fold (wt value)” entries indicate the fold changes observed in *grhl* mutant aerial hyphe and conidia (relative to wild type); wild-type microarray fluorescence values are shown in parentheses (the background level was ~100 units). “FDR” entries indicate the False Discovery Rate values calculated for each gene; only genes with FDR values less than 0.01 are shown. Columns 1–9 of the grid represent a simplification of the FunCat classification system; solid-colored blocks indicate those genes are classified in the corresponding FunCat categories; dashes indicate that we found evidence in the literature to suggest these genes belong in the corresponding categories. Column 10 of the grid indicates whether the encoded proteins are predicted to be secreted, according to the SignalP (S) or TargetP (T) prediction algorithms.

Y29 in the *D. mel* GRH DNA-binding domain; see Figure S4 for details; this mutation is consistent with a functional null phenotype for *ghIM* and the lack of GRH protein detection with a C-terminal specific antibody [6]. See the Materials and Methods and Text S1 for more details on the *Drosophila* microarrays and data analyses. Besides the mutation in *gh*, the control and mutant embryos differed slightly with respect to their genetic backgrounds, as the wild-type strain contained the *yellow* allele, which has an adult pigmentation defect, while the *ghIM* embryos were *yellow*. However, we do not believe this significantly influenced our results since our microarray data indicate that *yellow* is expressed at extremely low levels during the embryonic stages we tested; furthermore, the *yellow* transcript expression levels were not significantly different between the *ghIM* and control embryos on the microarrays.

### Highly Enriched Gene Ontology Categories of the Misregulated Genes from the *Drosophila grh* Embryo Microarrays

Assayed at stages 16–17 of embryogenesis, zygotic loss of GRH function has a huge impact on the *Drosophila* transcriptome as a whole, as over 1,200 genes (FDR <0.01) were seen to be misregulated (up or down) in *ghIM* mutants compared with wild type (see the Materials and Methods section for the accession numbers of the *Drosophila* microarray datasets). Verification of microarray fold-change directionality for eight genes using quantitative RT-PCR is shown in Figure S5.

A search for enriched GO “Biological Process” (BP) and “Molecular Function” (MF) categories was performed (Text S1), and the top ten and eleven most significant classes, respectively, are shown in Table 1 (see Table S1 for the full lists of the significantly enriched GO-BP, GO-MF, and GO “Cellular Component” categories). As GRH is known to be very important for cuticle development and wound healing in *Drosophila*, we expected to see numerous genes involved in these processes misregulated on the microarrays. Indeed, four of the most significant GO-BP classes (e.g., “chitin metabolic process” and “aminoglycan metabolic process”) and five of the most significant GO-MF classes (e.g., “structural constituent of the cuticle” and “chitin binding”) are consistent with the known role of GRH in regulating the formation of chitin-based cuticular barriers. Surprisingly, the remaining six GO-BP categories (of the top ten) were all composed of genes involved in either innate immunity or the stress response (e.g., “defense response”, “immune response”, and “humoral immune response”). Similarly, the remaining six GO-MF categories (of the top eleven) were all composed of genes that encode products with either serine-protease or serine-protease-inhibitor activity. This was interesting because serine protease cascades are used to trigger the hemolymph melanization-reactions used in response to infection, and serine protease inhibitors (also known as serpins) are used to limit the spread of this reaction (for a review see [44]). This was initially puzzling, as most of these genes were seen to be up-regulated in the *ghIM* mutants (Table 2), and GRH has no known function as an inhibitor of the immune response.

From these we results, we conclude that in addition to the expected misregulation of genes involved in cuticle formation, late-stage *ghIM* embryos are experiencing a massive wound/immune response as well. During the stages they were collected (late-stage 16 or early-stage 17 of embryogenesis) the *ghIM* embryos have weaker and more permeable epidermal barriers [1,6,12], yet are still motile, which can cause their fragile cuticles to rupture. Consistent with this, the *pale* gene, which encodes tyrosine hydroxylase, is known to be up-regulated around sterile wound sites in a largely *gh*-independent manner [3,4], and in *ghIM* embryos, *pale* transcripts are significantly up-regulated. This is also consistent with the observation that clean puncture wounding of late-stage embryos (in the absence of intentional microbial infection) also induces the expression of large numbers of *Drosophila* genes involved in innate immunity and the stress response (R. Patterson & W. McGinnis, unpublished).

### Misregulated Genes from the *Drosophila grh* Embryo Microarrays Reflect the Role of GRH in Barrier Formation and Wound Healing

We carried out a manual classification of the genes both and up- and down-regulated on the *Drosophila ghIM* embryo microarrays, and select genes are shown in Table 2. We placed 64 genes in the category “Cuticle Formation/Cutin Metabolism”, including genes involved in the generation and degradation of chitin molecules, as well as genes for many cuticle proteins that are deposited into the cuticle to mediate aspects of cuticle-shape and elasticity [61,62]. The majority of these genes (42 of 64) were down-regulated, consistent with potential direct regulation by GRH, and consistent with the idea that GRH is a crucial regulator of physical-barrier formation in *Drosophila*. However, a subset of these “cuticle/chitin” genes (22 of 64) were seen to be up-regulated in the *gh* mutants. It is possible that these up-regulated cuticle genes are normally directly repressed by GRH in wild-type embryos, but we believe it more likely that they are being overexpressed to compensate for the lack of the GRH-activated cuticle proteins, or they are being overexpressed in response to cuticular damage in *ghIM* mutants (see above).

Interestingly, one of the most strongly down-regulated genes on the microarray was *chitinaise 3* (~13 fold down) (Table 2), which is a *Drosophila* homolog of *Neurospora chitinaise 1* – the most strongly down-regulated gene in the *Neurospora gh* mutants (see above). We identified three high-affinity GRH binding sites within the two kb region upstream of the *Drosophila chitinaise 3* transcriptional start site, and *chitinaise 3* is also extensively co-expressed with GRH throughout the *Drosophila* epidermis and tracheal system during embryogenesis (data not shown), consistent with direct regulation by GRH.
Table 2. Select misregulated genes from the late-stage grh<sup>IM</sup> embryo microarrays.

| CG #      | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR     |
|-----------|---------------------|----------------------|------------------|---------|
| CG2044    | Lcp4                | cuticle protein      | −2.36E - 07      |         |
| CG30163   | Cpr60D              | cuticle protein      | 1.20E - 06       |         |
| CG18066   | Cpr57A              | cuticle protein      | 4.27E - 06       |         |
| CG15515   | −                   | cuticle protein      | 7.15E - 06       |         |
| CG2043    | Lcp3                | cuticle protein      | 3.64E - 05       |         |
| CG18140   | Cutinase 3          | chitin metabolism    | 2.48E - 05       |         |
| CG7941    | Cpr67Fa1            | cuticle protein      | 4.05E - 05       |         |
| CG6955    | Lcp65Ad             | cuticle protein      | 5.02E - 04       |         |
| CG4052    | Cpr5C               | cuticle protein      | 7.31E - 05       |         |
| CG8697    | Lcp2                | cuticle protein      | 1.60E - 04       |         |
| CG32400   | Lcp65Ab1            | cuticle protein      | 1.65E - 04       |         |
| CG17052   | obstructor-A         | cuticle organization | 2.64E - 04       |         |
| CG8510    | Cpr49Af             | cuticle protein      | 2.75E - 04       |         |
| CG9070    | Cpr47Eg             | cuticle protein      | 2.79E - 04       |         |
| CG6217    | knickkopf           | cuticle organization | 3.19E - 04       |         |
| CG14250   | TweedleQ            | cuticle protein/body shape | 3.72E - 04 |         |
| CG7287    | Lcp65Aa             | cuticle protein      | 3.91E - 04       |         |
| CG4778    | obstructor-B         | cuticle organization | 3.88E - 04       |         |
| CG18773   | Lcp65Ab2            | cuticle protein      | 5.24E - 04       |         |
| CG7216    | Acp1                | cuticle protein      | 5.02E - 04       |         |
| CG14643   | TweedleG            | cuticle protein/body shape | 6.66E - 04 |         |
| CG9939    | miniature           | cuticle organization | 1.33E - 03       |         |
| CG14639   | TweedleF            | cuticle protein/body shape | 1.79E - 03 |         |
| CG10297   | Acp65Aa             | cuticle protein      | 1.38E - 03       |         |
| CG11650   | Lcp1                | cuticle protein      | 1.18E - 03       |         |
| CG10529   | Lcp65Ae             | cuticle protein      | 1.31E - 03       |         |
| CG5883    | −                   | chitin metabolism    | 1.38E - 03       |         |
| CG7548    | −                   | cuticle protein      | 2.01E - 03       |         |
| CG9535    | mummy               | chitin biosynthesis  | 2.37E - 03       |         |
| CG11142   | obstructor-E         | cuticle organization | 2.77E - 03       |         |
| CG5494    | Cpr92F              | cuticle protein      | 2.92E - 03       |         |
| CG33302   | Cpr31A              | cuticle protein      | 2.95E - 03       |         |
| CG18779   | Lcp65Ag3            | cuticle protein      | 3.08E - 03       |         |
| CG12009   | −                   | chitin metabolism    | 6.66E - 03       |         |
| CG7252    | −                   | chitin metabolism    | 4.48E - 03       |         |
| CG9295    | Cpr76Bc             | cuticle protein      | 4.82E - 03       |         |
| CG12755   | l(3)mbn             | cuticle protein      | 4.95E - 03       |         |
| CG32499   | Cda4                | chitin metabolism    | 6.23E - 03       |         |
| CG15008   | Cpr64Ac             | cuticle protein      | 5.95E - 03       |         |
| CG18778   | Cpr65Au             | cuticle protein      | 6.47E - 03       |         |
| CG32404   | Cpr65Aw             | cuticle protein      | 6.89E - 03       |         |
| CG5812    | TweedleT            | chitin metabolism    | 9.30E - 03       |         |
| CG9781    | obstructor-G         | cuticle protein/body shape | 9.48E - 03 |         |
| CG9307    | Cutinase 5          | cuticle organization | 8.48E - 03       |         |
| CG9079    | Cpr47Ea             | chitin metabolism    | 8.29E - 03       |         |
| CG8515    | Cpr49Ah             | cuticle protein      | 6.72E - 03       |         |
| CG2555    | Cpr11B              | cuticle protein      | 5.96E - 03       |         |
| CG6773    | sec13               | cuticle protein      | 4.89E - 03       |         |
| CG9665    | Cpr73D              | cuticle organization | 6.30E - 03       |         |
### Cuticle Formation/Chitin Metabolism (64)

| CG #  | Gene Name or Symbol | Protein Type/Process                  | Fold (wt value)* | FDR       |
|-------|---------------------|---------------------------------------|------------------|-----------|
| CG7876 | Muc18B              | cuticle protein                       | 2.12 (5161)      | 3.78E – 03|
| CG10725 | –                   | chitin metabolism                     | 2.22 (4127)      | 2.45E – 03|
| CG7539 | Edg91               | cuticle protein                       | 2.26 (20178)     | 2.19E – 03|
| CG4784 | Cpr72Ec             | cuticle protein                       | 2.29 (180)       | 2.77E – 03|
| CG10533 | Lcp65Af             | cuticle protein                       | 2.34 (61521)     | 2.70E – 03|
| CG15006 | Cpr64Aa             | cuticle protein                       | 2.46 (148)       | 1.48E – 03|
| CG10531 | Chitinase           | chitin metabolism                     | 2.46 (108)       | 1.43E – 03|
| CG10501 | a methyl dopa-resistant | dopamine synthesis            | 2.54 (15116)     | 3.05E – 04|
| CG1963  | Pcd                 | dopamine synthesis                     | 2.29 (2769)      | 1.65E – 03|
| CG42639 | prophenol oxidase A1 | melanization effector                | 2.29 (14798)     | 4.14E – 03|
| CG10244 | Cad96Ca/Stitcher   | atypical RTK/wound healing            | 1.91 (12245)     | 9.35E – 03|
| CG1102  | MP1                 | serine protease/melanization activator| 1.75 (4362)      | 9.94E – 03|
| CG10118 | Pale tyrosine hydroxylase/dopamine synthesis | 3.77 (6615) | 2.53E – 04|

### Melanization/Wound Healing (9)

| CG #  | Gene Name or Symbol | Protein Type/Process                  | Fold (wt value)* | FDR       |
|-------|---------------------|---------------------------------------|------------------|-----------|
| CG10501 | a methyl dopa-resistant | dopamine synthesis            | –5.46 (15116)     | 3.05E – 04|
| CG1963  | Pcd                 | dopamine synthesis                     | –2.92 (6569)     | 1.65E – 03|
| CG42639 | prophenol oxidase A1 | melanization effector                | –2.29 (14798)    | 4.14E – 03|
| CG10244 | Cad96Ca/Stitcher   | atypical RTK/wound healing            | –1.91 (12245)    | 9.35E – 03|
| CG1102  | MP1                 | serine protease/melanization activator| 1.75 (4362)      | 9.94E – 03|
| CG10501 | a methyl dopa-resistant | dopamine synthesis            | –5.46 (15116)     | 3.05E – 04|
| CG1963  | Pcd                 | dopamine synthesis                     | –2.92 (6569)     | 1.65E – 03|
| CG42639 | prophenol oxidase A1 | melanization effector                | –2.29 (14798)    | 4.14E – 03|
| CG10244 | Cad96Ca/Stitcher   | atypical RTK/wound healing            | –1.91 (12245)    | 9.35E – 03|
| CG1102  | MP1                 | serine protease/melanization activator| 1.75 (4362)      | 9.94E – 03|
| CG10118 | Pale tyrosine hydroxylase/dopamine synthesis | 3.77 (6615) | 2.53E – 04|

### Serine Proteases and Serpins (44)

| CG #  | Gene Name or Symbol | Protein Type/Process                  | Fold (wt value)* | FDR       |
|-------|---------------------|---------------------------------------|------------------|-----------|
| CG11912 | –                   | serine protease (6)                  | –42.3 (2411)*    | 2.37E – 06|
| CG7722  | Snp47C              | serpin                               | –22.9 (1389)*    | 8.99E – 06|
| CG16997 | –                   | serine protease (2)                  | –6.98 (9899)     | 1.38E – 04|
| CG16704 | –                   | serine protease (2)                  | –5.39 (973)      | 2.94E – 04|
| CG1342  | Spn100A             | serpin                               | –5.39 (18723)    | 2.94E – 04|
| CG4386  | –                   | serine protease                      | –4.35 (1386)     | 7.19E – 04|
| CG1200  | –                   | serine protease (2)                  | –4.05 (593)      | 6.74E – 04|
| CG11843 | –                   | serine protease (2)                  | –3.11 (212)*     | 1.63E – 03|
| CG2071  | Ser6                | serine protease                      | –2.66 (8292)     | 2.29E – 03|
| CG12172 | Snp43Aa             | serpin                               | –2.4 (3524)      | 3.63E – 03|
| CG12385 | thetaTry            | Trypsin                              | –2.36 (1322)     | 3.73E – 03|
| CG18477 | –                   | serine protease (6)                  | –2.17 (234)      | 5.14E – 03|
| CG33160 | –                   | serine protease                      | –1.99 (59006)    | 7.50E – 03|
| CG6483  | Jonah 65Aiii        | serine protease (5,6)                | –1.89 (29930)    | 9.49E – 03|
| CG33127 | –                   | serine protease                      | 1.81 (9020)      | 7.85E – 03|
| CG5246  | –                   | serine protease (2,6)                | 1.96 (77)*       | 6.13E – 03|
| CG9649  | –                   | serine protease (2)                  | 2.01 (553)       | 4.25E – 03|
| CG12388 | kappaTry            | Trypsin                              | 2.04 (2297)      | 4.13E – 03|
| CG3513  | –                   | serpin                               | 2.08 (151)       | 3.44E – 03|
| CG9456  | Spn1                | serpin                               | 2.14 (422)       | 4.06E – 03|
| CG33329 | Sp212               | serine protease                      | 2.15 (424)       | 3.84E – 03|
| CG #  | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR  |
|-------|---------------------|----------------------|------------------|------|
| CG3344 | —                   | serine protease (6)  | 2.19 (4432)      | 2.52E-03 |
| CG5639 | —                   | serpin                | 2.36 (8777)      | 1.79E-03 |
| CG8869 | Jonah 25Bii         | serine protease (1,3,5,6) | 2.4 (569) | 1.62E-03 |
| CG8871 | Jonah 25Biii        | serine protease (1,5) | 2.51 (4743)      | 1.43E-03 |
| CG9672 | —                   | serine protease       | 2.54 (292)       | 1.40E-03 |
| CG7754 | iotaTry             | Trypsin               | 2.57 (1749)      | 1.26E-03 |
| CG18180| —                   | serine protease (1,5) | 2.63 (65)*       | 1.07E-03 |
| CG1859 | Spn43Ad             | serpin (1,2)          | 2.79 (3765)      | 8.27E-04 |
| CG18681| epsilonTry          | Trypsin (6)           | 2.96 (122)       | 6.83E-04 |
| CG4998 | —                   | serine protease       | 2.97 (66250)     | 6.21E-04 |
| CG11668| —                   | serine protease       | 2.98 (282)       | 6.19E-04 |
| CG11911| —                   | serine protease (2)   | 3.12 (26537)     | 5.65E-04 |
| CG7432 | —                   | serine protease       | 3.15 (3722)      | 4.96E-04 |
| CG7754 | iotaTry             | Trypsin               | 3.17 (46409)     | 4.28E-03 |
| CG18180| —                   | serine protease       | 3.59 (1111)      | 3.56E-04 |
| CG1859 | Spn43Ad             | serpin (1,2)          | 3.68 (568)       | 2.96E-04 |
| CG18681| epsilonTry          | Trypsin (6)           | 3.7 (146)        | 2.80E-04 |
| CG12351| deltaTry            | Trypsin               | 3.93 (355)       | 6.21E-04 |
| CG33459| —                   | serine protease       | 6.68 (84)*       | 4.15E-05 |
| CG33028| gammaTry            | Trypsin               | 6.96 (181)       | 4.31E-05 |
| CG8867 | Jonah 25Bii         | serine protease (3,5) | 8.72 (109)       | 2.17E-05 |
| CG9733 | —                   | serine protease       | 9.14 (727)       | 1.81E-05 |
| CG18211| betaTry             | Trypsin               | 24.12 (106)      | 2.13E-06 |

Innate Immunity (37)

| CG #  | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR  |
|-------|---------------------|----------------------|------------------|------|
| CG18108| IM1                 | putative AMP (1,2,6) | −58.95 (3622)*   | 9.45E-07 |
| CG14823| —                   | lysozyme             | −9.02 (6023)     | 6.43E-05 |
| CG7709 | Mucin 91C           | ECM component        | −2.63 (46409)    | 2.48E-03 |
| CG7106 | lectin-28C          | putative PRR         | −2.31 (420)      | 4.25E-03 |
| CG30062| —                   | lysozyme             | −2.05 (293)      | 6.60E-03 |
| CG6124 | eater               | PRR/phagocytosis     | −1.94 (356)      | 8.79E-03 |
| CG1179 | LysB                | lysozyme             | 1.84 (382)       | 6.80E-03 |
| CG5008 | GNB3                | PRR (Fungi)/Toll-signaling | 1.96 (189) | 5.55E-03 |
| CG18279| IM10                | putative AMP (1,6)   | 1.97 (6532)      | 6.84E-03 |
| CG6426 | —                   | lysozyme             | 2.02 (26238)     | 4.31E-03 |
| CG10146| Attacin-A           | AMP (GN Bacteria) (1,2,3,4,6) | 2.02 (80)* | 5.19E-03 |
| CG16705| SPE                 | serine protease/Toll-signaling | 2.06 (3498) | 4.04E-03 |
| CG7876 | Mucin 18B           | ECM component        | 2.12 (5161)      | 3.78E-03 |
| CG14704| PGRP-LB             | catalytic PGRP (1,6) | 2.13 (240)       | 2.97E-03 |
| CG11159| —                   | lysozyme             | 2.24 (326)       | 2.52E-03 |
| CG1180 | LysE                | lysozyme             | 2.44 (439)       | 1.50E-03 |
| CG33717| PGRP-LD             | PRR                  | 2.63 (1653)      | 1.08E-03 |
| CG4432 | PGRP-LC             | PRR (GN Bacteria)/Imd-signaling (1) | 2.63 (474) | 1.06E-03 |
| CG15678| pirk                | response to symbiotic bacteria | 2.87 (998) | 7.21E-04 |
| CG9697 | PGRP-SB2            | catalytic PGRP       | 3.12 (67)*       | 5.07E-04 |
| CG8175 | Metchnikowin        | AMP (Fungi) (1,2,3,6) | 3.3 (296) | 4.05E-04 |
| CG15065| IM2-like            | putative AMP (1,2)   | 4.01 (1102)      | 1.95E-04 |
| CG1165 | LysS                | lysozyme             | 4.17 (204)       | 1.70E-04 |
| CG10794| Diptericin B        | AMP (GN Bacteria) (1,3,4) | 4.19 (193) | 1.70E-04 |
| CG #   | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR    |
|--------|---------------------|----------------------|------------------|--------|
| CG15231 | IM4                 | putative AMP (1,6)   | 4.35 (13040)     | 1.88E-04 |
| CG16844 | IM3                 | putative AMP (1,3,6) | 5.45 (10195)     | 1.74E-05 |
| CG2279  | drosomycin-2        | AMP (Fungi)          | 5.49 (177)       | 1.73E-05 |
| CG15066 | IM23                | putative AMP (1,6)   | 5.69 (853)       | 1.71E-05 |
| CG9120  | LysX                | lysozyme             | 6.62 (93)        | 1.70E-05 |
| CG18372 | Attacin-B           | AMP (GN Bacteria) (1,2,3,4,6) | 6.44 (77)* | 1.67E-05 |
| CG10810 | Drosomycin          | AMP (Fungi) (1,2,3)  | 6.60 (1095)      | 1.66E-05 |
| CG4740  | Attacin-C           | AMP (GN Bacteria) (1,3,4) | 6.63 (72)* | 1.66E-05 |
| CG13422 | PRR                | putative AMP (1,2,3,6) | 7.12 (64)* | 1.65E-05 |
| CG9118  | LysD                | lysozyme             | 9.12 (75)        | 1.64E-05 |
| CG10812 | drosomycin-5        | AMP (Fungi) (1,2)    | 13.8 (81)*       | 1.63E-05 |

**Cytoskeleton/Cell Adhesion/Apico-Basal Polarity (19)**

| CG #   | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR    |
|--------|---------------------|----------------------|------------------|--------|
| CG9379 | blistery            | tensin/focal adhesion component | 6.28 (13823)     | 1.91E-04 |
| CG31190| Dscam3              | homophilic cell adhesion | 6.21 (455)*      | 2.03E-04 |
| CG18250| Dystroglycan        | apico-basal polarity; anchoring to ECM | 4.81 (862) | 6.87E-04 |
| CG31009| Cad99C              | cadherin/actin organization | 4.01 (6509)     | 2.56E-03 |
| CG42610| Fhos                | actin organization | 3.67 (11613)     | 9.41E-03 |
| CG3320 | Rab1                | small GTPase/actin organization | 2.57 (15615) | 3.45E-03 |
| CG6445 | Cad74A              | cadherin/cell adhesion | 2.36 (2895)      | 4.05E-03 |
| CG5055 | bazooka             | Par3 homolog/apico-basal polarity | 2.31 (2298) | 3.96E-03 |
| CG17716| faint sausage       | epithelial morphogenesis | 2.29 (7220)     | 4.87E-03 |
| CG42734| Ankyrin 2           | microtubule organization | 1.98 (2520) | 7.00E-03 |
| CG12437| raw                 | epithelial morphogenesis | 1.75 (2601) | 9.66E-03 |
| CG42614| scribbled           | apico-basal polarity | 1.83 (2482)      | 7.13E-03 |
| CG17957| Sry-alpha           | actin organization | 1.87 (215)       | 6.86E-03 |
| CG6976 | Myo28B1             | myosin/molecular motor | 1.9 (1697)      | 5.60E-03 |
| CG4316 | Stubble             | serine protease/actin organization | 2.07 (587) | 4.89E-03 |
| CG33979| capulet             | actin organization | 2.13 (818)       | 3.60E-03 |
| CG10125| zero population growth | gap junction channel | 2.21 (127) | 2.63E-03 |
| CG8978 | Suppressor of profilin 2 | actin organization | 2.26 (13355) | 2.34E-03 |
| CG5178 | Act88F              | actin | 2.43 (95)*       | 1.53E-03 |

**Detoxification (44)**

| CG #   | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR    |
|--------|---------------------|----------------------|------------------|--------|
| CG1944 | Cyp4p2              | P450 (Fat Body (7))  | 23.79 (490)*     | 4.20E-07 |
| CG10241| Cyp6a17             | P450 (Hindgut (7))   | 66.78 (3284)*    | 8.68E-07 |
| CG33503| Cyp12d1-d           | P450 (Fat Body, Midgut, Malphigian Tubes (7)(8)) | 22.22 (1314)* | 9.99E-06 |
| CG18730| Amylase proximal    | detoxification (8)   | 11.54 (1153)     | 3.31E-05 |
| CG10842| Cyp4p1              | P450 (Midgut, Malphigian Tubes (7)) | 10.72 (5972) | 4.31E-05 |
| CG33546| gfzf                | glutathione S-transferase | 10.04 (12617) | 4.58E-05 |
| CG17876| Amylase distal      | detoxification (8)   | -5.86 (885)      | 2.39E-04 |
| CG9363 | –                   | glutathione S-transferase | -5.49 (6388) | 2.91E-04 |
| CG1488 | Cyp311a1            | P450 (Midgut (7))    | -4.11 (454)      | 6.29E-04 |
| CG30489| Cyp12d1-p           | P450 (Fat Body, Midgut, Malphigian Tubes (7)) | 4.02 (1004) | 7.24E-04 |
| CG8652 | Ugt37c1             | glucuronosyltransferase | -2.56 (1005) | 2.70E-03 |
| CG9362 | –                   | glutathione S-transferase | -2.33 (2238) | 3.84E-03 |
| CG31002| –                   | glucuronosyltransferase | -2.27 (1530) | 4.29E-03 |
| CG #  | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR     |
|-------|---------------------|----------------------|------------------|---------|
| CG17527 | GstE5               | glutathione S-transferase | −2.22 (3038)    | 6.11E−03|
| CG12242 | GstD5               | glutathione S-transferase | −2.21 (223)     | 4.83E−03|
| CG13271 | Ugt368b             | glucuronosyltransferase | −2.19 (240)     | 4.97E−03|
| CG17525 | GstE4               | glutathione S-transferase | −2.16 (1686)    | 5.26E−03|
| CG5137  | P450 (Gonads)       |                       | −2.11 (322)     | 5.94E−03|
| CG11289 | −                   | glucuronosyltransferase | −2.05 (997)     | 6.70E−03|
| CG8453  | P450 (Fat Body, Midgut, Malphigian Tubes (7)) | | −2.03 (463) | 7.11E−03|
| CG4688  | −                   | glutathione S-transferase | 1.78 (245)      | 8.52E−03|
| CG4026  | IP3K1               | oxidative stress response | 1.8 (2853)      | 8.64E−03|
| CG1829  | P450 (Gonads (7))   |                       | 1.78 (245)      | 8.38E−03|
| CG8587  | P450 (Hindgut (7))  |                       | 1.8 (463)       | 7.51E−03|
| CG4772  | Ugt86Dh             | glucuronosyltransferase | 1.8 (2621)      | 7.46E−03|
| CG6633  | P450 (Malphigian Tubes (7)) | | 1.85 (591) | 9.17E−03|
| CG31507 | Jheh2               | detoxification (8)    | 1.99 (2525)     | 4.67E−03|
| CG15661 | −                   | glucuronosyltransferase | 2.01 (846)      | 6.75E−03|
| CG3943  | kraken              | digestion; detoxification | 2.05 (8581)    | 4.02E−03|
| CG4485  | Cyp9b1              | P450 (7)              | 2.06 (533)      | 3.49E−03|
| CG5999  | −                   | glucuronosyltransferase | 2.28 (64)*      | 2.05E−03|
| CG1702  | −                   | glutathione S-transferase | 2.49 (2822)    | 1.49E−03|
| CG13270 | Ugt368a             | glucuronosyltransferase | 2.73 (6015)     | 9.76E−04|
| CG11012 | Ugt37a1             | glucuronosyltransferase | 4.47 (93)*      | 1.37E−04|
| CG3481  | Adh                 | alcohol dehydrogenase | 4.76 (21147)    | 1.21E−04|
| CG10248 | Cyp6a20             | P450 (7)              | 5.85 (2512)     | 6.56E−05|
| CG4302  | −                   | glucuronosyltransferase | 6.55 (2304)     | 4.20E−05|
| CG5724  | −                   | glucuronosyltransferase (8) | 9.13 (117) | 1.74E−05|
| CG8345  | Cyp6v1              | P450 (Fat Body, Midgut, Malphigian Tubes (7)) | 9.13 (128) | 1.67E−05|
| CG18539 | Cyp309a2            | P450 (Gonads (7))     | 28.2 (94)*      | 1.58E−06|

| CG #  | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR     |
|-------|---------------------|----------------------|------------------|---------|
| CG16954 | Hsp60D             | heat shock protein   | −5.85 (546)*     | 2.42E−04|
| CG33117 | Victoria           | Turandot-like        | −3.99 (500)      | 7.06E−04|
| CG6830  | Hsp60B             | heat shock protein   | −3.06 (6325)     | 1.97E−03|
| CG6404  | Gilal Lazarillo    | ApoD ortholog        | −2.78 (9228)     | 1.94E−03|
| CG40209 | Peroxidase         | ECM peroxidase (1,2,6)/ROS metabolism | −2.62 (6843) | 2.91E−03|
| CG6646  | DJ-1alpha          | oxidative stress response | −2.5 (686)      | 3.29E−03|
| CG7052  | TepII              | opsonization; humoral response (1,2,6) | −2.19 (6648) | 5.88E−03|
| CG6871  | Catalase           | ROS metabolism; hydrogen peroxide breakdown | 2.25 (19730) | 2.44E−03|
| CG31059 | Turandot A         | humoral stress response (6) | 2.51 (68)*      | 1.34E−03|
| CG6186  | Transferrin 1      | Iron sequestration (2) | 2.92 (105)      | 6.96E−04|
| CG4183  | Hsp26              | heat shock protein (1) | 3.76 (563)      | 2.47E−04|
| CG6489  | Hsp70Bc            | heat shock protein (1,3,4) | 4.75 (156) | 1.11E−04|
| CG31449 | Hsp70Ba/Bb/Bbb     | heat shock protein (4) | 5.51 (297)      | 7.73E−05|
| CG31508 | Turandot C         | humoral stress response (6) | 5.54 (70)      | 7.52E−05|
Table 2. Cont.

Cuticle Formation/Chitin Metabolism (64)

| CG # | Gene Name or Symbol | Protein Type/Process | Fold (wt value)* | FDR |
|------|---------------------|----------------------|------------------|-----|
| CG31366 | Hsp70Ab/Ab | heat shock protein (4) | 7.48 (1513) | 3.13E-05 |
| CG31359 | Hsp70Bb/Bbb | heat shock protein (4) | 7.54 (383) | 2.69E-05 |

Select significantly misregulated genes were manually classified into the following categories: Cuticle Formation/Chitin Metabolism; Melanization/Wound Healing; Serine Proteases/Serpins; Innate Immunity; Cytoskeleton/Cell Adhesion/Apical-Basal Polarity; Detoxification; and Defense/Stress Response. "CG #" refers to the accession numbers from FlyBase. “Gene Name or Symbol” refers to either the full gene name or the gene symbol on Flybase; this column is blank if no assigned gene name was found in Flybase. "Protein Type/Process" refers to experimentally verified or putative (most often based on homology) functions assigned to the genes. Numbers in curly brackets refer to studies in which these genes were also seen to be misregulated upon the following treatments: (1) bacterial infection [87-89]; (2) fungal infection [87,88,90]; (3) viral infection [90,91]; (4) Wolbachia infection [90,92]; (5) Microsporidia infection [90]; and (6) parasitoid infection [93,94]. Categories (1-6) were adapted from [66]. [7] refers to a systematic analysis of the expression patterns of the Drosophila p450 genes [66,81] refers to a systematic analysis of detoxification genes in Drosophila [69]. "Fold (wt value)*" refers to the fold changes seen in the expression of these genes relative to wild type. Absolute wild-type fluorescence values are shown in parentheses. An asterisk next to a value means the lowest value in the category "Melanization/Wound Healing" (Table 2), four of which were down-regulated. The three most strongly down-regulated genes in this class (alpha methyl dopa-resistant, Ped, and prophenol oxidase A1) are known or suspected to be directly involved in the cuticular melanization/sclerotization pathway. Stitches/Cad/96Ca, a wound-induced gene known to be directly activated by GRH [5], was significantly down-regulated on our microarrays. Dopa decarboxylase, another gene known to be directly dependent on GRH for its expression during development [1] and the epidermal wound-response [3], was down-regulated (~1.7 fold down), although it did not pass the stringent FDR threshold we set and is not shown in Table 2.

Also consistent with the grh mutant phenotypes, 19 genes known or predicted to be involved in a category we called “Cell Adhesion/Apical-Basal Polarity/Cytoskeleton” were significantly misregulated (Table 2). This category includes three Cadherin-domain-containing protein genes, including Dystroglycan (~5 fold down), which is required for maintaining the apical-basal polarity of epithelial cells and anchoring the intracellular actin cytoskeleton to the extracellular matrix [65]. Another gene required for apical-basal polarity and adhesion of epidermal cells that was significantly down-regulated in grhIM mutants (~2.3 fold down) is bazooka [64]. Previous studies by Narasimha et al. [13] have shown that two genes encoding components of the Drosophila septate junction – coracle and Fasciclin 3 – were expressed at reduced levels in grh mutant clones in imaginal disc epithelia, and that coracle and Fasciclin 3 gene expression could be activated by ectopic GRH protein in embryonic amnioserosa cells. As measured by our late-stage embryonic grh mutant microarrays, coracle transcript levels were very slightly lowered, but the difference compared to wild-type levels was not statistically significant. Fasciculin 3, which was assessed by eleven different sequences on the microarray chip we used, was reproducibly reduced in grh mutants; we found that the expression of this gene was ~25% lower than wild type in every experimental and biological replicate, although this difference was never significant enough to reach the stringent FDR threshold we set. Therefore, it is important to note that since we used whole embryos as the RNA source for our microarray experiments there are likely to be many true GRH-regulated genes that were not identified as significant in our analyses, such as coracle and Fasciculin 3. It is likely that this category will include genes whose expression is only quantitatively changed in grh mutant backgrounds, or whose expression is limited to only a subset of the cells that produce GRH protein, and thus would not pass our FDR threshold.

We placed nine genes in the category “Melanization/Wound Healing” (Table 2), four of which were down-regulated. The three most strongly down-regulated genes in this class (alpha methyl dopa-resistant, Ped, and prophenol oxidase A1) are known or suspected to be directly involved in the cuticular melanization/sclerotization pathway. Stitches/Cad/96Ca, a wound-induced gene known to be directly activated by GRH [5], was significantly down-regulated on our microarrays. Dopa decarboxylase, another gene known to be directly dependent on GRH for its expression during development [1] and the epidermal wound-response [3], was down-regulated (~1.7 fold down), although it did not pass the stringent FDR threshold we set and is not shown in Table 2.

Misregulated Genes from the Drosophila Microarrays Indicate Mutation of grh Triggers Innate-immune and Stress-response Pathways

While a comprehensive analysis of all the genes involved in innate immunity, stress, and detoxification that were seen to be misregulated in grhIM mutants is beyond the scope and focus of this paper (and because many are very likely to be misregulated due to indirect effects of the grhIM phenotype), we will only briefly review the major classes of genes.

Thirty-seven genes in the category “Innate Immunity” (Table 2) were misregulated in grhIM embryos, and they included genes from nearly every aspect of Drosophila innate immunity [44]. One innate-immune gene, IM1, was strongly down-regulated, suggesting the potential for direct activation of IM1 by GRH in the epidermis. Consistent with this possibility, there is a near perfect palindromic GRH binding site (AACTGGTTT) found less than 600 bp upstream of the IM1 gene. Other down-regulated immunity genes potentially under the direct control of GRH include lectin-28C, Metchnikowin, and two putative lysozymes. However, the majority of innate-immune genes that were misregulated in grhIM mutants were up-regulated (31 of 37), and they include known antimicrobial peptides (e.g., Attacin, Drosomycin, Diptericin B, and Metchnikowin), lysozymes, Pattern-Recognition Receptors, and the Toll-signaling activator Spaetzle-Processing Enzyme (SPE).

Eighteen genes in the “Defense/Stress Response” category were misregulated in grhIM embryos (Table 2). The two most strongly down-regulated genes were methuselah-like 8 and 3. Mutations in a paralogous gene (methuselah) have been correlated with longer lifespan and increased resistance to stress in Drosophila [65], so it possible that the observed down-regulation of mthl-8 and -3 was a response to tissue damage and stress in these embryos. Seven heat shock protein (hsp) genes were also misregulated in grhIM embryos, five of which were up-regulated and have been shown elsewhere to be differentially expressed upon infection of adult flies with microbes (Hsp26, 70Bc, 70Bb, 70Bbb, and 70Da) [66]. Tardatol A, C, and Victoria were also seen to be up-regulated, which are believed to act as extra-cellular chaperones, binding to denatured proteins in the hemolymph that are released upon tissue damage or stress [67].
modifying toxic compounds in the cytoplasm or hemolymph in order to render them less active [69]. The misregulation of these genes (which were both up- and down-regulated in nearly equal proportions) is still somewhat unclear, although we propose that their expression levels are altered in response to the release of toxic endogenous compounds during tissue damage in grh	extsuperscript{emb} mutants. Taken together, these results indicate that late-stage grh	extsuperscript{emb} embryos have reduced expression of a wide variety of extracellular cuticular-barrier genes as well as a number of cell-cell adhesion genes [13] (Tables 1, 2, and S1); in addition they are experiencing a massive wound/immune response and are undergoing extreme stress, likely due to global tissue damage in response to cuticular tearing or epidermal-barrier permeability [6].

**GRH is Required for Epidermal Integrity During Larval Stages of Drosophila Development**

We wished to more fully characterize the phenotypes of grh null mutants at later stages of the *Drosophila* life cycle. However, as grh null-mutant embryos die at the embryonic/larval transition, this prohibited us from determining the function of GRH in grh	extsuperscript{emb} larvae and adults. To circumvent this, we produced a *Drosophila* strain in which GRH is knocked down in the larval epidermis (e13C>GRH	extsuperscript{RNAi}) by crossing a transgenic UAS-GRH	extsuperscript{RNAi} line with a strain containing the larval driver e13C-GAL4, which produces GAL4 in the larval epidermis, fat body, gut, imaginal discs, and salivary glands [70]. By immunostaining, we observed that GRH protein was undetectable in the epidermal tissues of late third instar salivary glands [70]. By immunostaining, we observed that GRH protein was undetectable in the epidermal tissues of late third instar salivary glands [70]. By immunostaining, we observed that GRH protein was undetectable in the epidermal tissues of late third instar salivary glands [70]. By immunostaining, we observed that GRH protein was undetectable in the epidermal tissues of late third instar salivary glands [70].

While generally healthy, wandering third-instar e13C>GRH	extsuperscript{RNAi} larvae all developed melanized clots of diverse size and distribution, which were never observed in control larvae (Figures 4A and B) or in e13C>GRH	extsuperscript{RNAi} larvae during the earlier first, second, or foraging third-instar stages (data not shown). Upon dissection it was found that these melanotic spots were tightly associated with epidermal cells of the body wall, similar to the phenotypes seen when both Dorsal and Dif transcription factors are knocked down during larval stages [70]. Furthermore, all e13C>GRH	extsuperscript{RNAi} larvae died at the prepupal stage with noticeably decreased body size compared with wild type, despite the fact that experimental and control larvae appeared similar in size prior to wandering (data not shown).

A lack of GRH function during *Drosophila* embryogenesis leads to a fragile cuticle [11,12] and increased epidermal permeability [6]. Therefore, it seemed likely that knocking down GRH during larval stages would have similar effects, which could explain both the presence of randomly localized melanized spots as well as the decreased body size (presumably due to fluid loss). In *Drosophila*, melanized spots have been associated with hyperactivation of the immune system [71], loss of both Dorsal and Dif transcription factors [70], and such dark spots also appear at wound sites, apparently to strengthen clots and prevent body-fluid loss following physical injury.

We propose that upon leaving the moist food source, wandering e13C>GRH	extsuperscript{RNAi} larvae develop multiple “micro-wounds” (as evidenced by the ectopic melanotic spots) due to the fragility of their cuticles, which is exacerbated by the dry conditions on the vial walls. Furthermore, we hypothesized that the decreased body size observed in these larvae is a result of fluid loss due to increased epithelial permeability as well as a loss of hemolymph following micro-wounding. These observations suggest that during larval stages, GRH is required for the maintenance of epidermal/cuticular-barrier integrity.

To determine whether epidermal GRH activity is required in larvae to prevent body-fluid loss following wounding, we wounded wandering third-instar larvae with a sterile needle and let them recover in either moist or dry conditions. Under moist conditions, both control and e13C>GRH	extsuperscript{RNAi} larvae maintained approximately the same body mass, even after clean injury (compare Figures 4C and D). However, when injured e13C>GRH	extsuperscript{RNAi} larvae were placed in dry conditions, they showed an obviously decreased body size 7 h after injury (Figures 4F). Injured control, uninjured control, or uninjured e13C>GRH	extsuperscript{RNAi} larvae did not show any obvious decreases in body size under dry conditions after 7 h (Figures 4E, E’, F). These results suggest that GRH activity in the epidermis is needed for properly repairing wounds and preventing catastrophic body-fluid loss following wounding under dry conditions. These effects could be due to failures or delays in wound healing in the knockout larvae, possibly caused by weakened cuticles due to lower GRH protein levels during larval stages.

We were curious to see if epidermal GRH knockdown could lead to desiccation in the absence of injury after longer periods (>7 h) in dry conditions. We incubated both injured and uninjured control and e13C>GRH	extsuperscript{RNAi} larvae for ~24 h under dry conditions. Although most uninjured (96.2±0.6%) and injured (65.6±3.9%) control larvae reached the prepupal stage without any obvious decreases in body size, all uninjured and injured e13C>GRH	extsuperscript{RNAi} larvae (100%) showed decreases in body size and died before initiating the pupariation process (data not shown). These results indicate that the larval function of GRH is crucial for avoiding excessive body-fluid loss under dry conditions, and it is necessary for viability even in the absence of major injury.

**Silencing of GRH in Adult Drosophila Increases their Susceptibility to Septic Injury**

Due to the relatively short time-course of larval development, and the fact that e13C>GRH	extsuperscript{RNAi} larvae do not develop past the prepupal stage, examining the role of epidermal GRH expression in larval microbial resistance is problematic. Therefore, we focused on analysis of clean or septic injury in *Drosophila* adults in which grh function was knocked down by heat shock induced RNAi. We found that heat-shock driven expression of a UAS-GRH	extsuperscript{RNAi} (hs>GRH	extsuperscript{RNAi}) can efficiently eliminate GRH protein in most cells of the adult epidermis compared with similar treated control flies containing only the hs-GAL4 construct (Figures 5A and B). These hs>GRH	extsuperscript{RNAi} flies were completely viable and did not develop the drying phenotypes observed in e13C>GRH	extsuperscript{RNAi} larvae.

The hs>GRH	extsuperscript{RNAi} flies did not show any reduction in normal life span compared with control flies, indicating that GRH is not required for the homeostatic maintenance of adult viability, at least under laboratory conditions (Figure 5C, and data not shown). Next, we challenged knockout and control flies with either clean or septic injury using Ecc15 (a gram-negative bacterium) or *M. luteus* (a gram-positive bacterium), and their survival was monitored over a 10 day period. Although control and hs>GRH	extsuperscript{RNAi} flies showed similar survival curves after clean injury (Figure 5D), hs>GRH	extsuperscript{RNAi} flies showed decreased survival after *Ecc15* and *M. luteus* infection than controls. Ten days after septic injury with *Ecc15* or *M. luteus*, 40 and 67% of the GRH knockdown adults...
survived, respectively, compared with 57 and 80% of control adults that were heat shocked without GRHRNAi knockdown (Figures 5E and F). These results suggest that GRH plays some protective role in Drosophila adults following microbial infection. However, our embryonic microarrays indicate that ghr is not required to activate the standard repertoire of antimicrobial peptide genes, so it seems unlikely that GRH is crucial for activating the canonical genes of the innate immune response in adults [44]. Therefore, the exact nature of the protective effects imparted by GRH during Drosophila adulthood remains to be identified.

Discussion

Is there an ancestral connection between the transcriptional control of many fungal cell walls and animal epidermal extracellular barriers? This question is probably impossible to answer definitively, given the vast evolutionary distances between extant fungal and animal lineages and the loss of so many transitional states. However, because of the high-level conservation of GRH-family function in animal epidermal barrier formation, we believed that by studying the function of transcription factors related to the GRH family in the filamentous ascomycete fungus Neurospora crassa we might shed some light on this question. We find that with respect to several amino acid residues predicted to be important for DNA-binding specificity, fungal GRH-like proteins are more similar in sequence to the GRH family than to the LSF family of transcription factors. Consistent with this, we show that the Neurospora GRHL protein can bind to the same DNA consensus site as metazoan GRH-like proteins in vitro (albeit with a lower affinity). Therefore, we believe the last common ancestor of Fungi and Metazoa was likely to have contained at least one CP2 superfamily protein that was more related, both structurally and functionally, to existing animal GRH family proteins than to existing animal LSF family proteins.

Based on previously published GRH studies and the phenotype of the Neurospora ghl/csp-2 mutant, as well as a comparison of the transcriptome profiles of a Neurospora ghl knockout and a Drosophila ghl mutant, we present a model for the evolution of GRHL/CP2-family transcription factor function in the opisthokont lineage (Figure 6). We propose that the function of GRHL/CP2 proteins in the single-celled opisthokont last common ancestor was to regulate genes that contributed to both the formation and remodeling of an extracellular physical barrier (e.g., structural-biopolymer modifying enzymes and cell wall-associated proteins), and that it may also have regulated some genes that contributed to a defense-virulence “barrier”.

Strong evidence has accumulated that animal GRH-like proteins have a conserved function in the regulation of physical extracellular-barrier formation and wound healing in a wide variety of animal epithelial and epidermal tissues. For example, Drosophila GRH regulates the levels of genes encoding enzymes involved in cuticle melanization and chitin metabolism, cell adhesion proteins, and protein components of the cuticle. In mice, Grhl3 regulates the levels of genes that encode structural-barrier proteins in keratinocytes and the enzymes that crosslink such proteins, as well as cell-adhesion proteins and proteins that modulate the lipid composition of the epidermis [1,3,6,13–15,19–21,24,72,73]. We propose that the original functions of Grainy head-like proteins in the opisthokont last common ancestor predisposed GRH-like proteins to regulate many aspects of extracellular-barrier formation and wound healing in early animals, as well as to evolve the related ability of regulating cell-cell adhesion genes in many epithelial tissues.

In the metazoan lineage, many types of epidermal barriers have evolved over time, including epithelia with chitin-based extracellular barriers (e.g., the arthropod epidermis). We believe that chitin is one of the few extracellular structural biopolymers common to both fungi and animals. While chitin synthase itself does not appear to be regulated by GRH-like proteins in any system yet studied, it appears that GRH and GRH-like proteins of the CP2 superfamily regulate the expression of many genes involved in the formation and remodeling of chitin-based barriers, at least in Neurospora and Drosophila. It is also intriguing that chitinase I in Neurospora and chitinase 3 in Drosophila both appear to be strongly regulated by GRHL and GRH, respectively, consistent with an ancestral transcriptional control of chitinase expression by GRH-like proteins in the opisthokont last common ancestor. We believe it is possible that components of the ancestral opisthokont cell wall were repurposed (or redeployed) during the evolution of chitin-based apical extracellular barriers in some basal multicellular animals (Figure 6), with GRH proteins maintaining a role in barrier formation and remodeling during the process. A similar process may have occurred during the evolution of multicellular volvocine algae, as it has been proposed that the outer (tripartite) cell wall of unicellular algae evolved to become part of the apical extracellular barrier of multicellular algae [74]. This would have been independent of control by CP2 superfamily proteins, as sequenced genomes in the algal lineage do not encode recognizable members of this superfamily [28].

The evolution of multicellularity in fungi was presumably less complicated than in metazoans, as one can invoke incomplete cell division creating syncytial colonies of fungi. In this evolutionary scenario, the conservation of ancestral GRHL function with respect to barrier formation and remodeling would be straightforward, as the cell walls of the unicellular opisthokont last common ancestor and extant multicellular fungi would be very similar in structure and function. In addition to the greatly lowered expression of the chitinase I gene (which is likely to be partially responsible for the conidial separation phenotype observed in ghl strains), we also found evidence that Neurospora GRHL plays a role in the expression of enzymes involved in the synthesis and remodeling of another key biopolymer of the fungal cell wall – beta-1,3-glucan. GRHL may turn out to have a more general role in promoting cell wall development, although we were unable to uncover phenotypic evidence for this, despite testing the growth of ghl mutant strains under several conditions shown elsewhere to inhibit the growth of S. cerevisiae strains with compromised cell walls (e.g., high-osmolality media, high-temperature incubation, and media containing the chitin-binding molecule Calcofluor-
White) [75] (data not shown). However, it is important to note that most cell wall integrity assays in *Neurospora* are based on mycelial cell wall growth, and if the *grhl* phenotypes manifest mainly in their non-dispersing conidia, the assays we used would probably not uncover them.

Dispersing conidia are a cell type very likely to encounter novel and dangerous environments, and one could imagine that a fast growing organism such as *Neurospora* would devote more resources towards protecting their spores than their mycelia. With this in mind, it was very interesting to see that many of the down-regulated genes with known or predicted functions on the *Neurospora grhl* AHC microarrays were classified as defense and virulence genes, and that many of the proteins encoded by these genes are predicted to be secreted. Extracellular barriers (such as the fungal cell wall or animal epidermis) act as passive defense mechanisms against infection, but they can also contain molecules that are actively hostile to pathogens [44,45]. Furthermore, the distinction between defense and virulence in pathogenic fungi can be semantic – one way to become more virulent is to better defend yourself against your host, and vice versa. The deposition of

---

**Figure 5.** Loss of GRH function in adult flies increases their susceptibility to bacterial infection. (A and B) Whole-mount preparations of dissected adult abdominal epidermal tissue from control flies (*hs-GAL4*) and GRH knock-down flies expressing a *UAS-GRH*RNAi transgene driven by *hs-GAL4* (*hs>GRHRNAi*). Antibody stains for GRH (green) and Fasciclin 3 (red) are shown. (C–F) Survival curves from *hs-GAL4* and *hs>GRHRNAi* adults after mock treatment (C), clean injury (D), injury with a needle coated with gram-negative *Ecc15* bacteria (E), or injury with a needle coated with gram-positive *M. luteus* bacteria (F). The survival of adult flies was measured over the 10 day period after injury. The average values of three independent experiments are shown along with the standard errors of the mean.

doi:10.1371/journal.pone.0036254.g005
defense-virulence factors into the fungal cell wall could be analogous to how many epithelial barriers throughout the animal and plant kingdoms produce antimicrobial peptides, both proactively and in response to infection (e.g., the *Drosophila* trachea and epidermis, mammalian lung and skin, and plant cuticles) [44,45]. Unfortunately, *Neurospora crassa* does not have any characterized host-pathogen interactions, so we were unable to directly test the function of any of these genes in terms of their effects on virulence or defense. Experimental testing of the potential for GRHL proteins playing a direct role in defense and/or virulence will have to await studies in other ascomycete species with gene-knockout technology and well-characterized host-pathogen interactions.

While regulation of antimicrobial defense does not appear to be a major function of *Drosophila* GRH (at least in embryos), we did find a few innate immune genes that were significantly down-regulated on the *Drosophila grh* knock-down microarrays. We also found that knocking down GRH function in adult *Drosophila* increased susceptibility to septic (bacterial) wounding, without other discernable effects on overall health. Therefore, it is possible that GRH proteins might mediate some aspects of epidermal antimicrobial defense in *Drosophila*. There is as yet no functional evidence suggesting a role for mammalian GRH-family genes in epithelial antimicrobial defense, although the embryonic skin of mouse *Grhl3* mutants shows greatly reduced expression of one of the antimicrobial defensin genes, *Defa15* [21]. Although CP2 superfamily transcription factors with GRH-like properties were apparently encoded by the genome of the opisthokont last common ancestor, CP2/GRH-like proteins have
been lost in many fungal lineages and, so far, have only been found in the genomes of a subset of the Ascomycota and Zygomycota [20]. On the face of it, this seems at odds with our proposal that GRH-like proteins are crucially linked to the regulation of extracellular-barrier formation, since many fungi with perfectly functional extracellular barriers (e.g., the well-studied ascomycete *Saccharomyces cerevisiae*, and basidiomycete mushrooms) lack any detectable genes of the CP2 or GRHL types. This discrepancy could be explained by the fact that, in Fungi, transcriptional batteries of genes that produce identical biological outputs can evolve to be regulated by different combinations of upstream transcription factors. For example, mating type in most ascomycetes is regulated by the a2 transcription factor; however, this protein was lost in the lineage leading to *Saccharomyces cerevisiae*, which evolved a different combination of transcription factor inputs to determine mating type [76]. However, it is equally true that many animal transcription factor families, for hundreds of millions of years, have been regulating very similar developmental patterning or cell-type-specific properties during development [77] – a striking example of which is the conservation of GRH family function with respect to epithelial barrier formation in animals. It may be that the functions of animal transcription factors are somewhat more evolutionarily constrained than those of Fungi (perhaps due to differences in generation time, population size, or morphological complexity), and that Fungi are more likely to evolve new combinations of transcription factors to regulate core biological functions.

**Materials and Methods**

**Neurospora Stocks, grhl Knockouts, and grhl/csp-2 Complementation Assays**

Wild-type strains [FGSC2489 (74-OR23-1V, mat A) and FGSC4200 (ORS-SL6, mat a)], *grhl* knockout strains [FGSC13563 (ΔNCU06095, mat A) and FGSC13564 (ΔNCU06095, mat a)], and the NHEJ-deficient strain [FGSC9720 (Δmuw-52::bar; his-3, mat A)] were obtained from the Fungal Genetics Stock Center (FGSC) [78]. Stocks were maintained on minimal Vogel’s agar medium (VWR, 100357–652). After 48 h at 30°C under a 12 h light/dark cycle, and conidia were harvested by plating conidia on minimal Vogel’s agar medium. As the *grhl* knockout strains were derived from the NHEJ-deficient strain [FGSC9720 (Δmuw-52::bar; his-3, mat A)], and the NHEJ-deficient strain [FGSC9720 (Δmuw-52::bar; his-3, mat A)] were obtained from the Fungal Genetics Stock Center (FGSC) [78]. Stocks were maintained on minimal Vogel’s agar slants with 1.5% sucrose and appropriate supplements [36]. Genomic DNA for PCR analysis was obtained according to [79].

The isolation of the independently derived *grhl* deletion strains was performed by transforming a NCU06095-targeted hygromycin replacement cassette (courtesy of the Dunlap lab, Dartmouth) into FGSC9720, as described elsewhere [80]. Hygromycin-resistant colonies were selected, and homokaryotic *grhl* knockout strains were tested using PCR to verify loss of the *grhl* locus. All strains missing the *grhl* locus displayed the conidial separation phenotype. These new strains (Δmuw-52::bar; Δ*grhl*:hyg++; his-3, mat A) were also used in the complementation assay fusions described below. The primer sequences for verifying the *grhl* knockout and for detecting *grhl* transcripts (Figures 2A and B) were as follows: *grhl*-For – CACCATGTTCAGTCAACGAACAAG – and *grhl*-Rev – GCCCTTATGTCGCTGCTTTTC. Positive control primers were as follows: actin-For – ATCCGACAC-CGGCGCGAGGACCTGCACGATTCAGTCAACGAACAAG – and actin-Rev – TGGCAACAACCGCC-TCTCAAG.

Genetic complementation assays between *grhl* and *csp-2* were carried out by fusing one of the independently derived *grhl* deletion strains described above (Δmuw-52::bar; Δ*grhl*:hyg++; his-3, mat A) to ten different isolates of *csp-2*; *bd; ino*. The *csp-2*; *bd; ino* strains were created using standard crossing methods (csp-2; *bd* × *ino*). Exactly as expected, only half (five) of the fusings were viable on minimal media (due to opposite mating-type incompatibility), all of which displayed the conidial separation phenotype. Using PCR, the *csp-2*/*grhl* heterokaryons were verified as positive for the *grhl* locus, which is consistent with the deletion/stop codon in *csp-2* strains (data not shown).

**Neurospora GRHL and Drosophila GRH Protein Production and Gel-shifts**

The full-length *Drosophila grh* coding sequence was cloned into the plasmid pCNA3.1/myc-His (-) (Invitrogen) as described elsewhere [4]. The full-length *Neurospora grh* coding sequence was amplified using the Phusion polymerase (New England Biolabs) from an oligo-dT-primed cDNA library (RETOscript kit, Ambion). The primers grhl5’XbaIKozak – CGCGTCTAGAGCCACGATGTTCAGTCAACGAACAAG – and grhl3’HindIII – CGCCAGCTTTGTAGACGACCAGTCGCAGTTATGTCGCTGCTTTTC – were used to introduce a Kozak sequence (for efficient translation) and restriction endonuclease sites. The fragment was cloned into pCNA3.1/myc-His(-) A using the XbaI and HindIII sites in the multiple cloning site. The insert was fully sequenced and was found to be identical in sequence and exon structure to that predicted by the Broad Institute *Neurospora* database. GRH and GRHL proteins were translated using the TNT T7 Quick Coupled Transcription/Translation System (Promega) by adding 1 µg of template to each master mix aliquot, according to the manufacturer’s instructions. Protein expression levels were assayed by Western blotting, using antibodies against the C-terminal Myc tags, as described elsewhere [4]. The translated proteins were directly used in the gel-shift assays, as freezing was found to negatively affect DNA-binding activity. For each oligonucleotide pair, 500 pmol of each were annealed in a final volume of 100 µl in annealing buffer (10 mM Tris-HCl pH 7.5, 20 mM NaCl) by heating to 95°C for 5 min and slowly cooling to 25°C. Then, 5 pmol of double-stranded oligonucleotides was labeled with polynucleotide kinase (New England BioLabs) in the presence of ATP-32P] for 30 min at 37°C. The double-stranded probes were purified using the QIAquick Nucleotide Removal Kit (Qiagen). Next, 10-20 fmol of radiolabeled double-stranded oligonucleotides and 1.5 µl of protein from the in vitro transcription/translation reactions were added to 10 µl binding buffer [25 mM Hepes pH 7.9, 100 mM KCl, 1 mM DTT, 1% polyvinylalcohol, 1% Nonidet P-40, 0.1% BSA, 10% glycerol, and 20 µg/ml poly(dI-dC)] and incubated with DNA for 30 min at 4°C. The binding reaction was then electrophoresed through a 4% native polyacrylamide gel in 0.5x TBE at 4°C. Gels were dried and autoradiographed with the use of intensifying screens.

**Neurospora and Drosophila Microarray Sample Collection**

*Neurospora* samples for microarray analysis were collected according to the following procedures. Seeder slants of wild-type (FGSC2489) and *grhl* (FGSC13563) strains were grown for 3 days at 30°C under a 12 h light/dark cycle, and conidia were harvested in 1 ml H2O. As the *csp-2* phenotype makes homogenous resuspension of *grhl* conidia impossible, accurate conidial counts of the suspensions could not be obtained. Therefore, plates and flasks were inoculated with approximately equivalent masses of conidia and *grhl* samples. 1) “ALL” samples were collected by densely plating conidia on minimal Vogel’s agar medium +1.5% sucrose in 10 cm Petri dishes that had been overlain with disks of cellophane (VWR, 100357–652). After 48 h at 30°C under a 12 h light/dark cycle, the plates were densely covered with conidiating colonies.
Samples were scraped off the cellophane using cell-scrapers, submerged in 5 ml Trizol (Invitrogen), and quickly frozen in liquid nitrogen. 2) “AHC” samples were collected by densely plating conidia on minimal Vogel’s agar medium +1.5% sucrose in a deep 10 cm Petri dishes. Disks of medium gauge wire mesh were suspended ~0.5 cm above the surface of the agar using a ring of plastic tubing around the periphery of each Petri dish. After 48 h at 30°C under a 12 h light/dark cycle, the aerial hyphae and conidia had grown abundantly through the mesh. The mesh disks were carefully peeled off, and the adherent cells were harvested in H2O using cell-scrapers. Samples were dried by vacuum filtration, removed from the filter paper using cell-scrapers, submerged in 5 ml Trizol, and quickly frozen in liquid nitrogen. 3) “MYC” samples were collected by inoculating 25 ml of liquid Vogel’s medium +1.5% sucrose in 125 ml Erlenmeyer flasks with sponge stoppers. After 28 h at 28°C with constant shaking in the dark, log-phase mycelial mats were recovered by vacuum filtration, removed from the filter paper using cell-scrapers, and frozen in 2 ml Trizol with liquid nitrogen.

To obtain total RNA from the Neurospora samples for microarray analysis, we followed a protocol similar to that reported elsewhere [81]. Samples in Trizol were thawed and quickly homogenized by vortexing and passing through a P1000 pipet tip multiple times to break up large clumps. Approximately 100 µl of cells were placed in an eppendorf tube with 1 ml Trizol and 200 mg of 0.5 mm Zirconia/Silica Beads (Biospec). Samples were disrupted twice with a MiniBeadBeater (Biospec) at maximum speed for 30 s each time. RNA was then extracted using standard Trizol procedures and resuspended in 100 µl H2O. RNA was quantified in 10 mM Tris pH 7.5, and 50 µg of total RNA was cleaned further using the RNeasy miniprep kit (Qiagen). RNA integrity was assayed by gel electrophoresis, RT-PCR against several developmentally dynamic genes [81], and Bioanalyzer (Agilent) analysis (data not shown).

The following Drosophila embryo collection procedures were carried out in duplicate. To aid in the collection of homozygous grh-deficient embryos, the cn, grhM, bw, sp chromosome [2] was placed over the fluorescent balancer GY0, Knüppel-GFP (CGK) [B2]. Heterozygous collections of embryos (+; cn, grhM, bw, sp/GCK, +) were allowed to develop at 25°C until 15–18 h of age. Embryos were aligned on a thin agar slab on a slide, and GFP-negative embryos (grhM homozygotes) were selected using epifluorescent microscopy. Gut autofluorescence in the GFP channel allowed for the selection of viable and properly aged embryos (late stage 16). Gut autofluorescence in the GFP channel allowed for the selection of viable and properly aged embryos (late stage 16).

Excess abdominal fat, the flies were obtained from the Bloomington Stock Center. The el3C-GAL4 [70] flies were a gift from Dr. Norbert Perrimon at Harvard University. The UAS-GR144 drives were obtained from the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at/control/main).

**Microarray Design and Analysis**

**Neurospora** microarrays were custom synthesized by Agilent using the sequences from the Neurospora crassa array available from the FGSC [B1, B3]. All probe sequences were shortened from 70 mers to 60 mers by removing the first ten 5’ nucleotides. A total of 10,526 unique spots were printed on each chip, corresponding to predicted genes from several databases. Once a finalized list of significant genes was obtained, probe sequences were BLASTed against the Neurospora crassa genome to verify the Broad (or MIPS) gene ID annotations.

Predesigned Drosophila melanogaster arrays were ordered from Agilent (Design ID # 18972). A total of 43,603 spots were printed on each chip, which mapped to ~13,000 unique FlyBase genes.

Fluorescence values from redundant probes (or unique probes targeting the same gene) were grouped, and only the highest fold-change values were used in these analyses. Once a finalized list of significant genes was obtained, probe sequences were BLASTed against the Drosophila melanogaster genome to verify the FlyBase CG # annotations.

**RNA labeling, hybridizations, fluorescent quantification, data normalization, FDR calculations** [84], and GO annotations (Tables 1 and S1) were carried out by the Biogem Core facility (UC San Diego); see Text S1 for an in-depth description of the microarray analyses. Manual Neurospora gene classifications (Figure 3) were carried out by consulting the Broad Institute Neurospora crassa database (www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html), the MIPS Neurospora crassa database (mips.helmholtz-muenchen.de/genome/proj/ncrassa), and the Functional Catalog (FunCat) [41] classifications found in the MIPS database (mips.helmholtz-muenchen.de/genome/proj/ncrassa/). To search Catalogs, we used the search function (searchCatFirstFun.html), as well as with literature and homology searches. Both the SignalP (www.cbs.dtu.dk/services/SignalP) and TargetP (www.cbs.dtu.dk/services/TargetP) servers were used to look for secretion signals in the down-regulated Neurospora proteins. Manual Drosophila gene classifications (Table 2) were carried out by consulting Flybase (flybase.org) and The Gene Ontology (www.geneontology.org), as well as with literature and homology searches. The NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) and JGI (genome.jgi.doe.gov/gene-project) search tools were used extensively in these analyses. The normalized microarray results have been deposited in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), and the accession numbers for the Neurospora and Drosophila datasets are GSE35017 and GSE34997, respectively.

**Drosophila RNAI Strains and Conditions**

Flies were raised on the standard Drosophila medium at 25 or 29°C. The hs-GAL4 driver was located on the third chromosome, and the flies were obtained from the Bloomington Stock Center. The el3C-GAL4 [70] flies were a gift from Dr. Norbert Perrimon at Harvard University. The UAS-GR144 drives were obtained from the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at/control/main).

**Epidermal Tissue Preparation and Immunostaining**

Wandering third-instar larvae were rinsed in phosphate-buffered saline (PBS), dissected in Brower Fixative (0.15 M PIPES pH 6.9, 3 mM MgSO4, 1.5 mM EGTA, and 1.5% NP40) with 4% formaldehyde (ultrapure, methanol-free from Polysciences Inc.), and fixed for 2 h at 4°C. To visualize melantoic spots, fixed larval tissues were washed in PBS with 0.1% Tween 20 and 0.1% Triton X-100 (PBTwix), mounted in Prolong Gold anti-fade reagent (Invitrogen), and imaged using phase-contrast microscopy on a Leica DM 2500 microscope. Epidermal tissues from adult abdomens were also dissected in Brower Fixative with 4% formaldehyde, fixed for 2 h at 4°C, and excess abdominal fat was removed by gentle aspiration.

For immunostaining, fixed epidermal tissues were washed in PBTwx, then incubated in a blocking solution of PBTwx with Western blocking reagent (WBR; Roche) for 1 h at room temperature. Incubations with primary antibodies were performed in PBT + WBR at 4°C overnight, and incubations with fluorescently labeled secondary antibodies were performed in PBT + WBR at room temperature for 2 hours. Primary antibodies utilized in this paper were as follows: guinea pig anti-GRH at a 1:300 dilution [6] and mouse anti-Fasciclin 3 (7G10 concentrate, from the Developmental Studies Hybridoma Bank) at a 1:400 dilution.
Figure S2 Quantitative RT-PCR verification of the fold changes observed on the Neurospora grhl AHC microarrays. Quantitative RT-PCR (qPCR) was carried out on a selection of ten genes (five up- and five down-regulated) seen to be misregulated on the Neurospora grhl AHC microarrays. Genes were chosen to span a wide range of fold changes. The qPCR results verify the directionality of the fold changes seen on the microarrays, as well as in (most cases) the approximate fold-change values. Results were analyzed using two different housekeeping genes as controls – actin and chp. Labels correspond to the following genes: NCU04883–chitinase 1; NCU04850–exo-beta-1,3-glucanase; NCU07387–creatininase; NCU00151– fumarohydrinase; NCU07382–dimethylallyl monooxygenase; NCU04533–abundant perithelial protein; NCU07610–taurine dioxygenase; NCU07819–alpha-ketoglutarate-dependent taurine dioxygenase; and NCU07232–heat shock protein 30. Primer sequences were as follows: NCU04883–TA-CCTCTGCTGACACCCAAGC and CTTTGGGTTGGCAAGGAG; NCU04850–TCTCTACAGCGGTGTCGTC and CCGACCATATATATATGAC; NCU07387–ATTACGTCC-CAGCCTTTTTAC and GTTCGATAGCCTTGTCTTAG; NCU00151–ATTCGTAGTTGGGGCTAAG and CCGTACGAAAAGCTCTCAAG; NCU07610–CTTAAAGGGGTGGACAC and GAGCGACCCAACTCTGCTTCTG; NCU07819–AGTTTGGGTTGGGCAAGGAG and ACGACGGCCATACCTGCTTCTC; NCU07232–GCTGGGCGCGGGTATAG and ATCCAAACCGTACGATTCCG; NCU07819–AAGACATTGTGGGTTGAATCG and TCAGAAT-CAATCGTTCCGTCG; and NCU07232–AGCGACGATGAGAGTGTTC and TATCCGTATCCACCGGAGTC. (TIF)

Figure S3 Up-regulated genes from the Neurospora grhl AHC Fly Hyphae and Conidia microarray samples. A manual classification of the significantly up-regulated genes from the Neurospora grhl AHC microarrays. “Broad ID” entries correspond to the gene IDs found in the Broad Institute Neurospora crossa database. Italicized entries in this column refer to probes that do not correspond to genes in the Broad database, but which correspond to genes in the MIPS database. “Gene name or Description” entries were based on the annotations found in the Broad and MIPS databases, as well literature and homology searches. “Fold (wt value)” entries indicate the fold changes observed in grhl mutant aerial hyphae and conidia relative to wild type; wild-type microarray fluorescence values are shown in parentheses (the background level was ~100 units). “FDR” entries indicate the False Discovery Rate values calculated for each gene; only genes with FDR values less than 0.01 are shown. Columns 1–9 of the grid represent a simplification of the FunCat classification system; solid-colored blocks indicate those genes are classified in the corresponding FunCat categories; dashes indicate that we found evidence in the literature to suggest these genes belong in the corresponding categories. Column 10 of the grid indicates whether the encoded proteins are predicted to be secreted, according to the TargetP (T) prediction algorithm. (TIF)

Figure S4 The lesion responsible for the grhIM allele is a stop-codon introduction shortly into the DNA-binding domain. A) RT-PCR demonstrates that grhIM embryos still produce grh transcripts at roughly the same levels as wild-type embryos; RT-PCRs were carried out with biological replicates. (B) A schematic showing the location of the TAT>GAA stop-codon introduction in the grhIM mRNA, shortly after the start of the DNA-binding domain (tyrosine Y29, from the “D.mel GRH”

Drying Assays for Wandering Third-instar Drosophila Larvae

To cause clean injury, larvae were first rinsed in PBS, placed in a small drop of PBS on a black rubber block, and were punctured with a sterile 0.125 mm tungsten needle (Fine Science Tools) through their posterior-lateral epidermis, as described elsewhere [85,86]. After epidermal injury, larvae were placed into either a Petri dish (60×13 mm) containing Whatman 3 MM chromatography paper moistened with PBS (moist conditions), or an empty Petri dish (dry conditions), and raised at 25°C. Mock-treated larvae were treated as above, except without any epidermal injury. Individual drying assays were performed using at least 40 larvae for each genotype, and each assay was repeated at least three times. Images of wandering third-instar larvae were obtained using a SteREO Discovery.V12 stereomicroscope (Zeiss), and images of representative larvae are presented in Figure 4.

Clean and Septic Epidermal Injury Experiments in Adult Drosophila

Approximately 24 h old male flies were heat shocked for 3 h at 37°C once a day for four consecutive days; flies were raised at 29°C between and after heat shock treatments. Clean or septic injuries were performed on the fifth day after eclosion using a 0.25 mm tungsten needle to puncture their dorsal abdomens, as described elsewhere [85,86]. For septic injury, needles were dipped in gram-negative Escherichia coli carotovora 15 (Eec15) or gram-positive Micrococcus luteus (M. luteus) bacterial solutions prior to injury. Images of wandering third-instar larvalvae were obtained using a SteREO Discovery.V12 stereomicroscope (Zeiss), and images of representative larvae are presented in Figure 4.

Supporting Information

Figure S1 The mutation responsible for the csp-2FS590 allele is in the grhl gene (NCU06095). (A) The wild-type amino acid sequence of GRHL/Csp-2. The serine whose codon is mutated in the csp-2FS590 allele is highlighted in blue. The generation and initial characterization of this UV-induced mutation is described in Selitrennikoff et al. (1974). The residues of the DNA-binding domain are shown in bold. (B) Codon S509 contains a one bp deletion (-) in the csp-2FS590 allele, which results in a frame-shift mutation leading to a premature stop codon (*) after 14 out-of-frame codons. This mutant allele is predicted to encode a truncated version of the GRHL/Csp-2 protein lacking the proper 285 C-terminal amino acids. We also identified a C>A mutation in codon 510, although whether this mutation existed in the parental strain prior to mutagenesis is unclear. (TIF)

Grainy Head-Like Function in Animals and Fungi
protein sequence in Figure 1B). (C–F) Sequencing reactions from both RNA and genomic DNA templates unambiguously verify this mutation: homozygous deficiency (cn, grhIM, bw, sp) RNA from embryos (C), wild-type (y; cn, bw, sp) RNA from embryos (D), heterozygous (cn, grhIM, bw, sp/G, Krippel-GFP) genomic DNA from adults (E), and wild-type (y) DNA from adults (F).

**Figure S5** Quantitative RT-PCR verification of the fold changes observed on the *Drosophila grhIM* embryo microarrays. Quantitative RT-PCR (qPCR) was carried out on a selection of eight genes (three up- and five down-regulated) seen to be misregulated on the *Drosophila grhIM* microarrays. Genes were chosen to span a wide range of fold changes. The qPCR results verify the directionality of the fold changes seen on the microarrays, as well as (in most cases) the approximate fold-change values. Results were analyzed using the housekeeping gene *Rp49* (CG7939) as a control. Primer sequences were as follows: Lp6– TCTGAGATCGTGGTTCGCTC and GACATGCTGAGGTTGAGAC; Rp49– GCCAGGCAAAGATGTCGCG and ATGAGGACCACGGTGTTCC; TephII– GAATCATGAACTGATCCCGAAG and TCCGTCTTGTCATCGCTCAGCAG; Cyp6w1– GCAGCTCCT; TepII– CTGGTTTGATCCCAATGAGG; and Cpr67Fa1– GAAGATTGGAAAGAACTTGCAG and CGGGAGCATA– GGATGGCCATGAAAAGAGTG and CAGGTCTCGTTGTCCCAGAC.

**References**

1. Bray SJ, Kafatos FC (1991) Developmental function of Elf-1: an essential transcription factor during embryogenesis in *Drosophila*. Genes Dev 5: 1672–1683.
2. Nüsslein-Volhard C, Wieschaus E, Kluding H (1994) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. Wilhelm Roux' Archiv 193: 267–282. doi:10.1007/BF00848136.
3. Mace KA, Pearson JC, McGinnis W (2005) An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. Science 308: 381–385. doi:10.1126/science.1107573.
4. Pearson JC, Juarez MT, Kim M, Drivenes Ø, McGinnis W (2009) Multiple transcription factor codes activate epidermal wound-response genes in *Drosophila*. Proc Natl Acad Sci USA 106: 2224–2229. doi:10.1073/pnas.0901291106.
5. Wang S, Tiazoulas V, Vlongudis N, Sahni N, Takwi K, et al. (2009) The tyrosine kinase Sticher activates Grainy head and epidermal wound healing in *Drosophila*. Nat Cell Biol 11: 890–895. doi:10.1038/ncb1988.
6. Kim M, McGinnis W (2011) Phosphorylation of Grainy head by ERK is essential for wound-dependent regeneration but not for development of an epidermal barrier. Proc Natl Acad Sci USA 108: 650–655. doi:10.1073/pnas.1013689108.
7. Almeida MS, Bray SJ (2005) Regulation of post-embryonic neuroblasts by *Drosophila* Grainyhead. Mech Dev. 122: 1282–1293. doi:10.1016/j.mod.2005.08.004.
8. Cenci C, Gould AP (2005) *Drosophila* Grainyhead specifies late programmes of neural proliferation by regulating the mitotic activity and Hox-dependent apoptosis of neuroblasts. Development 132: 3833–3843. doi:10.1242/dev.01932.
9. Huang JD, Dubnicoff T, Lawg GJ, Bai Y, Valentine SA, et al. (1995) Binding sites for transcription factor NTF-1/Elf-1 contribute to the ventral repression of decapentaplegic. Genes Dev 9: 3177–3189.
10. Lawg GJ, Rudolph KM, Huang JD, Dubnicoff T, Courney AJ, et al. (1995) The torso response element binds GAG and NTF-1/Elf-1, and regulates tailles byrel dependence. Genes Dev 9: 3163–3176.
11. Hemphla J, Uv A, Cantera R, Bray S, Samakovlis C (2003) Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling. Development 130: 249–258.
12. Ostrowski S, Dierick HA, Bejozer A (2002) Genetic control of cuticle formation during embryonic development of *Drosophila* melanogaster. Genetics 161: 171–182.
13. Narasimha M, Uv A, Krejci A, Brown NH, Bray SJ (2008) Grainy head promotes expression of septate junction proteins and influences epithelial morphogenesis. *Genes Dev* 22: 747–752. doi:10.1101/gad.159422.
14. Venkatassan K, McManus HR, Mello CC, Smith TF, Hansen U (2003) Functional conservation between members of an ancient duplicated transcription factor family, NSF/Grainyhead. Nucleic Acids Res 31: 4304–4316.

15. Tao J, Kulyev E, Wang X, Li X, Wilanowski T, et al. (2005) BMP1-dependent expression of Xenopus Grainyhead-like 1 is essential for epidermal differentiation. Development 132: 1273–1284. doi:10.1242/dev.014642.
16. Ting SB, Wilanowski T, Auden A, Hall M, Voss AK, et al. (2005) Inositol- and folate-resistant neural tube defects in mice lacking the epithelial-specific factor Grhl-3. Nat Med 9: 1513–1519. doi:10.1038/nm1361.
17. Kudryavtseva EI, Sugihara TM, Wang N, Lasso RJ, Gudanov VN, et al. (2003) Identification and characterization of Grainyhead-like epithelial transactivator (GET-1), a novel mammalian Grainyhead-like factor. Dev Dyn 226: 604–617. doi:10.1002/dvdy.10255.
18. Auden A, Caddy J, Wilanowski T, Ting SB, Cunningham JM, et al. (2006) Spatial and temporal expression of the Grainyhead-like transcription factor family during murine development. Genes Expr Patterns 6: 964–970. doi:10.1016/j.genepr.2006.03.011.
19. Ting SB, Caddy J, Hulop N, Wilanowski T, Auden A, et al. (2005) A homolog of *Drosophila* grainy head is essential for epidermal integrity in mice. Science 308: 411–413. doi:10.1126/science.1107511.
20. Ting SB, Caddy J, Wilanowski T, Auden A, Cunningham JM, et al. (2005) The epidermis of grhl3-null mice displays altered lipid processing and cellular hyperproliferation. *Organogenesis* 2: 33–35.
21. Yu Z, Lin KK, Bhandari A, Spencer JA, Xu X, et al. (2006) The Grainyhead-like epithelial transactivator Grtl-1/Grhl3 regulates epidermal terminal differentiation and interacts functionally with LMO1. *Dev Biol* 299: 122–136. doi:10.1016/j.ydbio.2006.07.013.
22. Yu Z, Bhandari A, Mannik J, Pham T, Xu X, et al. (2008) Grainyhead-like factor Get1/Grhl3 regulates formation of the epidermal leading edge during eyelid closure. Dev Biol 319: 56–67. doi:10.1016/j.ydbio.2008.04.001.
23. Gustavsson P, Copp AJ, Greene NDE (2008) Grainyhead genes and mammalian neural tube closure. Birth Defects Res Part A Clin Mol Teratol 82: 726–735. doi:10.1002/bdra.20494.
24. Yu Z, Mannik J, Soto A, Lin K, Anderson B (2009) The epidermal differentiation-associated Grainyhead gene Get1/Grhl3 also regulates urothelial differentiation. *Embo J* 28: 3163–3176.
25. Wilanowski T, Caddy J, Ting SB, Hulop N, Cerruti L, et al. (2008) Perturbed desmosomal cadherin expression in grainy head-like 1-null mice. EMBO J 27: 896–897. doi:10.1038/embj.2008.24.
26. Pyrgaki C, Liu A, Niswander L (2011) Grainyhead-like 2 regulates neural tube closure and adhesion molecule expression during neural fold fusion. *Dev Biol* 353: 38–49. doi:10.1016/j.ydbio.2011.02.037.
27. Bogley W, Wilanowski T, Caddy J, Parekh V, Auden A, et al. (2011) The unique and cooperative roles of the Grainy head-like transcription factors in epidermal development reflect unexpected target gene specificity. *Dev Biol* 349: 512–522. doi:10.1016/j.ydbio.2011.10.011.
28. Traylor-Knowles N, Hansen U, Dubuc TQ, Martindale MQ, Kaufman L, et al. (2010) The evolutionary diversification of NSF and Grainyhead transcription...
factors preceded the radiation of basal animal lineages. BMC Evol. Biol. 10: 101. doi:10.1186/1471-2148-10-101.

29. Veljkovic J, Hansen U (2004) Lineage-specific and ubiquitous biological roles of the mammalian transcription factor LSF. Gene 343: 23–40. doi:10.1016/j.gene.2004.03.046.

30. Uv AE, Thompson CR, Bray SJ (1994) The Drosophila tissue-specific factor ELPS2812 is coexpressed with the early hemocyte marker P-cadherin during embryonic development. Proc Natl Acad Sci USA 91: 7334–7338. doi:10.1073/pnas.91.16.7334.

31. Shirra MK, Hansen U (1998) LSF and NTF-1 share a conserved DNA recognition motif yet require different oligomerization states to form a stable protein-DNA complex. J Biol Chem 273: 19260–19268.

32. King N, Westbrook MJ, Young SL, Kuo A, Abedin M, et al. (2008) The genome of the chesnookflagellate Monosiga brevicollis and the origin of metazoans. Nature 451: 783–788. doi:10.1038/nature06617.

33. Schel-Prê-ois D, de Mendoza A, Lang BF, Degnan BM, Ruíz-Trillo I (2011) Unexpected repertoire of metazoan transcription factors in the unicellular holozoan Cuspazas owczarzaki. Mol Biol Evol 28: 1241–1254. doi:10.1093/molbev/msr309.

34. Kosokoszka K, Ostrowski J, Rychlewski L, Wyrwicz LS (2008) The fold recognition of CP2 transcription factors gives new insights into the function and evolution of tumor suppressor p53. Cell Cycle 7: 2907–2915.

35. Brody S, Ochman K, Schneider K, Perrino S, Grote A, et al. (2010) Circadian rhythms in Neurospora crassa: Downstream effectors. Fungal Genet Biol 47: 159–168. doi:10.1016/j.fgb.2009.09.006.

36. Davis R (2000) Neurospora: Constructions of a Model Organism. Oxford: Oxford University Press.

37. Springer ME, Yoon D (1989) A morphological and genetic analysis of conidioaphore development in Neurospora crassa. Genes Dev 3: 559–571.

38. Selitrennikov CP, Nelson RE, Siegel RW (1974) Phase-specific genes for macroconidiation in Neurospora crassa. Genetics 78: 679–690.

39. Lambreghts R, Shi M, Belden WJ, Decaprio D, Park D, et al. (2009) A high-density single nucleotide polymorphism map for Neurospora crassa. Genetics 181: 767–781. doi:10.1534/genetics.108.091922.

40. Perkins D (2000) Neurospora crassa genetic maps and mapped loci. Fungal Genet Newslet 47: 40–56.

41. Roux A, Zolotov A, Maier D, Albermann K, Hani J, et al. (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res 32: 5359–5365. doi:10.1093/nar/gkh974.

42. Tang H (2009) Regulation and function of the melanization reaction in Drosophila. Biopolymers 88: 303–115.

43. Zelante T, Fallarino F, Bistoni F, Puccetti P, Romanini L (2009) Endoelodea 2.3: dioxygenaze in infection: the paradox of an evasive strategy that benefits the host. Microbes Infect 11: 133–141. doi:10.1016/j.micinf.2008.10.007.

44. Lemaitre B, Hoffmann J (2007) The host defense of Drosophila melanogaster. Annu Rev Immunol 25: 697–743. doi:10.1146/annurev.immunol.25.022006.141615.

45. Schauer J, Gallo RI (2009) Antimicrobial peptides and the skin immune defense system. J Allergy Clin Immunol. 124: R13–8. doi:10.1016/j.jaci.2009.07.014.

46. Maddi A, Bowman SM, Free SJ (2009) Trifluoromethanesulfonic acid-based proteomic analysis of cell wall and secreted proteins of the ascomycetous fungus Neurospora crassa and Candida albicans. Fungal Genet Biol 46: 786–791. doi:10.1016/j.fgb.2009.06.005.

47. Brunoe JM, Magnin T, Tagat E, Legrand R, Bernard M, et al. (2000) Proteome analysis of Aspergillus fumigatus identifies glycosylphosphatidylinositol-anchored proteins associated to the cell wall biosynthesis. Electrophoresis 22: 2818–2823. doi:10.1002/1522-2683(200108)22:15<2818::AID-ELPS2812>2.0.CO;2-Q.

48. Yin QY, de Groot PWJ, de Koster CG, Klis FM (2008) Comprehensive proteomic analysis of Saccharomyces cerevisiae cell walls using mRNA profiling and comparative genomics approaches. Eukaryotic Cell 7: 1549–1564. doi:10.1128/EC.00195-08.
82. Caso D, Ramírez-Weber F, Kornberg TB (2000) GFP-tagged balancer chromosomes for Drosophila melanogaster. Mech. Dev. 91: 451–454.

83. Kasuga T, Townsend JP, Tian C, Gilbert LB, Mannhaupt G, et al. (2005) Long-oligomer microarray profiling in Neurospora crassa reveals the transcriptional program underlying biochemical and physiological events of conidial germination. Nucleic Acids Res 33: 6469–6485. doi:10.1093/nar/gki953.

84. Sašek R, Woelk CH, Corbeil J (2004) Microarray truths and consequences. J Mol Endocrinol 33: 1–9.

85. Tzou P, Meister M, Lemaitre B (2002) Methods in Microbiology. Elsevier. 507–529 pp doi:10.1016/S0580-9517(02)31028-6.

86. Romeo Y, Lemaitre B (2008) Drosophila immunity: methods for monitoring the activity of Toll and Imd signaling pathways. Methods Mol. Biol. 415: 379–394. doi:10.1007/978-1-59745-570-1_22.

87. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B (2001) Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA 98: 12590–12595. doi:10.1073/pnas.221436998.

88. Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, et al. (2001) A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci USA 98: 15119–15124. doi:10.1073/pnas.221573998.

89. Boutros M, Agaisse H, Perrimon N (2002) Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev Cell 3: 711–722.

90. Roxström-Lindquist K, Terenius O, Faye I (2004) Parasite-specific immune response in adult Drosophila melanogaster: a genomic study. EMBO Rep. 5: 207–212. doi:10.1038/sj.embor.7400573.

91. Dottert C, Jouangay E, Irving P, Troxler L, Galiana-Arnoux D, et al. (2005) The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nat. Immunol. 6: 946–953. doi:10.1038/njil237.

92. Xi Z, Gavotte L, Xie Y, Dobson SL (2008) Genome-wide analysis of the interaction between the endosymbiotic bacterium Wolbachia and its Drosophila host. BMC Genomics 9: 1. doi:10.1186/1471-2164-9-1.

93. Wertheim B, Kraaijeveld AR, Schuster E, Blanc E, Hopkins M, et al. (2005) Genome-wide gene expression in response to parasitoid attack in Drosophila. Genome Biol 6: R94. doi:10.1186/gb-2005-6-11-r94.

94. Schedeke TA, Moralez J, Govind S, Clark AG (2007) Contrasting infection strategies in generalist and specialist wasp parasitoids of Drosophila melanogaster. PLoS Pathog. 3: 1486–1501. doi:10.1371/journal.ppat.0030158.