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Identification of Drosophila Zfh2 as a Mediator of Hypercapnic Immune Regulation by a Genome-Wide RNA Interference Screen

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Hypercapnia, elevated partial pressure of CO2 in blood and tissue, develops in many patients with chronic severe obstructive pulmonary disease and other advanced lung disorders. Patients with advanced disease frequently develop bacterial lung infections, and hypercapnia is a risk factor for mortality in such individuals. We previously demonstrated that hypercapnia suppresses induction of NF-κB–regulated innate immune response genes required for host defense in human, mouse, and Drosophila cells, and it increases mortality from bacterial infections in both mice and Drosophila. However, the molecular mediators of hypercapnic immune suppression are undefined. In this study, we report a genome-wide RNA interference screen in Drosophila S2* cells stimulated with bacterial peptidoglycan. The screen identified 16 genes with human orthologs whose knockdown reduced hypercapnic suppression of the gene encoding the antimicrobial peptide Diptericin (Dipt), but did not increase Dipt mRNA levels in air. In vivo tests of one of the strongest screen hits, zinc finger homeodomain 2 (Zfh2; mammalian orthologs ZFHX3/ATBF1 and ZFHX4), demonstrate that reducing zfh2 function using a mutation or RNA interference improves survival of flies exposed to elevated CO2 and infected with Staphylococcus aureus. Tissue-specific knockdown of zfh2 in the fat body, the major immune and metabolic organ of the fly, mitigates hypercapnia-induced reductions in Dipt and other antimicrobial peptides and improves resistance of CO2-exposed flies to infection. Zfh2 mutations also partially rescue hypercapnia-induced delays in egg hatching, suggesting that Zfh2’s role in mediating responses to hypercapnia extends beyond the immune system. Taken together, to our knowledge, these results identify Zfh2 as the first in vivo mediator of hypercapnic immune suppression. The Journal of Immunology, 2016, 196: 655–667.

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cercapnia, elevation of blood and tissue levels of CO2, develops in many patients with severe chronic obstructive pulmonary disease (COPD), currently the third leading cause of death in the United States (1), and other advanced lung diseases. Hypercapnia has long been recognized as a risk factor for mortality in patients with acute exacerbations of COPD (2–6), and recently it was shown that use of nocturnal ventilatory support to decrease CO2 levels improved survival of hypercapnic patients with COPD (7). Acute exacerbations of COPD, which are linked to mortality, are most commonly triggered by bacterial or viral respiratory infections (8–10). Hypercapnia is also a risk factor for mortality in patients hospitalized with community-acquired pneumonia (11, 12), children with adenoviral lung infections (13), and cystic fibrosis patients awaiting lung transplantation (14). These observations suggest that hypercapnia may contribute to poor clinical outcomes by increasing susceptibility to pulmonary infections.

Consistent with this hypothesis, we and others have shown that hypercapnia inhibits expression of IL-6, TNF, and other cytokines linked to mortality, are most commonly triggered by bacterial or viral respiratory infections (8–10). Hypercapnia is also a risk factor for mortality in patients hospitalized with community-acquired pneumonia (11, 12), children with adenoviral lung infections (13), and cystic fibrosis patients awaiting lung transplantation (14). These observations suggest that hypercapnia may contribute to poor clinical outcomes by increasing susceptibility to pulmonary infections.

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species, and autophagy (15–17), all key phagocyte antimicrobial functions. Furthermore, we showed that hypercapnia reduces bacterial clearance and increases mortality in a mouse model of Pseudomonas pneumonia (16). Despite these observations, the molecular mechanisms by which elevated CO₂ levels are sensed and transduced in immune cells are not yet understood.

One way that elevated CO₂ might influence immune responses is by reducing extracellular and/or intracellular pH; indeed, there is evidence that acidosis may decrease the function of various immune cells (20). However, in our in vitro studies, suppression of cytokine gene expression, phagocytosis, and autophagy by elevated CO₂ levels was independent of acidosis (15–17). Furthermore, hypercapnia increased the mortality of bacterial pneumonia in mice with acute and chronic hypercapnia to the same degree, despite more severe acidosis in animals with acute hypercapnia compared with chronic hypercapnia (due to renal compensation in the latter group) (16). Taken together, these results suggest that molecular CO₂ has immune suppressive effects that are experimentally and physiologically distinguishable from the effects of acidosis.

To investigate the molecular mechanisms of hypercapnic immune suppression, we studied Drosophila as a tractable molecular genetic model system for nonneuronal responses to elevated CO₂ (21). The response to hypercapnia in Drosophila and mammals has multiple parallels (15, 16); elevated CO₂ levels suppress production of Drosophila immune response genes in vivo and in vitro at the transcriptional level via a mechanism that is downstream of NF-κB proteolytic activation. As in mammalian macrophages, downregulation of immune response genes was independent of pH. Hypercapnia also significantly decreases resistance of adult flies to bacterial infections. The similarities of hypercapnic immune suppression in flies and mammals, in combination with evidence that a conserved JNK pathway controls Na,K-ATPase endocytosis during hypercapnia (22), suggest that elevated CO₂ acts via specific evolutionarily conserved pathways to downregulate host defenses.

In this study, we describe the results of a genome-wide RNA interference (RNAi) screen in Drosophila S2* cells to identify mediators of CO₂-induced immune suppression. The screen identified >16 genes with human orthologs whose knockdown attenuates hypercapnic suppression of the antibacterial peptide (AMP) gene in peptidoglycan (PGN)-stimulated S2* cells. In vivo characterization of one of these genes, the transcription factor zinc finger homeodomain 2 (zfh2; mammalian orthologs ZFHX3/ATF8F1 and ZFHX4), shows that reducing zfh2 levels in the major immune organ of the fly, the fat body, improves resistance and survival to Staphylococcus aureus infection during hypercapnia. To our knowledge, this is the first description of a component of CO₂ response pathways that mediate the in vivo effects of hypercapnia on immune responses.

## Materials and Methods

### CO₂ treatment for cell lines and flies

Exposures to air (normocapnia: 0.039% CO₂, 21% O₂, 78% N₂) and elevated CO₂ were carried out in BioSpherix C- Chambers fitted with ProCO₂ regulators and CO₂ and O₂ sensors. CO₂ (100%) was injected into the chambers to raise the ambient CO₂ levels to either 5% CO₂ (mild hypercapnia: 5.0% CO₂, 20% O₂, 75% N₂) or 13% CO₂ (hypercapnia: 13.0% CO₂, 18% O₂, 68% N₂). For fly experiments, humidity was maintained at ~60% using Drierite.

### RNAi screening

Pilot screening was performed at Northwestern University’s High Throughput Analysis Laboratory using the S2* Diptericin-luciferase (Dipt-luc) cell line (gift from N. Silverman). The Dipt-luc reporter consists of 2.2 kb of the Dipt promoter (23) driving firefly luciferase in the pGL3 vector (Promega) (24), A Renilla luciferase-Pol III (polIII-luc) reporter, in which a fragment of the promoter for the RNA polymerase 128 subunit drives Renilla luciferase (25) (gift of the Drosophila RNAi Screening Center [DRSC], Harvard Medical School, Boston, MA), was transfected into the Dipt-luc cell line using Effectene (Qiagen). Selected PCR amplons were obtained from the DRSC and in vitro transcribed using the T7 MEGAscript kit (Ambion, Applied Biosystems) into dsRNAs using DRSC protocols (26). Cells were maintained in 1 ml/mg G418 and 200 μg/ml hygromycin in Schneider’s insect medium (Sigma-Aldrich) containing 10% FBS. All liquid handling was performed using a Matrix WellMate dispenser (Thermo Scientific). Five hundred cells per well in 384-well plates were bathed with preplated dsRNAs (~1–5 μg total dsRNA) for 3 d prior to CO₂ exposure to ensure effective gene knockdown. Cells were harvested 24 h postinfection (after CO₂ exposure), cells were primed with 1 μM 20-hydroxyecdysone (Sigma-Aldrich) to improve immune responsiveness. Cells were exposed to 13% CO₂ for 10 h in media neutralized with 25 mM NaOH to maintain pH at 7.1 as previously described (21). Five hours after the start of CO₂ exposure, cells were challenged with 100 ng/ml E. coli PGN (E. coli 0111:B4, InvivoGen) to induce Dipt. Firefly and Renilla luminescence was measured sequentially using Dual-Glo luciferase reagent (Promega) with an Analyst GT plate reader (Molecular Devices). Data were analyzed using DRSC software and Microsoft Excel. Primary RNAi screening was carried out in duplicate in 13% CO₂; these experiments were performed on-site at the DRSC. The raw data from the primary screen are available from the DRSC under project ID 128 at http://www.flymaze.org/cgi-bin/DRSC_screen_csv.pl?project_id=128, and the normalized and formatted data are shown in Supplemental Table II.

### luminescence

dsRNAs of interest were identified based on their Z-score, which represents the number of standard deviations the signal from one well on a plate is above or below the plate mean. Z-scores were calculated by normalizing the Dipt-luc signal from each well to the polIII-luc signal from the same well, calculating the average Dipt-luc/polIII-luc ratio on a plate, and determining the deviation of each well from the plate average.

Secondary screening was performed in 13% CO₂ in duplicate wells of 96-well plates filled using multichannel pipettes. A Z-score was calculated for each dsRNA by averaging the Z-scores for the dsRNAs on each of the two independent plates. Tertiary screening was performed as per secondary screening, except that duplicate plates with two dsRNAs on a plate were each screened in air and 13% CO₂.

### Fly stocks and maintenance

Drosophila stocks were kept on cornmeal food at room temperature or at 25°C. Stocks were obtained from the Bloomington Stock Center unless otherwise specified. zfh2M3209 (27), zfh2M350R (28), and zfh2M4090R (28) were backcrossed five times to w1118 prior to infection experiments (zfh2M3209 and zfh2M4090R were provided by S. Elgin). The w1118 stock used for backcrossing was used as a control for zfh2 mutant experiments. For RNAi knockdown of zfh2, CG-GAL4 (29) and C754-GAL4 (30) were used to drive GAL4 in fat body. Control experiments used the w1118 isogenic strain w600005, which uses UAS-zfh21305 was derived.

### Western blotting

Western blotting was performed using ~10⁴ S2* cells per sample, 20 fly heads, 20 dissected male abdominal fat bodies, or 20 carcasses after head and fat body removal. Fat bodies were dissected using the protocol of Krupp and Levine (32). For S. aureus challenge, samples were collected 4 h postinfection. Samples were run on 4% acrylamide gels and transferred to nitrocellulose membrane by electroblotting at 100 V for 80 min. Membranes were blocked with 5% skim milk in TBST (20 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.1% Tween 20), incubated overnight with 1:500 rat anti-Zfh2 sera no. 205 (33), then 45 min with 1:10,000 goat anti-rat IgG-HRP (sc-2065, Santa Cruz Biotechnology). Actin (as a loading control) was detected using 1:200 mouse mAb IL20 (Developmental Studies Hybridoma Bank) and 1:10,000 goat anti-mouse IgG-HRP (170-6516, Bio-Rad Laboratories). Amersham ECL Prime Western blotting detection reagent (RPN2232, GE Healthcare Life Sciences) was used for detection, and chemiluminescence of bands was quantitated with the Odyssey Fc imaging system (LI-COR Biosciences).

### Fly infection assays

Fly infection and mortality tests were performed on adult male flies as described in Helenius et al. (21). Bacterial load assays for Fig. 4E were performed as described in Helenius et al. (21), and for Fig. 4G were determined 16–18 h postinfection by washing single flies with 75% ethanol, rinsing with Luria–Bertani media, and then homogenizing in 200 μl fresh
Luria–Bertani media. Homogenates were centrifuged on a bench-top centrifuge for 3 min at 2000 rpm, and 100 μl bacterial supernatant was transferred to 1.9 ml fresh Luria–Bertani media and then shaken at 37°C for 8 h, at which time OD_{600} was determined.

**Ex vivo fat body culture**

For each experiment, three dissected fat bodies (see above) were placed in 1 ml S2* media (described above) and cultured in one well of a 24-well plate for 24 h in media equilibrated with air or 13% CO₂. PGN treatment and media conditions were as for S2* cell induction (21). Total RNA was obtained from fat bodies using TRIzol LS (no. 10296-028, Life Technologies). Quantitative PCR (qPCR) was performed per the manufacturer’s protocol using the iScript cDNA synthesis kit (no. 170-8891, Bio-Rad Laboratories), the iTaq Universal SYBR Green Supermix (no. 172-5124, Bio-Rad Laboratories), and the Dipt primers 5'-ACCGCAGTACCCACT-CAATC-3’ and 5’-ACTTTCCAGCTCGTGTTCTGA-3’. Primers for Atta
cin (Arti), Cecropin (Cecr), Drosomycin (Drs), and Metchnikowin (Mkh) were as previously described (21).

**Statistical analysis**

Data are presented as means ± SEM. GraphPad Prism (version 5.04) and SigmaPlot (version 11.0) were used for statistical analysis. Differences between two groups were assessed using a Student t test. Differences between multiple groups were assessed by ANOVA and the Tukey range test. For comparison of bacterial CFU data, the log_{10} values were used in the analysis. For mortality experiments, the Gehan–Breslow–Wilcoxon test was used. Significance was accepted at p < 0.05.

**Results**

**Dipt-luc is a CO₂-responsive reporter of innate immune responses**

We had previously established *Drosophila* S2* cells as a model for investigating hypercapnic immune suppression by showing that *E. coli* PGN-stimulated induction of the mRNA for the anti-

microbial peptide Dipt was suppressed by hypercapnia in a concentration-dependent manner, without any cytotoxicity (21). In principle, this suppression could be the basis for a genome-wide screen to identify genes that mediate CO₂-induced immune suppression. However, the previously used qPCR-based approach was poorly suited to high-throughput assays. We therefore tested whether the firefly *Dipt-luc* construct containing 2.2 kb of the *Dipt* promoter (24) would show the same CO₂-induced suppression as the endogenous *Dipt* gene. We also tested whether expression of a *Renilla luciferase* driven by the promoter for the RNA polymerase III 128 subunit (*polIII-luc*) (25) was independent of CO₂ levels, and two dsRNAs per gene. The dsRNA library was tested in du-

plets of the immune deficiency (Imd) pathway that regulates AMP expression (34), typically upregulated expression of the *Dipt-luc* reporter by hypercapnia (Fig. 1D). Similar negative results were obtained with the other 34 candidate genes tested, including components of NO and hypoxia response pathways. Components of the Imd pathway, and *Gr63a/Gr21a* that comprise the fly neuronal CO₂ sensor (Supplemental Table I). That none of the dsRNAs targeting candidate genes caused significant differential effects in air versus elevated CO₂ suggests that hypercapnic immune suppression is mediated by novel mechanisms and und-

erscores the need for discovery-based approaches to identify components of CO₂ response pathways.

**A genome-wide RNAi screen for components of CO₂ response pathways**

We next performed a genome-wide screen at the Harvard DRSC, using the DRSC 2.0 library that covered 13,900 of the ~14,000 annotated *Drosophila* genes (36), at an average of between one and two dsRNAs per gene. The dsRNA library was tested in du-

plicate in 384-well plates in 13% CO₂ in media adjusted to pH 7.0 (see Fig. 1E for screen workflow; additional details are in Materials and Methods). The primary genome-wide screen was performed only in 13% CO₂. The full results of the screen have been deposited with the Harvard DRSC and are available at http://www.

flyrnai.org/screensummary. As described below, top hits from the primary screen were further tested to identify those that differenti-

ally regulated *Dipt-luc* in elevated CO₂ versus in air.

Genes of interest from the primary screen were identified as those whose knockdowns most increased *Dipt-luc* levels, after normalization with *polIII-luc*. This was quantified using the Z-score, which is the number of standard deviations that the normalized *Dipt-luc* level in a well treated with a given dsRNA is above or below the mean normalized *Dipt-luc* levels of all wells of the plate in which the dsRNA was arrayed (see Materials and Methods). The primary screen identified a total of 126 dsRNAs targeting 123 genes that had an average Z-score from duplicate plates of ≥2.5 and 496 genes that had an average Z-score of ≥1.5 (Supplemental Table II). The ability of the screen to identify bi-

ologically relevant regulators of immune function was confirmed.
Zfh2 mediates CO2 immune suppression.

Functional classification, based on gene ontology biological processes, of the genes of interest identified in the primary screen revealed that the distribution of functions of genes with a Z-score of ±1.5 or ≤−1.5 from the screen is different from the fly genome as a whole (Supplemental Fig. 1). Furthermore, the genes whose knockdown upregulates the reporter fall into different functional groups than those whose knockdown downregulates the reporter. The group of genes that negatively regulate Dipt expression in hypercapnia is enriched for genes that are involved in responding to external stimuli or are involved in movement, morphogenesis, or differentiation, whereas the group of genes that positively regulate Dipt expression is enriched in spindle and centrosome functions (Supplemental Fig. 1). Notably, of the 126 primary hits, 51% have human orthologs based on analyses using HomoloGene (http://www.ncbi.nlm.nih.gov/homologene/) and InParanoid (45). Of those with orthologs, 25 have a Z-score of >3 (Supplemental Table II), suggesting the screen may have identified conserved genes that mediate the effects of hypercapnia on immune gene expression.

Secondary screening identifies 17 candidate mediators of CO2-induced immune suppression

Based on strength of Z-score, presence of a human ortholog, and possible likelihood of functional significance as a CO2 pathway signal transducer (e.g., a kinase), 192 dsRNAs (representing 192 genes) were selected for further screening (Supplemental Table II). Secondary screening was performed using the same
protocols as the primary screen, except dsRNAs were assayed in 96-well plates in quadruplicate, rather than in duplicate (see Materials and Methods). Of the 192 dsRNAs tested in secondary screening, 39 were found to upregulate Dipt-luc by at least 1 SD above the mean of all wells in at least one 96-well secondary screening plate (Supplemental Table II). Thus, secondary screening delineated 39 dsRNAs that had the strongest effects in a head-to-head comparison of the 192 dsRNAs selected from the primary screen. These 39 dsRNAs were then further tested at two different RNA concentrations in 13% CO₂ and ambient air to determine the CO₂ specificity of the upregulation of Dipt-luc (Fig. 2, Supplemental Table III). Candidate CO₂-response pathway components were identified as those whose knockdown preferentially upregulated Dipt-luc in hypercapnia but not in ambient air (Fig. 2). For 17 of the 39 candidate genes, induction of the Dipt-luc reporter was ≥2-fold higher in 13% CO₂ than in air at one or both of the dsRNA concentrations tested (Fig. 2, Supplemental Table III). Thus, these 17 genes, 16 of which have human orthologs, were preferentially required for hypercapnia to suppress Dipt induction and were designated as candidate CO₂ mediator genes.

Putative functional categories of the 17 candidate CO₂ mediators include a transcription factor (zfh2), chromatin-associated proteins (zormin, ffl, rpd3, and trx), regulators of signal transduction (ffl, ras85D, rhogap15b), a cell surface protein (corin), and an RNA-binding protein (scaf6). Interestingly, two of the candidate genes, ffl and ras85D (37), had previously been implicated in interacting with the Imd pathway, although they do not appear to act in linear order to control Imd signaling, and most proteins in the pathways with which these genes are associated did not score as hits. Gene ontology and STRING analyses of the functions and interactions of these candidate mediators did not reveal an obvious CO₂ sensor candidate or a common pathway or function among all of the genes. However, analysis of the top 17 candidate CO₂ mediators using STRING revealed known connections between Rho-GAP15B, Ras85D, Rpd3, and trx (Fig. 2C). Allowing the STRING program to search for potential connections by adding 10 nodes between the top 17 hits produced an interaction network connecting zormin and zfh2 to the Rpd3 group, however, only one of these new intervening nodes, brm, had a Z-score of ≤−1 in the primary screen. These results do not support the existence of a simple single linear pathway mediating the effects of CO₂ on immune responses. Further characterization of the candidate CO₂ mediators will be required to identify those that have instructive rather than permissive roles in responses to elevated CO₂.

**FIGURE 2.** Secondary screening identifies putative CO₂-response mediator genes. (A) Normalized Dipt-luc reporter expression in control S2* cells and in cells treated with dsRNAs targeting rpd3, zormin, and zfh2. Dashed line indicates expression in control cells in air; solid line indicates expression in control cells in 13% CO₂ in pH 7.1 media. n.t., control nontargeting dsRNA. Statistical significance was determined using a Student t test: *p < 0.05, **p < 0.01, ***p < 0.001. (B) The ratio of the expression of the normalized Dipt-luc reporter in CO₂ versus air, for cells treated with dsRNAs at low and high concentrations. Shown are the 17 genes with the highest CO₂/air ratios. The results for all 39 candidate genes tested in air and CO₂ are shown in Supplemental Table III. (C) An interaction network of genes in (B) predicted by the STRING program (44) using medium confidence settings. A subset of candidate CO₂ mediators have previously identified interactions. (D) An interaction network of genes in (B) predicted by the STRING program with extended connections that include known interactions that could relate zormin and zfh2 to other candidate CO₂ mediators.
zfh2 encodes a large conserved transcription factor expressed in S2* cells and adult Drosophila

To begin validation of candidate effectors of CO2-induced immune suppression, we selected zfh2 for further analysis. zfh2 was a strong candidate because two separate dsRNAs targeting zfh2 in the primary screen had average Z-scores from duplicate plates in the top 10 of all dsRNAs (amplicons DRSC17178 and DRSC28010, Supplemental Table II), and further screening revealed robust upregulation of the Dipt-luc reporter upon zfh2 knockdown in 13% CO2 (3.9- and 2-fold at low and high dsRNA concentrations, respectively) but not in air (1.1- and 0.4-fold) (Fig. 2A, Supplemental Table III). Furthermore, as detailed below, zfh2 encodes a transcription factor, which we expect to have a more specific role in CO2 signal transduction than other candidate genes such as trx or rpd3 that encode chromatin-modifying proteins. Finally, previous work on zfh2 has generated important reagents, including mutant lines, UAS-RNAi lines, and Abs.

The zfh2 gene encodes three isoforms of a large, ~330-kDa protein containing 13 zinc fingers and three homeodomains. Two of the isoforms, Zfh2-PA and Zfh2-PB, are 3003 and 3005 aa long, respectively, and differ by only two amino acids. A third isoform, Zfh2-PC, is generated by an alternative splice near the 3’ end of the coding sequence that truncates the C-terminal 200 aa of the A and B isoforms, including part of the C-terminal homeodomain (Fig. 3A). The amplicons and UAS-RNAi constructs used in the RNAi screen and in vivo assays (see below) target all splice forms of zfh2. Zfh2 is part of a large family of zinc finger homeodomain proteins present in most metazoans (Fig. 3B) (46), with the two clearest human orthologs of Zfh2 being ZFHX3 (also known as ATBF1, ATBT, ZNF927) (47–54) and ZFHX4 (55–57). Human ZFHX3 and ZFHX4 also encode large ~400-kDa proteins containing 23 zinc fingers and four homeodomains (Fig. 3A, 3B). Zfh2 is 25 and 24% identical to ZFHX3 and ZFHX4, respectively (58). The first three homeodomains of ZFHX3 share 77, 69, and 61% identity with the corresponding homeodomains of Zfh2, and several of the zinc finger motifs in ZFHX3 and ZfHX4 share 52–71% identity (59). Notably, the 200 aa deleted in the Zfh2-PC isoform have 30% similarity to the C termini of ZFHX3 and ZFHX4, suggesting that Zfh2-PC may have significant functional differences from Zfh2-PA and Zfh2-PB. A third potential human ortholog of Zfh2 is ZFHX2, but ZFHX2 appears to be further diverged from Zfh2 than either ZFHX3 or ZFHX4, sharing only 16% amino acid identity with Drosophila Zfh2 (58). Furthermore, using the DIOP ortholog search tool (60), ZFHX2 is identified as a Zfh2 ortholog by only three of nine prediction programs (Homologene, Inparanoid, Isobase, OMA, OrthoDB, OrthoMCL, Phylome, RoundUp, TreeFam), whereas ZFHX3 and ZFHX4 are identified by seven and six databases, respectively. zfh2 has previously been shown to have an important role in neural and epithelial development and in regulating cell death (27, 61–65).

FIGURE 3. zfh2 encodes a large conserved transcription factor expressed in immune tissues. (A) Domain structure of Drosophila (dm) Zfh2 isoforms A, B, and C, and human (hs) ZFHX3 and ZFHX4. Zfh2 isoforms A and B differ by only 2 aa and are shown together. Black bar indicates predicted zinc finger; magenta bar indicates predicted homeodomain. (B) A phylogenetic tree showing the relationships between Drosophila (dm) Zfh2 and human (hs), xenopus (xl), Ciona, and C. elegans (ce) zinc finger homeodomain proteins. The tree was generated using Ensembl release 81, June 2015 (76), which does not use branch length to represent evolutionary distance. (C) Western blot using rat polyclonal anti-Zfh2 antiserum (33) shows that Zfh2 is expressed strongly in neural tissue (head) and at lower, but easily detectable, levels in the fat body and the S2* cell line. Zfh2 levels in S2* cells and all tissues appear similar following exposure to air or 13% CO2 and after immune challenge (PGN for S2* cells, S. aureus inoculation for adult flies; see Materials and Methods). Actin was used as the loading control; carcass indicates remaining fly body after removal of the head and the abdominal fat body; fat body indicates fat body tissue dissected from the abdomens of adult flies (20lanes). (D) Western blot comparing Zfh2 expression in the fat bodies, carcasses, and heads of control (c) and zfh2209 (209) mutant flies. Blot was performed using rat polyclonal anti-Zfh2 antiserum (33), with chemiluminescence detection imaged by an Odyssey Fc imaging system (LI-COR Biosciences). Shown blot is representative of triplicate experiments. (E) Quantification of the bands from the Western blot in (D) and two replicates using the LI-COR Odyssey imaging system reveals that in zfh2209 flies (209), Zfh2 levels are reduced ~2-fold in the fat body, but similar in the carcass and head, compared with control flies (c, w1118). *p < 0.05.
ZFHX3 is involved in neural and epithelial development (47–50), implicated by GWAS in atrial fibrillation (51), and it has roles in multiple types of cancer (52–54). Similarly, ZFHX4 is involved in epithelial and glial cancers (55, 56) and in neuronal development (57). However, neither Zfh2 nor its human orthologs ZFHX3 and ZFHX4 had previously been shown to regulate immune gene transcription or gas sensing. Taken together, these observations suggest that Zfh2, ZFHX3, and ZFHX4 could define a family of conserved, but previously unidentified, proteins that mediate hypercapnic immune suppression.

To investigate a potential role for Zfh2 in immune regulation, we determined where Zfh2 was expressed and whether its levels were altered by immune stimulation or by hypercapnia. FlyBase listed Zfh2 as being enriched in the nervous system and not expressed significantly in other tissues or S2 cells (66). Western blotting (Fig. 3C) confirmed strong expression of Zfh2 in neural tissue (adult fly heads), and in addition revealed Zfh2 expression in both S2* cells and in the adult abdominal fat body, the major immune and metabolic organ in Drosophila. In adult flies, a predominant band of Zfh2 immunostaining at ~300 kDa is observed; however, lower molecular mass bands are also present. In S2* cells, multiple higher molecular mass bands are observed that appear considerably >300 kDa. The larger molecular mass bands may represent posttranslationally modified Zfh2, because they are strongly reduced in RNAi and CRISPR experiments, but are not consistent with the predicted sizes of Zfh2 isoforms (Supplemental Fig. 1). PGN challenge of S2* cells and S. aureus infection of adult flies did not appear to alter Zfh2 protein levels or electrophoretic mobilities (Fig. 3C). Likewise, exposure to 13% CO2, as compared with air, did not dramatically alter Zfh2 protein levels in S2* cells or adult fly tissues (Fig. 3C–E).

Zfh2 is required for elevated CO2 levels to decrease resistance to bacterial infection in vivo

To determine whether Zfh2 mediates hypercapnic immune suppression in vivo, we tested whether zfh2 mutant flies were protected from the increase in mortality caused by elevated CO2 in an S. aureus infection assay (Fig. 4A; see Materials and Methods). We were unable to assess the impact of complete Zfh2 deficiency because flies homozygous for strong zfh2 mutations, such as zfh213305 (27), do not survive to adulthood (data not shown). Transheterozygous combinations of zfh21-M707.R and weaker alleles, such as zfh2MS209 (27), are also not viable as adults (data not shown). However, we were able to analyze animals homozygous for the zfh2MS209 mutation, which is a hypomorphic allele resulting from a transposon insertion in the 3′ region of zfh2 (67). zfh2MS209 homozygotes have wing and leg developmental defects (67), but are viable for use in infection assays. zfh2MS209 mutant flies (w1118;Zfh2MS209) and control flies (w1118) maintained in air experience approximately the same mortality postinfection with S. aureus (Fig. 4B; red zfh2MS209 line above and below black control line, p = 0.18). However, when exposed to 13% CO2 for 48 h prior to infection, zfh2MS209 mutants exhibit significantly reduced mortality compared with w1118 controls (Fig. 4C; red zfh2MS209 line above black w1118 control line, p < 0.0005). Note that flies were exposed to elevated CO2 before infection only and placed in air in infection to avoid confounding effects of simultaneously exposing the pathogen as well as the host to hypercapnia. Consistent with the fact that zfh2MS209 is a partial loss-of-function mutation, Zfh2 levels in zfh2MS209 homozygotes are not different from control flies in the head and carcass, but are reduced ~2-fold in the fat body (Fig. 3D, 3E, 4F). zfh2MS209 mutants are not completely protected from the increased postinfection mortality caused by elevated CO2 (Fig. 4D, p = 0.009), although the deleterious effect of hypercapnia is less than in control flies (compare Fig. 4A and 4D). Thus, mutation of zfh2 allows flies to better survive infection after exposure to hypercapnia.

We next investigated whether the improved survival of hypercapnia-exposed zfh2MS209 flies resulted from increased resistance (the ability to limit pathogen burden) (68), or increased tolerance (the ability to limit the health impact of a given pathogen burden) (68), of the bacterial infection. The zfh2MS209 mutation appears to increase resistance because hypercapnia leads to increased bacterial load in control animals, but not in in zfh2MS209 homozygotes (Fig. 4E). Taken together, these results indicate that zfh2 is required for CO2 to suppress antibacterial host defense in vivo.

To confirm the above results and to identify tissues in which zfh2 acts to mediate CO2-induced immune suppression, we used the GAL4/UAS system (69) to express a zfh2-targeted short hairpin RNA construct (31) under the control of tissue-specific drivers. Consistent with the lethal phenotype of strong zfh2 mutations, da-GAL4-driven ubiquitous expression of the zfh2 RNAi construct UAS-zfh213305 caused lethality during embryonic and larval stages, and no pupa were observed (data not shown). However, embryonic, larval, and pupal development were not overtly perturbed by using the CG-GAL4 or C754-GAL4 drivers to express UAS-zfh213305 in the fat body, the major AMP-producing organ in the fly. Importantly, CG-GAL4-driven knockdown of zfh2 by UAS-zfh213305 reduces Zfh2 protein levels in the fat body, but not the rest of the body or head (Fig. 4F), and it provides protection against hypercapnic immune suppression (Fig. 4I). Whereas exposure to 13% CO2 increases mortality of S. aureus infection in CG-GAL4 control adults (Fig. 4H; red CO2 line below the black air line, p = 0.0001 for CG-GAL4/CG-GAL4; p = 0.03 for CG-GAL4/+; data not shown), hypercapnia does not increase the mortality of infection in zfh213305/CG-GAL4 adults (Fig. 4I; red CO2 line superimposed on black air line, p = 0.44). Expression of UAS-zfh213305 using another fat body driver, C754-GAL4, also improved survival of infected flies exposed to elevated CO2 (Fig. 4J, 4K). Improved survival of adult flies with fat body–specific knockdown of zfh2 is due at least in part to increased resistance to infection because bacterial load in UAS-zfh213305/CG-GAL4 was not increased in flies exposed to 13% CO2 compared with air (Fig. 4G). Importantly, no difference in bacterial loads was observed between control flies and flies with reduced Zfh2 levels in the fat body exposed to air alone (Fig. 4G), indicating that Zfh2 is not a major regulator of immune responses in ambient air. Taken together, to our knowledge, these results identify Zfh2 as the first in vivo mediator of hypercapnic immune suppression. Furthermore, the data provide strong evidence that the immunosuppressive effects of hypercapnia in Drosophila are mediated by a specific genetic pathway that functions in the fat body.

Zfh2 influences a nonimmunological function affected by hypercapnia

We next asked whether Zfh2 was a global mediator of hypercapnic responses, or whether it acted specifically in the immune system. We assayed the ability of zfh2 mutations to prevent the hypercapnia-induced reductions in egg laying and delays in hatching that we had previously observed (21). The partial loss-of-function zfh2MS209 and zfh2MS968R mutations do not significantly alter the suppression of egg laying by 13 or 19.5% CO2 (Fig. 5A), but they can partially mitigate the delay in egg hatching by 24 h in 13% CO2 and by 48 h in 19.5% CO2 (Fig. 5B, 5C). These results provide evidence that zfh2 mediates additional deleterious effects of elevated CO2 on a process not directly related to immune function in tissues other than the fat body.
Zfh2 mediates ex vivo hypercapnic suppression of multiple antimicrobial peptides

To determine whether increased expression of AMPs by the fat bodies of zfh2 knockdown flies could be at least partially responsible for the improved survival of zfh2 knockdown flies in elevated CO₂, we developed an ex vivo system for culturing fat bodies (see Materials and Methods). In this system, abdominal fat bodies are dissected from adult males and cultured in S2* media for 24 h in air or 13% CO₂. PGN treatment and media conditions were the same as for S2* cell culture, using fat bodies from three flies per experimental condition. In this assay, PGN causes a 4-fold induction of endogenous Dipt mRNA levels that is not suppressed by culture in pH 6.5 media, but is suppressed 3-fold by culture in 13% CO₂ (media pH 6.5) and 10-fold by culturing in 13% CO₂ in pH-neutralized media (pH 7.1, Fig. 6A).

When cultured in air, Dipt induction by PGN was not different in zfh2 knockdown fat bodies than in control fat bodies (Fig. 6B). Notably, culture in 13% CO₂ at pH 6.5 did not reduce induction of Dipt in zfh2 knockdown fat bodies (Fig. 6B), in marked contrast to results in control fat bodies (Fig. 6A). This finding corroborates...
the protective effect of zfh2 knockdown in the infection assays (Fig. 4I, 4K) and the CO2-specific increases in Dipt-luc expression observed in zfh2 RNAi-treated S2* cells (Fig. 2). Culture of zfh2 knockdown fat bodies in 13% CO2 in media buffered to pH 7.1 did reduce Dipt induction compared with culture in air; however, the reduction was significantly less than in control fat bodies, with zfh2 knockdown fat bodies inducing Dipt 3.6-fold more than control fat bodies under these culture conditions (Fig. 6B). Thus, Zfh2 mediates an immune suppressive effect of elevated CO2 on induction of Dipt transcription that is not dependent on extracellular acidosis.

To investigate whether Zfh2 regulates expression of AMPs other than Dipt, we determined the effects of zfh2 knockdown on Att and Cec, which can be induced by the Imd/Rel pathway (70), Drs, which is predominantly induced by the Toll pathway (71), and Mtk, which can be induced by both the Imd and Toll family receptors (71). Whereas knockdown of zfh2 had no effect on PGN-stimulated induction of these AMPs in fat bodies cultured ex vivo in air (Fig. 6D), zfh2 knockdown significantly attenuated hypercapnic suppression of Att and Mtk, in addition to Dipt (Fig. 6E), in fat bodies cultured in 13% CO2. Knockdown of zfh2 may have also attenuated hypercapnic suppression of Cec; however, Cec mRNA levels were more variable than those of the other AMPs, such that the increase in expression in CO2 relative to air did not reach statistical significance (Fig. 6E). Expression of Drs in elevated CO2 was not affected by zfh2 knockdown (Fig. 6E), but as Drs was not actually induced by PGN (Fig. 6D), this experiment does not address whether zfh2 modulates induction of genes by the Toll pathway. However, the results do indicate that Zfh2 does not control baseline (uninduced) levels of at least one Toll-regulated gene. Taken together, these findings demonstrate that zfh2 mediates hypercapnic suppression of multiple Imd/Rel-regulated immune genes in the fat body, and that the suppressive effect of zfh2 is specific to conditions of elevated CO2.

The ex vivo fat body experiments also provided insight into the detrimental effects of zfh2 knockdown in flies maintained in air, both in the absence and presence of infection. Flies in which zfh2 has been knocked down in the fat body take ~4 d longer to reach adulthood than do control animals (data not shown), and mortality of zfh2 fat body knockdown flies after infection in air is increased compared with controls (Fig. 4J, 4K). This may be explained by the finding that fat bodies from zfh2 knockdown flies raised in air had smaller volumes than fat bodies from control flies. Also, induction of Dipt was reduced in zfh2 knockdown fat bodies compared with control fat bodies, when cultured in air at pH 6.5 or at pH 7.1 with hypertonic media (25 mM NaCl) (Fig. 6C). Given the delayed growth of the zfh2 knockdown flies and the adverse effects of culture in low pH or hypertonic media in air on Dipt induction in cultured fat bodies from these animals, it is striking that induction of Dipt in cultured zfh2 knockdown fat bodies is not suppressed by elevated CO2 and that zfh2 knockdown in adult flies prevents 13% CO2 from increasing the mortality of S. aureus infection. Taken together, these results show that Zfh2 has multiple functions in the adult fat body, one of which is to mediate the immunosuppressive effects of elevated CO2.

**Discussion**

Although several CO2 sensing pathways have been defined in neuronal tissues, it has largely been assumed that the nonneuronal physiological effects of CO2 are exerted indirectly, either by H+ and HCO3− or by neuronal signaling. However, a growing body of evidence indicates that multiple nonneuronal cell types respond to elevated CO2 levels under neutral pH conditions, and responses to CO2 can be distinguished from responses to acidosis or bicarbonate (reviewed in Ref. 72). The molecular basis of these responses is a focus of current research, however, to date the mechanisms by which CO2 influences immune systems have not been elucidated. In this study, we report a genome-wide RNAi screen that used Drosophila S2* cells to identify genes that mediate CO2-induced suppression of Dipt, an important antimicrobial peptide. We identified 16 genes with human orthologs whose knockdown could cause the PGN-induced levels of a Dipt reporter to increase ≥2-fold more in 13% CO2 than in air. In vivo analysis of one of these genes, which encodes the transcription factor Zfh2, demonstrates that reducing zfh2 function in adult fat bodies by RNAi does not alter PGN-induced expression of AMP mRNAs during culture in air, but increases expression of Dipt and several other AMPs when cultured in elevated CO2. Correspondingly, reducing zfh2 function in whole flies by mutation, or more specifically in the fat body by RNAi knockdown, improves the ability of adult flies to clear and survive infection with S. aureus.

**Implications of Zfh2 as a CO2 mediator**

The identification of Zfh2 as a CO2 mediator is important for guiding further investigation of hypercapnic immune suppression in Drosophila. In particular, the ability of fat body–directed knockdown of zfh2 to prevent hypercapnic immune suppression identifies a site of action for this CO2 response pathway. Because...
the fat body is the major immune organ of the fly, it is not surprising that it is an important target site of action for immunomodulatory effects of CO₂; however, it was nonetheless critical to establish this fact experimentally. Moreover, when combined with conditional knockdown tools (31, 69), identification of a site of action enables the study of other putative CO₂ response genes that have essential roles in embryonic development and thus are difficult or impossible to study in simple homozygous mutants.

Identification of Zfh2 is also a critical step in understanding the molecular mechanisms of hypercapnic immune suppression. zfh2 knockdown in vivo almost fully rescues resistance to bacterial infection during hypercapnia, thus identifying zfh2 as a major mediator of hypercapnic immune suppression. In S2* cells and in ex vivo fat bodies, knockdown of zfh2 can eliminate up to 50% of the effect of elevated CO₂ on Dipt induction, suggesting that hypercapnia suppresses Dipt by acting on one or a small number of signaling pathways rather than by nonspecific mechanisms affecting the activity of many targets. This hypothesis is bolstered by our finding that increases in Dipt expression resulting from zfh2 knockdown are specific for hypercapnia and do not occur during culture in air or in acidified media without hypercapnia. zfh2 knockdown in fat bodies not only fails to mitigate the effects of nonhypercapnic acidosis, it makes fat bodies more sensitive to acidosis and less sensitive to elevated CO₂ levels. An important implication of the suggestion that the immune suppressive effects of hypercapnia are mediated by one or a small number of pathways is that, in principle, it should be possible to therapeutically block the effects of elevated CO₂ using small molecules.

Perhaps the most important aspect of the identification of Zfh2 as a mediator of hypercapnic immune suppression is that it may

**FIGURE 6.** zfh2 mediates hypercapnic suppression of AMPs in adult fat bodies cultured ex vivo. (A) Hypercapnia, but not normocapnic acidosis, suppresses Dipt induction in ex vivo cultured control fat bodies to a similar extent as does the suppression of Dipt induction observed in S2* cells (21). Bars show Dipt mRNA levels (normalized to RP49 mRNA) in fat bodies from control adults (w¹¹¹B, CG-Gal4/+ , solid bars) that have been cultured ex vivo in media equilibrated with air or 13% CO₂; uninduced and PGN-induced, at pH 7.1 (media without additions for air, or media plus 25 mM NaOH for 13% CO₂) or pH 6.5 (media plus 19 mM MOPS for air, or media without additions for 13% CO₂). (B) Fat body–specific knockdown of zfh2 does not significantly increase Dipt induction when fat bodies are cultured in media equilibrated with air, but does prevent or reduce hypercapnia-induced suppression of Dipt induction in fat bodies cultured in media equilibrated with 13% CO₂ at pH 6.5 or pH 7.1. (B) shows data from the same experiment as in (A), and in addition shows results obtained using fat bodies from adults in which zfh2 had been knocked down using a UAS-shRNA construct and the fat body driver, CG-Gal4 (genotype, w¹¹¹B, UAS-zfh2¹³³O/C-Gal4, stripped bars). (C) Induction of Dipt is suppressed in zfh2 knockdown, but not control, fat bodies cultured in media equilibrated with air at pH 6.5 or supplemented with 25 mM NaCl. (D) zfh2 knockdown (purple bars) does not alter induction of Dipt, Att, Cec, or Mtk mRNAs in ex vivo–cultured fat bodies cultured in media equilibrated with air (gray bars, control [c], w¹¹¹B, CG-Gal4/+ ; purple bars, zfh2 knockdown, w¹¹¹B, UAS-zfh2¹³³O/C-Gal4). n = 3. (E) zfh2 knockdown attenuates hypercapnic suppression of PGN-induced mRNA for Dipt, Att, and Mtk in ex vivo–cultured fat bodies. AMP mRNA levels in fat bodies cultured in 13% CO₂ in neutral media (pH7.1) are expressed as a percentage of the corresponding mRNA level in fat bodies cultured in air. For example, Mtk was suppressed ∼80% by hypercapnia in control fat bodies, but only ∼25% in zfh2 knockdown fat bodies. Note that Drs, the expression of which is not suppressed by 13% CO₂, was not induced by PGN (D). Genotypes: same as in (D). n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.
provide an entry point to understanding the molecular mechanism of hypercapnic immune suppression in humans. Zfh2 has established orthologs in mammals, and we have previously shown that there are strong parallels between hypercapnic immune suppression in Drosophila and mammals (15, 16, 21). Although it is possible that Zfh2 acquired a role as a CO2 mediator after flies and mammals diverged, strong conservation of the innate immune pathways, other gas-sensing pathways, as well as most other signaling systems, suggests that mammalian ZFHX3 and/or ZFHX4 could also mediate immunomodulatory effects of CO2. Additionally, given that zfh2 mutations partially blocked the effects of elevated CO2 on egg hatching, it is also possible that ZFHX3 and ZFHX4 could act outside the immune system to mediate nonimmunological effects of elevated CO2, such as hypercapnia-induced muscle wasting (73).

Possible models for how Zfh2 acts

How does Zfh2 mediate hypercapnic immune suppression? Zfh2 has 13 zinc fingers and three homeodomains, and it has been demonstrated to bind DNA. Although it is therefore likely that Zfh2 acts as a transcription factor in transducing CO2 responses, zinc fingers can also mediate protein–protein interactions, raising the possibility that a nontranscriptional function of Zfh2 mediates CO2 responses. In either case, the genetic data are consistent with Zfh2 acting as a CO2-specific negative regulator of AMP induction, either by directly binding AMP promoters or by binding and inhibiting positive regulators of AMP induction. At this point, it is unclear whether Zfh2 is the sole mediator of the immunosuppressive effects of CO2 because the lethality of Zfh2 mutants made it impossible to completely remove Zfh2 function. However, it seems likely that additional CO2-responsive factors may suppress innate immune function in flies because the limited role of Zfh2 in CO2-induced suppression of egg laying indicates that other CO2-responsive pathways exist, and few immunological responses are regulated by a single pathway.

Zinc finger homeodomain proteins: a family of immune regulators?

In addition to zfh2/ZFHX3/ZFHX4, the Drosophila and human genomes encode a second family of zinc finger homeodomain-containing proteins (46). Members of this family, which includes Drosophila zfh1 and human ZEB1 and ZEB2, are about one third the size of the Zfh2/ZFHX3/ZFHX4 protein family (~1100 aa), and each contains one homeodomain and seven to nine zinc finger domains. Notably, despite the considerable evolutionary divergence between these families, both Zfh1 and ZEB1 have been shown to also modulate NF-kB-regulated innate immune responses (74). Similar to Zfh2, Zfh1 is a negative regulator of Dipt and other Imd-responsive AMPS, including Att and Cec. Interestingly, epistasis experiments indicated that Zfh1 acts downstream of or in parallel to Rel, similar to our findings that elevated CO2, apparently via Zfh2, suppresses AMPs downstream of Rel proteolytic activation (21). However, a critical difference between Zfh1 and Zfh2 is that whereas knockdown of zfh1 in normocapnia increases expression of multiple AMPs, knockdown of zfh2 only increases AMP expression in hypercapnia. These findings raise the possibility that an ancestral zinc finger homeodomain protein may have regulated immune responses, with the Zfh1/ZEB1/ZEB2 family evolving as general immune regulators, whereas Zfh2, and potentially other members of the Zfh2 family, evolved as CO2-responsive regulators of innate immune responses. This specialization also suggests that the ability to separately regulate immune responses in normocapnia and hypercapnia is biologically important.

An interesting question is whether the screen identified a CO2 sensor that controls hypercapnic immune suppression. The variety in known CO2 sensing mechanisms, particularly the recent identification of a connexin subunit as a CO2 sensor by carbamylation (75), indicates that, in principle, almost any protein could act as a sensor, thus making it difficult to predict CO2 sensing ability based on known function or sequence. Thus, the screen may well have identified the sensor, but we are unable to recognize it as such at this time. Although it is possible that Zfh2 is carbamylated in elevated CO2 levels and thus is both a sensor and effector, another likely scenario is that CO2 levels are sensed by an as yet unidentified upstream pathway component, leading to posttranslational modification of Zfh2 that alters its capacity for DNA binding or protein–protein interactions, thereby altering transcription of AMPs and possibly other immune genes. Alternatively, secondary screening may not have identified the CO2 sensor because we focused on dsRNAs whose knockdown in air had minimal effects, but in hypercapnia prevented suppression of Dipt-luc. If the sensor, or any part of a hypercapnia response pathway, is essential for Imd pathway signaling in air, it would not have scored as a positive in the screen. Further analysis of the hits from this screen, and the use of confirmed CO2 mediators in assays of pathway function, will ultimately define the pathway of hypercapnic immune suppression and the CO2 sensor that regulates it.

In conclusion, the goal of this work was to identify genes that mediate immune suppressive effects of hypercapnia. Our in vitro screen identified 16 candidate Drosophila CO2 mediator genes that have human orthologs, one of which was tested for immunomodulatory effects in adult flies. These experiments defined Zfh2, to our knowledge, as the first known mediator of hypercapnic immune suppression. The screen was also expected to identify components of the Imd pathway that regulates the Dipt product, and given that 12 previously known positive and negative regulators of the Imd pathway were identified, it is likely that several of the candidate CO2 mediators will be in vivo mediators of hypercapnic immune suppression. Thus, this screen is an important starting point in the quest to fully define the components of the signaling pathways that mediate hypercapnic immune suppression.

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Disclosures

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