Genetic Analysis of Pathways Regulated by the von Hippel-Lindau Tumor Suppressor in Caenorhabditis elegans

Tammie Bishop1, Kah Weng Lau1, Andrew C. R. Epstein1, Stuart K. Kim2, Min Jiang2, Delia O’Rourke3, Christopher W. Pugh1, Jonathan M. Gleadel1, Martin S. Taylor1, Jonathan Hodgkin3, Peter J. Ratcliffe1*

1 The Henry Wellcome Building of Genomic Medicine, University of Oxford, Oxford, United Kingdom, 2 Department of Developmental Biology and Genetics, Stanford University Medical Center, Stanford, California, United States of America, 3 Department of Biochemistry, University of Oxford, Oxford, United Kingdom

The von Hippel-Lindau (VHL) tumor suppressor functions as a ubiquitin ligase that mediates proteolytic inactivation of hydroxylated α subunits of hypoxia-inducible factor (HIF). Although studies of VHL-defective renal carcinoma cells suggest the existence of other VHL tumor suppressor pathways, dysregulation of the HIF transcriptional cascade has extensive effects that make it difficult to distinguish whether, and to what extent, observed abnormalities in these cells represent effects on pathways that are distinct from HIF. Here, we report on a genetic analysis of HIF-dependent and -independent effects of VHL inactivation by studying gene expression patterns in Caenorhabditis elegans. We show that VHL functions in pathways other than HIF and -independent effects of VHL-1 inactivation. Genomic clustering, predicted functional similarities, and a common pattern of dysregulation in both vhl-1 worms and a set of mutants (dpy-18, let-268, gon-1, mig-17, and unc-6), with different defects in extracellular matrix formation, suggest that dysregulation of these genes reflects a discrete HIF-1-independent function of VHL-1 that is connected with extracellular matrix function.

Introduction

The von Hippel-Lindau (VHL) gene is a tumor suppressor that is mutated in the majority of both hereditary and sporadic, clear-cell renal carcinomas (Kaelin 2002). In hereditary VHL disease affected individuals are also predisposed to pheochromocytomas and retinal/central nervous system hemangioblastomas and develop multiple benign lesions in the kidney and other organs. Despite more than a decade of intensive investigation following identification of the defective gene in 1993 (Latif et al. 1993), the nature of the VHL tumor suppressor mechanism and how it relates to the physiological function of VHL remains unclear (Kaelin 2002).

To date, the best-understood function of VHL is as a ubiquitin ligase that affects oxygen-dependent proteolytic targeting of the α subunits of hypoxia-inducible factor (HIF) (Maxwell et al. 1999; Ohh et al. 2000). Oxygen-dependent hydroxylation of two HIF-α prolyl residues by HIF prolyl hydroxylases (Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001) promotes interaction with VHL and targets HIF-α for degradation by the ubiquitin-proteasome pathway. In VHL-defective cells HIF-α subunits are stabilized and HIF is constitutively activated, resulting in the upregulation of HIF target genes (Maxwell et al. 1999). Whether this, or other putative VHL pathways, accounts for the tumor suppressor action is the subject of active investigation (Kondo et al. 2002, 2003; Maranchie et al. 2002). For instance, a number of different VHL-dependent cellular phenotypes have been defined by contrasting VHL-defective cells with transfectants re-expressing wild-type VHL (Kaelin 2002). These have highlighted effects of VHL on invasiveness, branching morphogenesis, and matrix assembly (Ohh et al. 1998; Koochekpour et al. 1999; Davidowitz et al. 2001; Kamada et al. 2001; Esteban-Barragan et al. 2002). However, mechanistic links to VHL function have not yet been defined and it is unclear whether or not these effects are secondary to dysregulation of HIF. This has led to attempts to define the existence, or otherwise, of non-HIF, VHL-regulated pathways by comparing patterns of gene expression induced by VHL inactivation with those induced by hypoxia (Wykoff et al. 2000; Zatyka et al. 2002; Y. Jiang et al. 2003). The observed patterns are not fully concordant, suggesting that there may be non-HIF, VHL-regulated pathways. However, these studies leave important uncertainties since HIF dysregulation might have secondary effects on pathways that are not themselves responsive to hypoxia and VHL might target hypoxia pathways other than HIF.

To address this we have used a genetic approach in Caenorhabditis elegans. Whereas mammalian cells possess three HIF-α isoforms that are targeted by VHL, C. elegans has a single HIF-1 homolog (HIF-1) and a single VHL homolog (VHL-1),
vhl-1 inactivation were not observed in homolog EGL-9. In contrast, the HIF-1–independent effects of vhl-1 inactivation on gene expression were also produced by inactivation of the HIF prolyl hydroxylase homolog EGL-9. As a first step in defining VHL-1–dependent pathways in *C. elegans*, a whole-genome microarray was probed to compare transcript patterns in *vhl-1* versus wild-type worms (*n* = 1).

### Table 1. Top 30 Upregulated Genes in the *vhl-1* versus Wild-Type Microarray Comparison and Confirmation of Selected Genes by RNase Protection Assays

| Gene Name | Description | Microarrays: *vhl-1*/Wild-Type | RNase Protection Assays: *vhl-1*/Wild-Type |
|-----------|-------------|---------------------------------|------------------------------------------|
| **Fold**  | **Regulation** | **Confirmation** | **Fold** | **Regulation** | **p Value** |
| F22S5.4   | —           | 25.3 Y | 31.7 | < 0.01 |
| C32H11.9  | —           | 8.6 Y | N | — |
| egl-9/S7/T05B4.2 | Member of the nuclear hormone receptor/zinc finger protein family | 8.0 Y | 7.3 | < 0.01 |
| C32H11.10 | —           | 6.4 Y | — | — |
| C55B7.4   | Member of the acyl-CoA dehydrogenase protein family | 6.2 Y | N | — |
| fmo-12/R08C7.5 | Member of the flavin-dependent monooxygenase family, which are xenobiotic-metabolizing enzymes; has moderate similarity to flavin-containing monooxygenase 5 (human FMO5) | 5.9 Y | 2.8 | < 0.05 |
| Y59A8B.19 | Protein with moderate similarity to alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB, human NAGLU), which catalyzes the stepwise degradation of heparan sulfate and is associated with Sanfilippo syndrome type B upon mutation | 5.0 Y | — | — |
| K09E4.4   | —           | 4.9 Y | N | — |
| T03F1.6   | G protein-coupled receptor, member of unnamed subfamily with distant homology to SRG subfamily, no homolog found in human or Drosophila melanogaster | 4.8 Y | — | — |
| W07A12.6  | —           | 4.8 Y | — | — |
| ZK969.4   | Dioxygenase, regulates HIF-1 by prolyl hydroxylation | 4.6 Y | — | — |
| egl-9/F22E12.4 | — | — | — | — |
| Y43F6C.2  | Neuropeptide neurotransmitter | 4.5 Y | 1.8 | < 0.01 |
| F31G1.8   | Protein containing glutathione S-transferase S-terminal and C-terminal domains, has low similarity to prostaglandin D2 synthase (human PGDS), which is a prostaglandin D synthase and glutathione S-transferase that regulates non-rapid eye movement sleep | 4.4 Y | N | — |
| Y51A2A.4  | —           | 4.3 Y | — | — |
| K06D6.1   | —           | 4.3 Y | — | — |
| phy-2/F35G2.4 | Glutathione S-transferase alpha subunit | 4.1 Y | 6.8 | < 0.01 |
| C06H8.3   | —           | 4.1 Y | — | — |
| Y54D9A.1  | —           | 4.1 Y | — | — |
| R09B3.3   | —           | 4.1 Y | — | — |
| F13B4.3   | Protein containing two epidermal growth factor-like domains, a von Willebrand factor type A domain, and a C-type lectin domain, which mediate calcium-dependent carbohydrate recognition | 4.0 Y | — | — |
| C31C1.9   | —           | 4.0 Y | — | — |
| T23F5.4   | —           | 4.0 Y | — | — |
| H14N18.4  | Member of the gamma-glutamyltransferase (tentative) protein family | 3.9 Y | N | — |
| C16C10.3  | Piwi-related protein with similarity over the C-terminal region to human and D. melanogaster Piwi proteins and C. elegans PRG-1 and PRG-2, all of which are involved in germ-line proliferation | 3.9 Y | N | — |
| F07C4.7   | Protein containing a protein kinase domain, has a region of moderate similarity to MAP kinase-interacting serine-threonine kinase 1 (human MKR1), which is a kinase activated by the ERK and p38 MAP kinase signaling pathways | 3.7 Y | — | — |
| R166.5    | —           | 3.4 Y | — | — |
| F16B4.1   | Protein containing three eukaryotic-type carbonic anhydrase domains, which catalyze reversible hydration of carbon dioxide; has low similarity to carbonic anhydrase 2 (human CAZ), which hydrates carbon dioxide to form a bicarbonate ion and a proton | 3.3 Y | — | — |
| cah-4/R01E6.3 | — | — | — | — |
| C04F6.3   | —           | 2.8 Y | 2.3 | < 0.01 |
| 268, gon-1, mig-17, and unc-6) bearing defects in genes involved in extracellular matrix function, supporting the existence of a conserved non-HIF pathway connecting VHL with an as yet unknown extracellular matrix function. | 2.8 Y | N | — |

**Results**

Effect of VHL-1 Inactivation on Gene Expression in *C. elegans*

As a first step in defining VHL-1–dependent pathways in *C. elegans*, a whole-genome microarray was probed to compare transcript patterns in *vhl-1* versus wild-type worms (*n* = 1).
From this array a set of genes (selected for amplitude of differential expression, signal intensity, quality of array signal, and putative function) was assayed quantitatively by ribonuclease (RNase) protection (Table 1). Of the 14 genes analyzed, six (F22B5.4, unknown function; nhr-57, predicted nuclear hormone receptor; fmo-12, predicted flavin mono-oxygenase; egl-9, HIF-1 prolyl hydroxylase [Epstein et al. 2001]; phy-2, procollagen prolyl 4-hydroxylase α subunit [Friedman et al. 2000]; and cah-4, predicted carbonic anhydrase) were strikingly downregulated by VHL-1 (Figure 1A; Table 2, column B). Further analysis in synchronized worm populations indicated that the VHL-1–dependent effects were observed in all stages (Figure 1B and unpublished data).

Analysis of the EGL-9/HIF-1 Pathway

To determine the extent to which disruption of the conserved EGL-9/HIF-1 pathway mediates these effects we studied wild-type, hif-1, vhl-1, and egl-9 single mutant worms and hif-1; vhl-1 and egl-9; hif-1 double mutant worms. Apart from the mild phenotype of the vhl-1 worms (slightly uncoordinated, slow growth, and reduced brood size) and the egg-laying defective phenotype of egl-9, none of the worm strains showed obvious phenotypic abnormalities. Interestingly, hif-1 corrected the phenotype of egl-9.

The findings indicate that all six genes are strongly regulated by the EGL-9/HIF-1 pathway (Figure 1A; Table 2). All six genes were inducible by hypoxia in wild-type worms.
suggesting that the VHL-1–independent repressive effects on
mimicked by the dioxygenase inhibitor 2,2-
(Figure 1A). Second, three genes
was conserved in
inactivation of
pathway (Figure 1C). Interestingly, the effects of genetic
RNAi. Both procedures increased
expression, indicat-
9
that these genes are direct HIF-1 transcriptional targets.

Though the six genes all conformed to the above patterns
to demonstrate regulation by the EGL-9/HIF-1 pathway
(Figure 1A; Table 2), there were differences. First, for some
genes (F22B5.4, nhr-57, fmo-12, egl-9, and cah-4) showed modest upregulation, and one gene (phy-2) showed
modest downregulation, by hypoxia that was independent of
HIF-1, VHL-1, and EGL-9 (Table 2, columns G–I). Finally, for
certain genes, upregulation was clearly greater in
9
than
vhl-1 worms, results being particularly striking for nhr-57
(Table 2, columns C and B). To pursue this, we created
9
vhl-1 double mutants and also exposed vhl-1 worms to egl-9
RNAi. Both procedures increased nhr-57 expression, indicat-
ing that EGL-9 has non–VHL-1–mediated effects on this
pathway (Figure 1C). Interestingly, the effects of genetic
inactivation of egl-9 in the vhl-1 background were not
mimicked by the dioxygenase inhibitor 2,2'-dipyridyl (DIP),
suggesting that the VHL-1–independent repressive effects on
nhr-57 may be nonenzymatic.

Evidence for a VHL-1–Dependent, HIF-1–Independent
Pathway
To address directly whether HIF-1-independent, VHL-1-
mediated pathways exist, we performed further microarray
comparisons of RNA from hif-1; vhl-1 and hif-1 worms (n = 3).
Fewer genes showed differential expression than in the
vhl-1 versus wild-type array; however, persisting differential
expression did suggest the existence of VHL-1 pathways that are
independent of HIF-1 (Table 4). To test this, a number of
genes were selected for further validation by RNase protec-
tion assay on the basis of amplitude of differential expression,
value, signal intensity, and quality of array signal. Of the 25
genes analyzed by RNase protection assay (Table 4), six
(C01B4.7, F56A4.10, C01B4.9, and C01B4.8, all predicted
transmembrane proteins belonging to the major facilitator
superfamily [InterPro: IPR007114 and IPR005828]; F56A4.2, a
predicted C-type lectin [InterPro: IPR001304]; and C01B4.6, a
predicted aldose epimerase [InterPro: IPR008183]) showed
clear downregulation by VHL-1 in a HIF-1-independent
manner (Figure 2A; Table 5, column C). These effects were
observed across essentially all developmental stages of the
worm (Figure 2B). Computational analysis revealed that only
one (C01B4.8) of the five HIF-1-independent, VHL-1-
dependent genes validated (C01B4.7, F56A4.10, C01B4.9,
C01B4.8, and C01B4.6; no single ortholog of F56A4.2 could
be identified in C. briggsae) contained a potential HIF-1
binding site (HBS) within an arbitrarily defined region that
was conserved in C. briggsae (see Table 3). This contrasts
with the HIF-1-dependent, VHL-1-dependent genes validated by
RNase protection assay (see Figure 1A), for which potential
HBSs could be defined for five of the six genes tested (see
Table 3).

Interestingly, all six genes validated by RNase protection
assay to be negatively regulated by VHL-1 in a HIF-1-
independent manner localize within 45 kb on Chromosome V
(although they were not situated in physical proximity on the
array). We applied single-linkage clustering (nearest-neighbor
method) (Sneath 1957; Dillon and Goldstein 1984; Roy et al.
2002) to identify spatial clusters of genes considered to be
negatively regulated by VHL-1 in a HIF-1-independent

Table 3. Evolutionarily Conserved HBS Consensus Sequences

| Gene Name | Organism | Relative Position | Orientation | Sequence |
|-----------|----------|-------------------|-------------|----------|
| F22B5.4   | C. elegans | -470/-482         | Antisense   | GACTACGTGAGGA |
|           | C. briggsae | -47h/-490        | Antisense   | GACTACGTGAGGA |
| nhr-57    | C. elegans | -271/-259         | Sense       | GCCTACGTGATTATA |
|           | C. briggsae | -274/-262        | Sense       | GCCTACGTGATTATA |
|           | C. elegans | -109/-121         | Antisense   | GTCTACGTGAGAGA |
|           | C. briggsae | -93/-105          | Antisense   | GCTCTACGTGAGAGA |
| fmo-12    | C. elegans | -203/-191         | Palindrome  | CCAACACGTGTTTCC |
|           | C. briggsae | -253/-241        | Palindrome  | CCAACACGTGTTTCC |
| egl-9     | C. elegans | -86/-98           | Antisense   | AACCAACGTGACAC |
|           | C. briggsae | -114/-126        | Antisense   | AACCAACGTGACAG |
|           | C. elegans | -69/-81           | Antisense   | GCTACACGTGACATAA |
|           | C. briggsae | -97/-109          | Antisense   | GCTACACGTGACAA |
| cah-4 (a) | C. elegans | -245/-233         | Palindrome  | GCACACCGTTTTTTT |
|           | C. briggsae | -236/-224        | Palindrome  | GCACACCGTTTTTTT |
| C01B4.8   | C. elegans | -300/-288         | Sense       | GATGACACGTGACTT |
|           | C. briggsae | -357/-345        | Sense       | GATGACACGTGACTT |
| Consensus motif | | | | RCCTG |

The sequence column shows alignments between C. elegans and C. briggsae that conserve a match (bold type) to the mammalian HBS consensus motif, RCCTG, where R = A or G. Matches were found on both the sense and antisense strands; matches that are perfect palindromes (CAGCTG) can be considered equally good matches to both the sense and antisense strand. The antisense matches are oriented to demonstrate alignment with the consensus motif. The positions of the aligned sequences are shown relative to the translation initiation site. The gene cah-4 has two alternate first exons (denoted “a” and “b” in WormBase); both were evaluated. In addition to the genes shown, C01B4.7, F56A4.10, C01B4.9, and C01B4.6 were screened for conserved RCCTG motifs, but none was found. DOI: 10.1371/journal.pbio.0020289.t003
Table 4. Upregulated Genes in the hif-1; vhl-1 versus hif-1 Microarray Comparisons and Confirmation of Selected Genes by RNase Protection Assays

| Gene Name             | Description                                                                 | Microarrays: hif-1; vhl-1[28] | RNase Protection Assays: hif-1; vhl-1[28] |
|-----------------------|------------------------------------------------------------------------------|-------------------------------|------------------------------------------|
|                       |                                                                              | Fold Regulation | p Value | Confirmation | Fold Regulation | p Value |
| F56A4.9/Y19D10A.7     | Member of glutathione S-transferase protein family, has similarity to human glutathione S-transferases | 16.6 < 0.01 | *       |             |                |        |
| F56A4.3/Y45G12C.2     | Member of an uncharacterized protein family with similarity to Entamoeba histolytica lysozymes | 7.7 < 0.05 | N       |             |                |        |
| F17E9.11              | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 6.6 0.06 | N       |             |                |        |
| F56F3.4               | Member of the sugar (and other) transporter family, has low similarity to solute carrier family 17 member 1 (mouse Slc17a1), which is involved in sodium-dependent phosphate transport and hepatic and renal anion drug transport | 5.2 0.08 | N       |             |                |        |
| Y19D10A.4/C01B4.7     | Member of the polypeptide chain release factor protein family | 5.1 < 0.05 | Y       | 4.5 < 0.01 |                |        |
| Y39C12A.4             | Protein containing a glutathione S-transferase N-terminal domain, has low similarity to C. elegans efn-4 (protein family) | 4.7 < 0.05 | N       |             |                |        |
| F56A4.10/Y19D10A.8    | Protein with a region of low similarity to a region of prostate-specific antigen (human PSA), which is a prostate-specific antigen and glutathione S-transferase that regulates non-rapid eye movement sleep | 4.0 < 0.01 | Y       | 3.7 < 0.01 |                |        |
| F35E8.8               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 3.8 < 0.05 | N       |             |                |        |
| Y19D10A.12/C01B4.9    | Protein with a region of low similarity to a region of monocarboxylate cotransporter domain 2, (rat Slc16a2), which mediates the transport of lactate and pyruvate across plasma membranes | 3.6 < 0.05 | Y       | 3.2 < 0.01 |                |        |
| Y11D7A.11             | Protein containing two collagen triple helix repeats, which are found in some extracellular proteins, and a nematode cuticle collagen N-terminal domain, has low similarity to C. elegans mec-5, which is a collagen family member | 3.6 0.07 | N       |             |                |        |
| F36D3.9               | Protein with high similarity to cathepsin B (human CTSB), which is a cysteine (thiol) protease that degrades cartilage matrix proteins and facilitates tumor invasion, member of the papain family of cysteine proteases | 3.6 0.07 | N       |             |                |        |
| F22D6.10              | Protein containing two collagen triple helix repeats and one nematode cuticle collagen N-terminal domain, has moderate similarity to C. elegans rol-6, which is a putative collagen that functions in morphogenesis of the epithelium and cuticle synthesis | 3.5 0.07 | N       |             |                |        |
| Y39G8B.8              | Protein containing a glutathione S-transferase N-terminal domain, has low similarity to C. elegans mec-5, which is a collagen family member | 3.4 < 0.01 | N       |             |                |        |
| C01B4.8/Y19D10A.5     | Member of the sugar (and other) transporter family, has low similarity to solute carrier family 17 member 1 (mouse Slc17a1), which is involved in sodium-dependent phosphate transport and hepatic and renal anion drug transport | 3.4 < 0.05 | Y       | 2.9 < 0.01 |                |        |
| F56A4.7/Y45G12C.12    | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 3.3 < 0.05 | —       |             |                |        |
| Y39C12A.5             | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 3.3 < 0.01 | N       |             |                |        |
| F35E8.8               | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 3.2 < 0.05 | —       |             |                |        |
| F56A4.2/Y19D10A.9     | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 3.1 < 0.05 | Y       | 3.3 < 0.01 |                |        |
| T11F9.6               | Putative zinc metalloprotease | 3.0 0.06 | N       |             |                |        |
| T05A10.3              | Member of the transferrin-like family | 2.9 0.08 | N       |             |                |        |
| F39F10.1              | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.8 < 0.05 | —       |             |                |        |
| F46G3.10              | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.8 < 0.05 | —       |             |                |        |
| C01B4.8/Y19D10A.4     | Member of the aldose 1-epimerase family, which interconvert aldoses between their alpha- and beta-forms; has low similarity to a region of Saccharomyces cerevisiae Gal1p, which is an UDP-glucose 4-epimerase that catalyzes a step in galactose metabolism | 2.7 < 0.01 | Y       | 2.7 < 0.01 |                |        |
| T06E4.8               | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.6 < 0.05 | N       |             |                |        |
| hif-1; rol-6          | Helix-loop-helix DNA-binding domain family member; involved in the development and differentiation of several different mesoderm-derived cells | 2.5 0.06 | N       |             |                |        |
| T05A8.3               | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.5 < 0.05 | —       |             |                |        |
| C46H11.7              | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.4 < 0.05 | —       |             |                |        |
| T06E4.9               | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.4 < 0.05 | —       |             |                |        |
| F58B3.2               | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.4 < 0.05 | N       |             |                |        |
| W02A2.3               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.2 0.05 | N       |             |                |        |
| ZK1307.2              | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.2 < 0.05 | —       |             |                |        |
| ZC169.5               | Protein containing an N-terminal domain, has low similarity to human glutathione S-transferase | 2.2 < 0.05 | —       |             |                |        |
| M04C3.1               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.2 < 0.05 | —       |             |                |        |
| Y43E8B.14             | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.2 < 0.05 | —       |             |                |        |
| Y40C7B.3              | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.2 < 0.05 | —       |             |                |        |
| C39H7.4               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.2 < 0.05 | —       |             |                |        |
| Y39S11A.6             | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 < 0.01 | —       |             |                |        |
| T19M3.3               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 0.05 | N       |             |                |        |
| T02E9.2               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 0.05 | N       |             |                |        |
| F23A7.5               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 0.05 | N       |             |                |        |
| Y2H9A.4               | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 0.05 | N       |             |                |        |
| Y39S4B.2              | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 0.05 | N       |             |                |        |
| C24A8.4               | Member of the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 0.05 | N       |             |                |        |
| Y45G12C.9/C13B7.4     | Protein containing the ubiquitin family, contains an AN1-like zinc finger domain | 2.1 < 0.01 | —       |             |                |        |
manner from the microarray data (Table 4) and random sampling to evaluate the significance of such clusters. Using a clustering threshold of 96,985 bp (see Materials and Methods), one cluster of ten genes and four clusters of two genes were identified (Figure 3A). On 100,000 simulated datasets of 57 randomly selected genes (equal number to that of VHL-1–dependent, HIF-1–independent genes; Table 4), the frequency of observed cluster sizes was as follows: one gene, 5,043,442; two genes, 298,425; three genes, 18,198; four genes, 1,190; five genes, 66; six genes, 4. No clusters of more than six genes were observed. Therefore, the cluster of ten VHL-1–regulated (HIF-1–independent) genes, which extends over 110 kb to include F56A4.9, Y45G12C.9, Y45G12C.12, and Y45G12C.2 in addition to the six genes validated by RNase protection assays, can be considered statistically significant to \( p < 10^{-5} \) (Figure 3B). Recent C. elegans genomic assemblies (for example, WS120) have shown that the entire 110-kb region containing the coregulated gene cluster is arranged in tandem with a second nearly identical segmental duplication of the locus (99.9% identical in alignment). At this level of identity, our microarray and RNase protection analyses cannot discriminate between the two copies of each gene, so for all of our analyses we have only used the names of the distal copy and genes from the proximal copy were excluded from computational analyses.

**Extracellular Matrix Link to Novel VHL-1 Pathway**

Since ubiquitin ligases commonly recognize more than one substrate, we considered whether these HIF-1–independent genes might be regulated by prolyl hydroxylases of another VHL-1 substrate by EGL-9. However, this was not supported by any differential expression in egl-9; hif-1 versus hif-1 worms (Figure 4A; Table 5, column E). Nevertheless, two genes, C01B4.7 and C01B4.8, were upregulated in hif-1 worms by hypoxia and the 2-oxoglutarate dioxygenase inhibitors, DIP and dimethyloxalylglycine (DMOG) (Figure 4; Table 5, column F), suggesting that another enzyme in this class might be involved. The procollagen prolyl hydroxylases DPY-18, PHY-2, and PHY-3 (Friedman et al. 2000; Riihimaa et al. 2002) and the procollagen lysyl hydroxylase LET-268 (Norman and Moerman 2000) were tested as potential candidates. A clear pattern was observed. All six VHL-1–regulated, HIF-1–independent genes were reproducibly downregulated by DPY-18 and LET-268 but not by PHY-2 or PHY-3 (Figure 5A; Table 5, columns H–K). The strain carrying the heterozygous let-268 mutation is heterozygous for unc-4, dpy-10, and unc-52; however, the VHL-1–dependent, HIF-1–independent genes were not differentially expressed in unc-4, dpy-10, or unc-52 worms, indicating that the effects were due to LET-268 (unpublished data). Further experiments on dpy-18; hif-1 double mutant worms clearly indicated that the effects of DPY-18 on this group of genes were (like the effects of VHL-1) HIF-1–independent (Figure 5C and unpublished data).

Downregulation by DPY-18 and LET-268 is consistent with the positive effects of hypoxia, DIP, and DMOG, since all these stimuli inhibit DPY-18 and LET-268. However, the involvement of a lysyl, as well as a prolyl, hydroxylase suggests that the effects were unlikely to arise from failure of hydroxylation of a second prolyl hydroxylation substrate recognized by VHL-1 and were more likely to be related to a common function of DPY-18 and LET-268, such as a function in extracellular matrix formation. To pursue this, we tested the effects of defects in proteins involved in other aspects of extracellular matrix formation (either in the cuticle or basement membrane) that are distinct from protein hydroxylation. These experiments indicated that the six genes were, to varying extents, upregulated in the basement membrane-associated gon-1 (heterozygote), mig-17, and unc-6 mutant worms but not in the cuticle-associated dpy-11, bli-4, or sqt-3 mutant worms (Figure 5B and unpublished data). In contrast, none of the HIF-1–dependent genes was upregulated in these mutants (Figure 5B and

### Table 4. Continued

| Gene Name | Description | Microarrays: hif-1;hif-1 | RNase Protection Assays: hif-1;hif-1 |
|-----------|-------------|-------------------------|------------------------------------|
| Y35C1B.3 | —           | 2.1                     | —                                 |
| Y37F11.1 | Putative component of the axonal cytoskeleton required for proper axon morphology; similar to the H. sapiens DFGY gene product | 2.1 | 0.09 |
| Y46G5A.26 | —           | 2.1                     | —                                 |
| F27C8.4 | Protein of unknown function, has weak similarity to human transmembrane mucin 1, MUC1 | 2.1 | <0.05 |
| ZK190.5 | —           | 2.1                     | —                                 |
| C45B2.2 | —           | 2.1                     | —                                 |
| C42D4.3 | Protein containing a DB module domain | 2.1 | 0.07 |
| Y43F1C.1 | Member of the transphrytin-like family | 2.1 | 0.05 |
| Y38C1BA.3 | —           | 2.1                     | —                                 |
| C46A5.3 | —           | 2.1                     | —                                 |
| R03D7.5 | —           | 2.1                     | —                                 |
| T11F9.8 | —           | 2.1                     | —                                 |
| ZK1010.7 | —           | 2.1                     | —                                 |

Confirmation of selected genes: Y, reproducible upregulation of gene in hif-1; hif-1/hif-1 worms as confirmed by RNase protection assays; N, no reproducible upregulation of gene as tested by RNase protection assays; asterisk, not assayed, riboprobe could not be constructed; NS, no signal by RNase protection assay; —, not determined. Microarrays and RNase protection assays were performed using worms cultured under normoxic conditions. C35B8.1, C46A5.3, R03D7.5, T11F9.8, and ZK1010.7 were also tested by RNase protection assay based on microarray data; these genes did not show reproducible upregulation by RNase protection assays.

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unpublished data). GON-1 and MIG-17 encode secreted metalloproteases and UNC-6 encodes a netrin; all are thought to be involved in basement membrane remodeling/cell migration during gonadal morphogenesis (Hedgecock et al. 1990; Blelloch and Kimble 1999; Nishiwaki et al. 2000). Conversely, DPY-11 (a thioredoxin) and BLI-4 (a serine endoprotease) are both involved in collagen formation in the worm cuticle (Thein et al. 2003) and SQT-3 encodes a

| Gene Name | A | B | C | D | E | F | G | H | I | J | K |
|-----------|---|---|---|---|---|---|---|---|---|---|---|
| hif-1 N | vhl-1 N | wild-type N | hif-1 N | vhl-1 N | wild-type N | hif-1 N | vhl-1 N | wild-type N | dpy-18 N | wild-type N | phy-2 N | wild-type N | phy-3 N | wild-type N | let-268 N | wild-type N |
| C01B4.7  | 5.1 (3) | * 9.0 (1) | 4.5 (11) | * 1.3 (3) | 1.4 (3) | 4.4 (5) | * 3.5 (4) | * 7.4 (3) | * 1.2 (3) | 1.0 (3) | 5.3 (3) | * |
| F56A4.10 | 4.0 (3) | * 2.6 (1) | 3.7 (8) | * 1.2 (3) | 1.3 (4) | 1.0 (3) | 4.1 (5) | * 3.9 (3) | * 1.8 (3) | 1.4 (3) | 3.4 (3) | * |
| C01B4.9  | 3.6 (3) | * 2.7 (1) | 3.2 (9) | * 1.0 (3) | 1.3 (3) | 1.3 (3) | 2.6 (5) | * 2.2 (3) | * 1.0 (3) | 0.9 (3) | 3.0 (3) | * |
| F56A4.2  | 3.4 (3) | * 5.0 (1) | 2.9 (8) | * 1.2 (3) | 1.0 (3) | 2.3 (3) | * 3.2 (5) | * 4.6 (3) | * 1.6 (3) | 1.2 (3) | 4.2 (3) | * |
| C01B4.6  | 3.1 (3) | * 11.1 | 3.3 (9) | * 1.1 (3) | 0.8 (4) | 0.7 (3) | 3.5 (3) | * 0.8 (3) | * 1.0 (3) | 1.0 (3) | 4.2 (3) | * |
| F21C3.5  | 2.7 (3) | * 16.1 | 2.7 (7) | * 1.7 (3) | 1.1 (3) | 1.1 (3) | 4.5 (4) | * 3.8 (3) | * 1.0 (3) | 0.9 (3) | 3.2 (3) | * |

Columns A and B, data from microarray comparisons; columns C to K, data from RNA protection assays. The figures represent the (fold) differences in expression averaged for the indicated number (n) of independent comparisons. Statistical analysis of differential expression was performed when n ≥ 3, * p < 0.05. N, normoxia; H, hypoxia (0.1% oxygen).

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cuticular collagen. These results therefore extend the characterization of the VHL-1–dependent, HIF-1–independent pathway and support a connection with extracellular matrix/basement membrane function.

**Discussion**

By comparing the effects of *vhl-1* inactivation in different genetic backgrounds, these data clearly distinguish HIF-1–dependent and –independent effects of VHL-1 on gene expression. Somewhat surprisingly, all of the VHL-regulated genes analyzed fell into one of two patterns: independent of HIF-1 and EGL-9 and dependent on DPY-18, LET-268, GON-1, MIG-17, and UNC-6, or the reverse, suggesting that they reflect perturbation of two discrete aspects of VHL-1 function.

The HIF-1–dependent expression pattern of all six genes chosen for detailed analysis from the *vhl-1* versus wild-type array underlines the importance of the HIF-1 pathway in VHL-1 function. Computational analysis revealed that five of these genes (*f22b5.4, nhr-57, fmo-12, egl-9*, and *cah-4*) have at least one HIF-1 binding core motif (RCGTG) that is conserved in *C. briggsae* within an arbitrarily defined (−1,000 to +250 nucleotides) promoter region, suggesting that they are direct HIF-1 transcriptional targets. Several genes (*egl-9, HIF prolyl hydroxylase; phy-2, procollagen prolyl 4-hydroxylase α subunit; and cah-4, carbonic anhydrase*) have mammalian homologs that are HIF targets (Ivanov et al. 1998; Takahashi et al. 2000; Epstein et al. 2001), emphasizing the extent of conservation of the pathway. Others, such as flavin monooxygenase *fmo-12* and the nuclear hormone receptor *nhr-57*, are apparently novel HIF-1 target genes. Interestingly, some of these HIF-1–dependent genes were partly downregulated by EGL-9 in a VHL-1– and iron-independent manner, suggesting that, in addition to the HIF-1/VHL-1 pathway, EGL-9 regulates HIF-1 transcriptional activity via a novel pathway.

Remarkably, among the candidate genes tested from the *hif-1; vhl-1* versus *hif-1* screens, all six that showed reproducible (HIF-1–independent) regulation by VHL-1 were located within 45 kb on Chromosome V. Analysis of the microarray data revealed that there was indeed a single, highly significant (*p* < 10^{-5}) chromosomal cluster of genes negatively regulated by VHL-1 in a HIF-1–independent manner and that in total this cluster extended over 110 kb to include *F56A4.9, Y45G12C.9, Y45G12C.12, and Y45G12C.2* in addition to the six genes validated by RNase protection assay. The chromosomal localization of genes in *C. elegans* is not random, with functionally related genes located close to one another (Roy et al. 2002) or even organized into operons (Blumenthal and Gleason 2003). Even though, based on the absence of spliced leader SL2 sequences (Blumenthal et al. 2002) and the presence of inverse transcriptional orientations, the genes in this cluster do not appear to be within the same operon, there may be a functional relevance to their physical proximity. Four of the six genes validated by RNase protection assay (*C01B4.7, F56A4.10, C01B4.9*, and *C01B4.8*) encode membrane transporters of the major facilitator

**Figure 3. Chromosomal Clustering of VHL-1–Dependent (HIF-1–Independent) Genes**

(A) Chromosomal localization of VHL-1–dependent, HIF-1–independent genes. The positions of the genes from Table 4 are indicated by vertical ticks along the *C. elegans* chromosomes (shown to scale). Where two such genes are too close to be clearly resolved, the tick is marked by an asterisk. The single significant spatial clustering of VHL-1–dependent, HIF-1–independent genes is indicated by a red rectangle. The histogram under each chromosome shows the gene density (deeper bar, greater density) calculated as a sliding window of 100,000 bp moving with 10,000-bp increments along each chromosome. Dark blue indicates total annotated gene density, and light blue indicates the density of genes from the microarray that passed preliminary quality control.

(B) Organization of the VHL-1–regulated (HIF-1–independent) gene cluster from Chromosome V. The relative positions and sizes of gene transcription units are shown to scale, with genes transcribed left to right above the horizontal line and right to left below the line. Names in black indicate genes that passed all selection criteria to be considered upregulated in *hif-1; vhl-1* versus *hif-1* worms (see Table 4). Genes with a mean >2.0-fold upregulation are indicated by green boxes, 1.5- to 2-fold are yellow, and <1.5-fold are red. Genes for which no data were obtained are shown as light grey.

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orthologous relationships for these genes between lineages. As such, it is not possible to define one-to-one rounds of gene duplication in the vertebrate and nematode (unpublished data). Both gene families have been subject to and C01B4.8 cluster with sodium phosphate transporters with monocarboxylate transporters, and C01B4.7, F56A4.10, transport of small solutes. C01B4.9 clusters phylogenetically superfamily, a family of transporters involved in passive transport of small solutes. C01B4.9 clusters phylogenetically with wild-type vhl, indicating that they are attributable, either directly or indirectly, to VHL loss of function. Furthermore, immunoprecipitation studies using renal carcinoma cell extracts have indicated that VHL binds to fibronectin (Ohh et al. 1998). Most tumor-associated VHL mutants, when transfected into VHL-defective renal carcinoma cells, are defective in both complementing HIF dysregulation and fibronectin binding (Kaelin 2002). However, mutations associated with type 2C (predisposition to pheochromocytoma only) VHL disease complement defective HIF regulation but bind fibronectin with lower affinity than wild-type VHL (Hoffman et al. 2001). Though the precise link to abnormal matrix assembly remains unclear, this has suggested a HIF-independent function of VHL. The present study supports the existence of a HIF-independent pathway connected with extracellular matrix function and suggests that this may be a highly conserved function of VHL that is potentially amenable to genetic analysis in model organisms.

**Materials and Methods**

**Strains and culturing conditions.** Worms were studied as mixed-stage populations or as synchronized populations following brief exposure to sodium hypochlorite. Exposure to hypoxia (2% or 0.1% oxygen), DIP (200 μM), and DMOG (1 mM) was for 18 h (Epstein et al. 2001). RNA interference (RNAi) was performed by feeding worms Escherichia coli strain HT115(DE3) expressing double-stranded (ds) RNA on Nematode Growth Medium containing 1 mM isopropyl-thiogalactopyranoside (ITPG) and 50 μg/ml ampicillin for 72 h. Plasmids for ds RNA production were derivatives of the L4440 vector (encoding the extracellular guidance protein, netrin) (Hedgecock et al. 1990)—suggest a related function for this HIF-1–independent VHL pathway. Interestingly, VHL-defective renal carcinoma cells demonstrate a variety of matrix-related abnormalities, including abnormal fibronectin assembly, defective formation of fibrillar adhesions, and changes in branching morphogenesis and migration (Ohh et al. 1998; Koochekpour et al. 1999; Davidowitz et al. 2001; Kamada et al. 2001; Esteban-Barragan et al. 2002). These abnormalities can be corrected by transfection of renal carcinoma cells with wild-type vhl, indicating that they are attributable, either directly or indirectly, to VHL loss of function.
under normoxic conditions using Tri-reagent (Sigma, Poole, Dorset, United Kingdom) and mRNA purified using oligo-dT beads (Qiagen, Crawley, West Sussex, United Kingdom). cDNA synthesis and microarray hybridization and scanning were performed as described previously (M. Jiang et al. 2001). Cy5-dUTP was used to label cDNA from wild-type and hif-1 worms and Cy3-dUTP was used to label cDNA from vhl-1 and hif-1; vhl-1 worms. The arrays were computer normalized by the default procedure in the Stanford Microarray Database (SMD); primary array data are available on the SMD (http:// genome-www.stanford.edu/microarray) and are also shown in Tables S1 through S4. Fold change was calculated as the ratio of the means of Cy3-dUTP intensity to normalized Cy5-dUTP intensity (normalized to correct for signal differences between Cy3-dUTP and Cy5-dUTP intensities across the whole array) with median background intensities subtracted from both signal intensities to correct for the background (see SMD). Genes with background-corrected signal intensities below zero or with array spots that were flagged in the SMD as being unreliable were discarded as a preliminary quality control. For the hif-1 versus hif-1; vhl-1 microarray comparisons (n = 3) the log2 fold change was calculated as the mean of the three log2 transformed fold changes. To test for significant upregulation, the mean log2 fold change was compared with zero using a Student’s t test. The genes were ranked by amplitude of fold upregulation and a subset of genes was selected for potential validation by RNase protection assays (see Tables 1 and 4) based on the following criteria: (a) t test, p < 0.10 (for the hif-1 versus hif-1; vhl-1 microarray comparisons, n = 3); (b) mean Cy3-dUTP and Cy5-dUTP background-corrected signal intensities exceeding 300 and 100 U, respectively (lower intensities than these were difficult to detect by RNase protection assay); and (c) high spot quality as judged by manual inspection. For the hif-1 versus hif-1; vhl-1 microarray comparisons (n = 3), genes (which had been filtered as described above) were considered to be differentially expressed if the mean fold change was greater than 2.0 (see Table 4).

RNase protection assays. Assays were performed on total RNA from mixed-stage populations of worm cultured under normoxic conditions, unless otherwise indicated. Details of riboprobe templates are provided in Table 7; details of genes tested are shown in Tables 1 and 4. Quantification was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, California, United States) and related to an internal control assay for the constitutively

Figure 5. Sensitivity of VHL-1–Regulated Genes to Defects in Extracellular Matrix-Associated Proteins
RNase protection assays showing altered expression of VHL-1–regulated genes that are HIF-1 independent (upper six panels) and HIF-1 dependent (F22B5.4) in worms bearing mutations affecting (A) procollagen prolyl and lysyl hydroxylases and (B) other extracellular matrix-associated proteins. A common pattern of upregulation is observed in hif-1; vhl-1, vhl-1, dpy-18, let-268, gon-1, mig-17, and unc-6 worms but not other mutants. This contrasts with the HIF-1–dependent gene F22B5.4, which is upregulated in vhl-1 worms but none of the other mutants. (C) RNase protection assay for C01B4.9 illustrating DPY-18–mediated changes in expression that are independent of HIF-1. DOI: 10.1371/journal.pbio.0020289.g005

VHL-1–Regulated Pathways in C. elegans
Table 6. C. elegans Strains and Alleles

| Gene Name | Strain | Genotype |
|-----------|--------|----------|
| egl-9     | CB4504 | gon-1(ok1254) egl-9
| F56A4.2   | CB4504 | gon-1(ok1254) egl-9
| F56A4.9   | CB4504 | gon-1(ok1254) egl-9
| F56A4.10  | CB4504 | egl-9(ok1254) F56A4.10

* Note that egl-9 is a null allele that renders the CB4504 strain temperature sensitive for the gon phenotype. DOI: 10.1371/journal.pbio.0020289.t007

Table 7. Sequence and Length of Riboprobes

| Gene Name | 5' End of mRNA | 5' End of mRNA | Protected Length (bp) |
|-----------|----------------|----------------|----------------------|
| C01B6     | 265            | 440            | 175                  |
| C01B7     | 123            | 319            | 196                  |
| C01B8     | 1139           | 1314           | 175                  |
| C01B9     | 35             | 200            | 165                  |
| C00F6.3   | 16             | 181            | 165                  |
| C16C10.3  | 112            | 291            | 179                  |
| C22H11.9  | 649            | 839            | 190                  |
| C35B8.1   | 118            | 292            | 174                  |
| C45A5.3   | 320            | 472            | 152                  |
| C53B7.4   | 517            | 690            | 173                  |
| cah-9     | 328            | 540            | 212                  |
| egl-9(5)  | 172            | 358            | 186                  |
| F01B9.11  | 399            | 576            | 177                  |
| F12C1.5   | 46             | 160            | 114                  |
| F22B5.4   | 195            | 441            | 246                  |
| F22D10.10 | 726            | 896            | 160                  |
| F35E8.8   | 132            | 281            | 149                  |
| F36D3.9   | 711            | 871            | 160                  |
| F37B1.8   | 137            | 306            | 169                  |
| F56A2.2   | 184            | 383            | 199                  |
| F56A4.10  | 75             | 270            | 195                  |
| F56F3.4   | 282            | 462            | 180                  |
| F58B3.2   | 187            | 363            | 176                  |
| fmo-1     | 173            | 320            | 147                  |
| H14N18.4  | 193            | 374            | 181                  |
| hif-1     | 85             | 280            | 195                  |
| K09D8.5   | 297            | 469            | 172                  |
| K09D4.4   | 537            | 715            | 178                  |
| nhr-57    | 359            | 526            | 167                  |
| phy-2     | 1400           | 1130           | 170                  |
| R03D7.5   | 285            | 484            | 199                  |
| T05A10.3  | 135            | 321            | 186                  |
| T06E4.8   | 50             | 199            | 149                  |
| T11F9.6   | 147            | 338            | 191                  |
| T11F9.8   | 303            | 452            | 149                  |
| W02A2.3   | 266            | 443            | 177                  |
| Y110A11.1 | 65             | 242            | 177                  |
| Y39G8.8   | 104            | 273            | 169                  |
| Y45G12C.2 | 25             | 205            | 180                  |
| ZK10I0.7  | 152            | 349            | 197                  |

* Note that the protected region of the egl-9 transcript does not overlap the sa307 deletion in the J3T07 egl-9 strain. DOI: 10.1371/journal.pbio.0020289.t008

Supporting Information

Primary microarray data can be viewed at http://genome-www.stanford.edu/microarray. Table S1. hif-1 versus Wild-Type Microarray Comparison

Supporting Information

Primary microarray data for the hif-1 (green, channel 1) versus wild-type (red, channel 2) comparison. Found at DOI: 10.1371/journal.pbio.0020289.s001 (6.5 MB XLS).

Table S2. hif-1; vhl-1 versus hif-1 Microarray Comparisons I

Primary microarray data for the three independent hif-1; vhl-1 (green, channel 1) versus hif-1 (red, channel 2) microarray comparisons. Continued in Tables S3 and S4. Found at DOI: 10.1371/journal.pbio.0020289.s002 (6.6 MB XLS).

Table S3. hif-1; vhl-1 versus hif-1 Microarray Comparisons II

Continuation of Table S2. Found at DOI: 10.1371/journal.pbio.0020289.s003 (6.6 MB XLS).

Table S4. hif-1; vhl-1 versus hif-1 Microarray Comparisons III

Continuation of Tables S2 and S3. Found at DOI: 10.1371/journal.pbio.0020289.s004 (6.6 MB XLS).

Accession Numbers

Primary array data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-SMBD-23. The H. sapiens VHL gene discussed in this paper can be found in Online Mendelian Inheritance in Man (OMIM) under accession number 608537 (http://www.ncbi.nlm.nih.gov/omim?term=VHL). The C. elegans genes discussed in this paper (hif-1, C01B4.6, C01B4.7, C01B4.8, C01B4.9, C01B4.10, egl-9, F21C3.5, F22B5.4, F56A2.2, F56A4.10, fmo-1, fog-2, gon-1, hif-1, let-266, mig-17, nhr-57, phy-2, phy-3, apt-3, unc-4, unc-6, unc-42, unc-51, vhl-1, Y45G12C.2, Y45G12C.9, and Y45G12C.12) can be found in the WormBase database by including the name of the gene at the end of the URL (e.g., for hif-1, http://wormbase.org/db/gene?gene=VHL-1).
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Conflicts of interest. The authors have declared that no conflicts of interest exist.

Author contributions. TB, KWL, ACRE, CWP, JH, and PJR conceived and designed the experiments. TB, KWL, ACRE, MJ, and DOR performed the experiments. TB, KWL, CWP, JH, and PJR analyzed the data. MST designed and implemented the computational analyses. SKJ, KWL, and PJR contributed reagents/materials/analysis tools. TB and PJR wrote the paper.

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