Diversification of a protein kinase cascade: IME-2 is involved in nonself recognition and programmed cell death in \textit{Neurospora crassa}

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ABSTRACT

Kinase cascades and the modification of proteins by phosphorylation are major mechanisms for cell signaling and communication, and evolution of these signaling pathways can contribute to new developmental or environmental response pathways. The *Saccharomyces cerevisiae* kinase Ime2 has been well characterized for its role in meiosis. However, recent studies have revealed alternative functions for Ime2 in both *S. cerevisiae* and other fungi. In the filamentous fungus *Neurospora crassa*, the IME2 homolog (*ime-2*) is not required for meiosis. Here we determine that *ime-2* interacts genetically with a transcription factor *vib-1* during nonself recognition and programmed cell death (PCD). Mutations in *vib-1* (∆*vib-1*) suppress PCD due to nonself recognition events, however, a ∆*vib-1* ∆*ime-2* mutant restored wild type levels of cell death. A role for *ime-2* in the post-translational processing and localization of a mitochondrial matrix protein was identified, which may implicate mitochondria in *N. crassa* nonself recognition and PCD. Further, ∆*vib-1* strains do not produce extracellular proteases, but protease secretion reverted to near wild type levels in a ∆*vib-1* ∆*ime-2* strain. Mass spectrometry analysis revealed that the VIB-1 protein is phosphorylated at several sites, including a site that matches the IME-2 consensus. The genetic and biochemical data for *ime-2* and *vib-1* indicate that IME-2 is a negative regulator of VIB-1 and suggests parallel negative regulation by IME-2 of a cell death pathway in *N. crassa* that functions in concert with the VIB-1 cell death pathway. Thus, IME2 kinase function has evolved following the divergence of *S. cerevisiae* and *N. crassa* and provides insight into the evolution of kinases and their regulatory targets.
INTRODUCTION

Cell-cell signaling via kinase cascades is an essential mechanism for communication within and between organisms. Protein kinases are one of the largest protein families in eukaryotes and as much as 30% of any given eukaryotic proteome is phosphorylated (DESHMUKH et al. 2010; MOSES and LANDRY 2010). Although kinases have constrained target specificities, these proteins are often structured in a modular way, such that they can evolve new functions via interaction with scaffolds, adapters or docking proteins (BHATTACHARYYA et al. 2006). In addition, duplication of kinase targets can result in reciprocal loss of phosphorylation sites and sub-functionalization of these targets, and/or a gain of new phosphorylation sites, resulting in neo-functionalization (AMOUTZIAS et al. 2010). Thus, changes in kinase structure, along with target duplication and divergence, can impact the structure and signaling output of kinase pathways. Although the major classes of kinases are conserved across fungal species, there is evidence for duplication, family expansion, and differences in domain organization, suggesting that fungi can change their kinase signaling pathways to accommodate changes in environment or developmental processes (KOSTI et al. 2010).

In Saccharomyces cerevisiae, Ime2 is a serine/threonine protein kinase involved in the induction of meiosis and sporulation (SMITH and MITCHELL 1989). Ime2 has both early and late roles in meiosis, including the initiation of meiosis, meiotic DNA replication, meiotic divisions I and II, and spore formation (BENJAMIN et al. 2003; BRUSH et al. 2012; HONIGBERG 2004). Nutritional signals for meiosis converge at Ime1, a transcriptional regulator of Ime2, as well as Ime2 itself, to coordinate meiotic initiation (HONIGBERG and PURNAPATRE 2003; KASSIR et al. 2003). One of the major roles of Ime2
is to contribute to activation of the major middle meiotic transcription factor \textit{NDT80} (PAK and SEGALL 2002a; SHIN \textit{et al.} 2010; SOPKO \textit{et al.} 2002), in part through phosphorylation of the repressor Sum1 (AHMED \textit{et al.} 2009; PAK AND SEGALL 2002B; WINTER 2012). In addition, Ime2 directly phosphorylates Ndt80, which is associated with an increased ability to activate transcription of Ndt80 target genes (SHUBASSI \textit{et al.} 2003; SOPKO \textit{et al.} 2002). Ndt80 binds to the middle sporulation element (MSE) and activates expression of middle meiotic genes (CHU \textit{et al.} 1998; CHU and HERSKOWITZ 1998); cells lacking Ndt80 arrest at pachytene, prior to nuclear division in meiosis I (XU \textit{et al.} 1995).

In the filamentous fungus \textit{Neurospora crassa}, there is one \textit{IME2} homolog (\textit{ime}-2), but three \textit{NDT80} homologs (\textit{female sexual development, fsd-1}; \textit{vegetative incompatibility blocked, vib-1} and \textit{NCU04729}) (BORKOVICH \textit{et al.} 2004; HUTCHISON and GLASS 2010). Homologs to \textit{IME1} and \textit{SUM1} are lacking in the \textit{N. crassa} genome. Recently, we showed neither \textit{ime-2}, nor the \textit{NDT80} homologs \textit{fsd-1}, \textit{vib-1} or NCU04729, are involved in meiotic functions in \textit{N. crassa} (HUTCHISON and GLASS 2010). Mutations in \textit{ime-2} did not affect the transcription or activity of \textit{fsd-1}, the homolog most closely related to \textit{S. cerevisiae} \textit{NDT80}. However, \textit{ime-2}, \textit{vib-1} and \textit{fsd-1} mutants were affected in the production of female reproductive structures, termed protoperithecia. The development of protoperithecia in \textit{N. crassa} is induced under conditions of nitrogen starvation (HIRSH 1954; WESTERGAARD and MITCHELL 1947). The \textit{Δfsd-1} and \textit{Δvib-1} mutants formed few protoperithecia under nitrogen starvation and an \textit{Δfsd-1 Δvib-1} mutant was female sterile. In contrast, an \textit{Δime-2} strain produced protoperithecia under conditions where development of these structures is normally suppressed (nitrogen sufficiency) and significantly more protoperithecia under nitrogen starvation conditions.
(HUTCHISON and GLASS 2010). A deletion of ime-2 restored protoperithecial development in an Δfsd-1 mutant, while an Δime-2 Δvib-1 mutant showed a Δvib-1 phenotype (few protoperithecia). These observations indicate a network of regulatory interactions between ime-2 and the NDT80 homologs fsd-1 and vib-1 during development of female reproductive structures in *N. crassa*.

The *vib-1* mutant was first identified in a search for mutations that alleviate heterokaryon incompatibility (HI) in *N. crassa* (XIANG and GLASS 2002). Heterokaryon incompatibility mediates nonself recognition and is a ubiquitous phenomenon in filamentous fungi (AANEN et al. 2010; CHOI et al. 2012). Within a single filamentous fungal colony, individual hyphae can fuse and form interconnected networks (FLEISSNER *et al.* 2008; READ *et al.* 2010). However, if fusion occurs between strains that contain alternative specificities at heterokaryon incompatibility (*het*) loci, either the fusion cell is walled off and rapidly killed or the growth of the heterokaryon is inhibited (AANEN *et al.* 2010; GLASS and KANEKO 2003) (Figure 1). In *N. crassa*, there are 11 *het* loci and genetic differences at any one of these 11 loci is sufficient to restrict heterokaryon formation (GLASS and DEMENTHON 2006). Strains carrying genetic differences at *het* loci, but also loss-of-function mutations in *vib-1* will form vigorous heterokaryons and do not show HI-associated PCD (LAFONTAINE and SMITH 2012; XIANG and GLASS 2002; XIANG and GLASS 2004). *vib-1* is also necessary for the expression of several genes known to be involved in cell death due to HI (Figure 1) (DEMENTHON *et al.* 2006). In *Aspergillus nidulans*, deletion of a *vib-1* homolog, *xprG*, prevents secretion of extracellular proteases upon nitrogen or carbon starvation (KATZ *et al.* 2006) and *vib-1* mutants exhibit this phenotype as well.
In this study, we show that in \textit{N. crassa}, a deletion of \textit{ime-2} restores HI-induced PCD in a Δ\textit{vib-1} strain and also restores the production of extracellular proteases. We further investigated the \textit{ime-2} phenotype using transcriptional profiling to assess physiological differences between \textit{ime-2} mutants and a wild type strain and identified a possible role for IME-2 in mitochondrial homeostasis. Our data suggests that IME-2 is a negative regulator of a cell death pathway that functions in parallel to the VIB-1 HI pathway to specifically regulate nonself recognition and cell death when strains carry incompatible specificities at \textit{het} loci.

\textbf{MATERIALS AND METHODS}

\textbf{Strains and growth conditions:} All strains used in this study are listed in Table 1. Deletion strains (FGSC 11308, FGSC 11309, FGSC 17936, and FGSC 17937) were constructed by the \textit{Neurospora} program project grant (COLOT \textit{et al.} 2006) and obtained from the Fungal Genetics Stock Center (FGSC) (MCCLUSKEY 2003). Strains were grown on Vogel’s minimal media (MM) (VOGEL 1956) unless otherwise specified, and crosses performed on Westergaard’s media (WESTERGAARD and MITCHELL 1947). Transformations were performed as previously described (MARGOLIN \textit{et al.} 1997). To obtain forced heterokaryons, conidial suspensions from strains of complementary auxotrophic markers were mixed and plated on minimal media. Growth rates were determined by growing strains in race tubes. Protoperithecial development was assessed over a 9 day time period of growth on water agar (HUTCHISON and GLASS 2010).

\textbf{Protease and cell death assays:} For extracellular protease assays, strains were grown (in triplicate) in MM (VOGEL 1956) overnight. Protease assays were performed as
described previously (DEMENTHON et al. 2006). Cell death frequency was measured by staining with methylene blue (HUTCHISON et al. 2009). Heterokaryons were inoculated onto MM overlaid with cellophane, grown for 2-3 days, and stained for 1-2 minutes with 0.003% methylene blue. Approximately 20 random images were taken and the percent of dead (blue) hyphal compartments was determined.

**RNA extraction and quantitative RT-PCR (Q-RT-PCR):** RNA extraction was performed on mycelia ground in liquid nitrogen, or on sections of mycelia grown on cellophane. Mycelia were mixed with 0.3 g of 0.5 mm silica beads and 1 mL of TRizol (Invitrogen) and disrupted using a bead-beater (Mini-BeadBeater-8, Biospec Products). RNA was extracted according to the manufacturer’s protocol for TRizol (Invitrogen). Samples were purified using an RNAeasy kit (Qiagen) and DNA removed with Qiagen DNase (cat. no. 79254) or Ambion Turbo DNase (cat. no. AM2238). RNA concentration and quality was assessed using a Nanodrop (Thermo Scientific) and gel electrophoresis. Q-RT-PCR was performed using an EXPRESS One-Step SYBR GreenER kit (Invitrogen) according to the manufacturer’s protocol, run on an ABI 7300 machine, and analyzed with ABI 7300 system software. Actin mRNA was used as the endogenous control, and reactions were performed in triplicate.

**Microarray analysis:** Microarray slide production, hybridization and analysis was performed as described (TIAN et al. 2007). Neurospora microarray slides are available from the FGSC (http://www.fgsc.net/). Approximately 10 µg of DNase-treated RNA was used as a template for cDNA synthesis (ChipShot Indirect cDNA Synthesis kit; Promega), and hybridizations were performed using ProntoPlus kits (Promega), according to manufacturer instructions. Slides were scanned using an Axon GenePix
4000B scanner, and analyzed using GenePix Pro 6 software (Molecular Devices Corporation). Three independent hybridizations pooled from 3 biological replicates were performed. Data was analyzed using BAGEL (Bayesian Analysis of Gene Expression Levels) (TOWNSEND and HARTL 2002). Microarray data was verified by Q-RT-PCR using template RNA from an independent experiment. All microarray data was deposited at the Filamentous Fungal Gene Expression Database (ZHANG and TOWNSEND 2010) and GEO database (ID GSE35905). Functional Category enrichment analysis was carried out through the MIPS database (http://www.helmholtz-muenchen.de/en/mips/projects/funcat) (RUEPP et al. 2004), which uses a hypergeometric distribution to calculate p values.

**Mitochondrial staining:** Mitochondria were visualized using 10 μM MitoTracker Red FM (Invitrogen; cat. no. M22425) (HICKEY et al. 2004). Approximately 10⁵ conidia were inoculated into a 30 mL flask of MM and shaken at 30° for approximately 6 hrs. MitoTracker Red FM was added to 1 mL aliquots of the conidia, followed by shaking at 30° for an additional 15-20 min. Conidia were pelleted by centrifugation and washed once with MM. Conidia were then spread on a MM plate and incubated at 30° for 5-10 min. Mitochondria were imaged using a Deltavision Spectris DV4 deconvolution microscope (Applied Precision Instruments). A stack of approximately 20 images were taken 0.2 μm apart, were deconvolved using SVI Huygens, and were visualized using Bitplane Imaris software.

**Protein extraction, Immunoprecipitation, and Western blotting:** Protein was extracted from mycelia for immunoprecipitation (IP) using a method adapted from the FGSC Neurospora protocol page (http://www.fgsc.net/neurosporaprotocols/Immunoprecipitation%20final.pdf). Briefly,
20-30 g of mycelia were ground in liquid nitrogen and homogenized using a 6770 Freezer/Mill from SPEX CertiPrep Group using 3 cycles of: 1 min pre-cool, 1 min run time, 1 min cool time, at a speed of 15 CPS. Homogenized mycelia were added to HEPES IP extraction buffer (50 mM HEPES (pH7.4), 137 mM NaCl, 10% Glycerol) containing Complete mini EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor (Roche) and vortexed to homogenization. Samples were centrifuged at 3400 rpm for 10 min. Supernatants were concentrated via centrifugation using Vivaspin 15R protein concentrators (10,000 MWCO; Sartorius Stedium Biotech). Four mL of each sample was immunoprecipitated using Protein G Dynabeads (Invitrogen), according to manufacturer’s instructions, with the following exceptions: mouse anti-GFP antibody (Roche) was incubated with the beads for 1 hr, and the sample was immunoprecipitated for 2 hrs at 4°. Protein was removed from the beads by boiling for 5 minutes, and samples were run on a 4-15% Criterion Tris-HCl gel (BioRad). Protein gels were either subjected to western blot analysis using standard methods, or stained with SimplyBlue SafeStain Coomassie G-250 stain (Invitrogen) to visualize protein, and gel bands of interest were extracted using a razor blade.

**Mass spectrometry.** Gel bands of interest from Coomassie-stained gels were cut out and minced into <1 mm² pieces. Protein bands were extracted following a protocol adapted from the UC Berkeley QB3 Proteomics/Mass Spectrometry Laboratory (http://qb3.berkeley.edu/pmsl/protocols.htm). Briefly, gel pieces were washed in 500 µL NH₄HCO₃ for 20 min. After discarding the NH₄HCO₃ wash, 150 µL of NH₄HCO₃ (100 mM) and 10 µL of DTT (45 mM) were added and samples were incubated for 15 min at 50 °C. After cooling to room temperature, 10 µL of iodoacetamide (100 mM) was added.
and samples incubated at room temperature for 15 min in the dark. The solvent was discarded and gel pieces were washed in 500 µL of a 1:1 mixture of acetonitrile and NH₄HCO₃ (100 mM) for 20 min. Then, gel pieces were incubated for 10-15 min in 50 µL of acetonitrile and dried in a speed vac. Gel pieces were re-hydrated in 10 µL of NH₄HCO₃ (25 mM) and digested overnight with either trypsin or endoproteinase C, according to the manufacturer. Proteins were extracted twice with a mix of 60% acetonitrile and 0.1% formic acid, and once with 100% acetonitrile. Extracted proteins were dried using a speedvac and 2D mass spectrometry analysis was performed at the QB3 Proteomics/Mass Spectrometry facility.

RESULTS

A deletion of ime-2 restores HI-mediated cell death in a Δvib-1 mutant. In N. crassa, if two individuals are genetically identical at all nonself recognition loci (termed het), they can undergo cell fusion to form a “compatible” heterokaryon that looks identical to a homokaryotic wild type strain. However, if individuals are genetically different at any one of eleven het loci, hyphal fusion results in compartmentalization of the fusion cell and death; these individuals are referred to as “incompatible” (GLASS and KANEKO 2003) (Figure 1). In addition to cell death, incompatible heterokaryons have a reduced growth rate and do not conidiate. Strains that contain loss-of-function mutations in vib-1 form vigorous heterokaryons even if they differ in het allelic specificity (Xiang and GLASS 2002; Xiang and GLASS 2004) (Figure 2C, D). Whereas compatible wild type heterokaryons conidiate upon contact with plate edges, Δvib-1 heterokaryons of
identical or alternate het-c pin-c haplotype display deregulated conidiation (Figure 2C, D).

Due to genetic interactions identified between ime-2 and vib-1 with regard to formation of female reproductive structures (Hutchison and Glass 2010), we evaluated whether IME-2 plays a role in HI by assessing the incompatibility phenotype of Δime-2 and Δvib-1 Δime-2 mutants as compared to wild type and Δvib-1 mutants. To evaluate HI, we used a forced heterokaryon approach using strains with the same het-c pin-c specificity (het-c1 pin-c1 and thus “compatible”) versus using strains containing alternate het-c pin-c haplotypes (het-c1 pin-c1 + het-c2 pin-c2 and thus “incompatible”) (Hall et al. 2010; Kaneko et al. 2006) (Figure 1; Table 1). Further, these strains have different, complementary auxotrophic markers; when isolates carrying different auxotrophic markers are placed on minimal media, only the heterokaryotic strain is able to grow.

Compatible heterokaryons of identical het-c pin-c haplotype of wild type, Δvib-1, Δime-2 or Δvib-1 Δime-2 strains were phenotypically identical to a homokaryotic wild type or mutant strain by itself (Figure 2A, C, E, G). Wild type heterokaryons with incompatible combinations of het-c pin-c haplotypes exhibited a severely decreased growth rate and lack of conidiation (compare Figure 2B with 2A). Heterokaryons carrying incompatible het-c pin-c haplotype and homozygous deletions of ime-2 also showed an identical incompatible phenotype to wild type (compare Figure 2F with 2B), indicating the ime-2 is not required for HI. In contrast, homozygous Δvib-1 heterokaryons of incompatible het-c pin-c haplotype showed a phenotype more similar to compatible Δvib-1 heterokaryons (compare Figure 2D to 2C); a deletion of vib-1 suppresses HI. However, a heterokaryon carrying incompatible het-c pin-c haplotypes, plus homozygous
\(\Delta vib-1 \Delta ime-2\) mutations showed significantly poorer growth and conidiation than a \(\Delta vib-1\) suppressed incompatible heterokaryon (compare Figure 2H to 2D). The \(\Delta vib-1 \Delta ime-2\) het-\(c\) pin-\(c\) incompatible heterokaryon had a growth rate of 2.80 cm/day ± 0.55 (Figure 2H), as compared to a wild type incompatible heterokaryon (1.16 cm/day ± 0.53; Figure 2B) and a \(\Delta vib-1\) incompatible heterokaryon (6.7 cm/day ± 0.40; Figure 2D). In addition, the \(\Delta vib-1 \Delta ime-2\) het-\(c\) pin-\(c\) incompatible heterokaryon produced fewer conidia as compared to a \(\Delta vib-1\) suppressed incompatible heterokaryon (compare Figure 2H to 2D) and exhibited an altered, dense growth pattern that did not resemble either single mutants (\(\Delta vib-1\) or \(\Delta ime-2\); Figure 2C, E) or the \(\Delta vib-1 \Delta ime-2\) compatible strain (Figure 2G). Thus, in a \(\Delta vib-1\) mutant, a deletion of \(ime-2\) partially restored the inhibited growth and aconidial phenotype associated with nonself recognition and HI.

In addition to growth inhibition and absence of conidiation, wild type heterokaryons carrying incompatible het-\(c\) pin-\(c\) haplotypes show substantial PCD, with ~30% of hyphal segments showing compartmentalization and death, which is assessed by staining with vital dyes such as methylene blue (Figure 1) (Glass and Kaneko 2003). The \(\Delta vib-1\), \(\Delta ime-2\) and \(\Delta vib-1 \Delta ime-2\) heterokaryons of identical het-\(c\) pin-\(c\) haplotype exhibited very little cell death, approximately 2-5%, which was similar to a wild type compatible heterokaryon (Figure 3A). Wild type heterokaryons carrying incompatible het-\(c\) pin-\(c\) haplotypes showed ~30% hyphal compartmentation and death (Xiang and Glass 2002). Similar to wild type incompatible heterokaryons and consistent with the HI phenotype (Figure 2F), the \(\Delta ime-2\) heterokaryon carrying incompatible het-\(c\) pin-\(c\) haplotypes showed ~27% hyphal compartmentation and death (Figure 3A). As observed previously (Xiang and Glass 2002), \(\Delta vib-1\) heterokaryons carrying incompatible het-\(c\)
pin-c haplotypes showed a substantially reduced cell death frequency that was similar to either Δvib-1 or wild type compatible heterokaryons (~2-5%). In contrast, the Δvib-1 ∆ime-2 heterokaryon carrying incompatible het-c pin-c haplotypes showed a cell death frequency similar to wild type incompatible heterokaryons (~25%) (Figure 3A). These data indicate that loss-of-function mutations in ime-2 restored hyphal compartmentation and death to Δvib-1 heterokaryons carrying incompatible het-c pin-c haplotypes.

Many proteins involved in HI, like PIN-C, contain a conserved protein domain of unknown function, termed HET (PF06985) (ESPAGNE et al. 2002). HET domains are filamentous fungal-specific protein domains that can cause an HI-like cell death when overexpressed (PAOLETTI and CLAVE 2007). Previously, it was shown that vib-1 is necessary for the expression of HET domain genes pin-c, tol and het-6, which are required for HI in N. crassa (DEMENTHON et al. 2006) (Figure 1). To test whether restoration of cell death in Δvib-1 ∆ime-2 strains correlated with HET domain gene expression, we performed quantitative RT-PCR for pin-c and tol in wild type, Δvib-1, ∆ime-2, and Δvib-1 ∆ime-2 strains (Figure 3B). As expected, expression of pin-c and tol was not detected in a Δvib-1 mutant. Although expression of pin-c and tol in ∆ime-2 strains was not significantly different than in wild type, expression of vib-1 was significantly increased, suggesting that IME-2 negatively regulates vib-1 expression levels. In the Δvib-1 ∆ime-2 strain, however, expression of pin-c and tol was similar to expression levels observed in the Δvib-1 strain (Figure 3B), indicating that restoration of cell death by ∆ime-2 mutations in a Δvib-1 strain carrying incompatible het-c pin-c haplotypes is not due to induced expression of pin-c.

Microarray analysis reveals that mutations in ime-2 affect mitochondrial
Because we identified a genetic interaction between *vib-1* and *ime-2* during HI, we evaluated what physiological processes were affected in the Δ*ime-2* mutant by performing gene expression profiling of wild type versus an Δ*ime-2* strain under nitrogen starvation conditions. The initiation of female reproductive structures (protoperithecia) in *N. crassa* is regulated by the availability of nitrogen (HIRSH 1954; WESTERGAARD and MITCHELL 1947), and we hypothesized that differences in gene expression between wild type and Δ*ime-2* deletion strains may be more pronounced under nitrogen starvation conditions (HUTCHISON and GLASS 2010). The wild type strain (FGSC 2489) and an Δ*ime-2* strain (FGSC 17937) were grown overnight (approximately 16 hrs) in minimal media. The mycelia was then washed and subsequently transferred to a flask containing minimal media without nitrogen and grown for an additional 4 hrs (see Materials and Methods). Mycelia from both strains were harvested for RNA extraction and microarray analysis. Three replicate microarrays, including dye swaps, were performed.

In the Δ*ime-2* strain, a total of 187 genes showed a significant decrease in expression level of at least 1.5 fold. Functional category analysis (RUEPP et al. 2004) of this gene set showed enrichment for Energy (p<0.0001), Transcription (p<0.05), Cellular Transport (p<0.005) and Transposable elements, viral and plasmid proteins (p<1e-11) (Figure 4). Many of the genes in the Energy functional category belonged to pathways involving electron transport and respiration. Furthermore, the enrichment of genes belonging “Transposable elements, viral and plasmid proteins” functional category was due almost exclusively to genes belonging to the mitochondrial genome. In fact, 13 of the 29 genes comprising the mitochondrial genome showed decreased expression levels in the Δ*ime-2* strain as compared to wild type (Table S1), a significant enrichment
A total of 506 genes showed increased expression levels in the Δime-2 strain as compared to wild type (Figure 4). These genes were enriched in a variety of functional categories (RUEPP et al. 2004), including Metabolism (p<1e-11), Energy (p<1e-17), Cell cycle and DNA processing (p<0.0001), Transcription (p<0.005), Protein synthesis (p<1e-24), Protein fate (p<1e-5), Protein with binding function or cofactor requirement (p<1e-44), Regulation of metabolism and protein function (p<0.0005), Cellular transport (p<1e-14), Cellular communication and signal transduction (p<1e-4), Cell rescue, defense, and virulence (p<0.0005), Interaction with the environment (p<1e-6), Cell fate (p<1e-8), Development (p<0.005), Biogenesis of cellular components (p<1e-12), and Cell type differentiation (p<1e-6). Although we hypothesized that the constitutive production of protoperithecia in Δime-2 strains (HUTCHISON and GLASS 2010) may be due to a defect in nitrogen sensing, we did not observe significant differences in gene expression with respect to nitrogen metabolism genes or genes involved in the metabolism of amino acids. Thus, the array data instead suggests that Δime-2 mutants are not deficient in nitrogen sensing specifically, but that these strains may have a more general nutrient-sensing defect.

Interestingly, genes involved in mitochondrial biogenesis (within the Biogenesis of cellular components category) were also significantly enriched among genes that showed increased expression levels in the Δime-2 mutant (p<0.0001). A recent study by KEEPING et al. (2010) used mass spectrometry as well as computational methods to
compile a comprehensive list of 738 genes that comprise the \textit{N. crassa} mitochondrial proteome. Using this dataset, we asked whether nuclear-encoded mitochondrial genes were expressed differently between wild type and Δ\textit{ime-2} strains. In fact, these genes were significantly enriched (p<1e-18) in the set of 506 genes that showed increased expression levels in the Δ\textit{ime-2} mutant. As previously mentioned, 13 of the 29 genes comprising the mitochondrial genome showed lower expression levels in the Δ\textit{ime-2} strain (Table S1). These data suggest that the Δ\textit{ime-2} mutant may have impaired mitochondria (as evidenced by decreased expression of genes within the mitochondrial genome), resulting in a regulatory feedback loop such that Δ\textit{ime-2} strains increase expression of nuclear-encoded mitochondrial genes to compensate for this defect.

Overall, the microarray data suggest that IME-2 plays a role in mitochondrial function in \textit{N. crassa}. We therefore assessed, via microscopy, whether mitochondrial morphology or protein localization was altered in Δ\textit{ime-2} mutants as compared to wild type and the Δ\textit{vib-1} mutants.

\textit{ime-2} mutants affect post-translational processing of the mitochondrial protein \textbf{ARG-4}. To evaluate the mitochondrial phenotype in wild type, Δ\textit{vib-1}, Δ\textit{ime-2}, and Δ\textit{vib-1} Δ\textit{ime-2} strains, we stained conidial germlings with MitoTracker Red FM and also transformed each strain with the nuclear encoded GFP-tagged mitochondrial marker gene encoding ARG-4 (BOWMAN \textit{et al.} 2009). The nuclear \textit{arg-4} locus encodes acetylornithine-glutamate acetyltransferase (arginine biosynthetic pathway) and which is imported into the mitochondrial matrix (CYBIS and DAVIS 1975). In wild type hyphae, mitochondria appear as long tubules in the apical regions of hyphae, and as more punctate structures further back from the hyphal tip (BOWMAN \textit{et al.} 2009). When stained
with MitoTracker Red FM, all strains (Δvib-1, Δime-2, and Δvib-1 Δime-2) looked identical to wild type (Figure 5A) and had brightly stained, long, tubular mitochondrial networks. Similarly, when mitochondria were visualized via ARG-4-GFP localization, wild type and the Δvib-1 mutant exhibited long, tubular mitochondria that looked identical to the MitoTracker Red FM-stained mitochondria (Figure 5B). However, Δime-2 strains exhibited an altered localization pattern. Instead of long, tubular structures, ARG-4-GFP localized either to vesicles, punctate structures, or was diffuse in the cytoplasm (Figure 5B). MitoTracker Red FM can permeate the cell membrane and accumulates in mitochondria based on membrane potential (MacHó et al. 1996; Poott et al. 1996), and thus it is likely that the mitochondria observed in all strains, including the Δime-2 strains, were active and have a functioning membrane potential. However, the lack of ARG-4-GFP localization in the Δime-2 strain suggested that IME-2 may play a role in protein targeting to the mitochondria. Interestingly, the Δvib-1 Δime-2 strain restored localization of ARG-4-GFP to tubular mitochondria (Figure 5B).

In yeast, the ARG-4 homolog Arg7 undergoes a post-translational autoproteolytic processing step that results in the formation of two smaller subunits, each of which localizes to the mitochondrial matrix where they associate in a complex (Abadjieva et al. 2000). The autoproteolytic activity of yeast Arg7 is dependent on a threonine residue, and this residue is conserved in N. crassa ARG-4. Therefore, we determined whether the defect in ARG-4-GFP localization in Δime-2 strains was due to a defect in protein processing. Two distinct bands for N. crassa ARG-4-GFP (~75 and 50 kDa) have previously been reported (Bowman et al. 2009), and which is expected if ARG-4 is proteolytically cleaved at the conserved threonine residue. In the wild type and Δvib-1
Strains, two ARG-4-GFP bands at approximately 75 kDa and 50 kDa were detected (Figure 6A, B). However, in Δime-2 strains much less ARG-4-GFP protein was present and only the 50 kDa ARG-4-GFP band was detected. Despite the lack of detectable full-length ARG-4 in the Δime-2 mutants, these mutants were not arginine auxotrophs. In S. cerevisiae, ARG7 mutants exhibit a leaky Arg phenotype (CRABEEL et al. 1997). Consistent with microscopy results, the Δvib-1 Δime-2 strain showed a wild type pattern for ARG-4 processing (Figure 6A, B). In fact, Δvib-1 Δime-2 strains appeared to produce more ARG-4-GFP than wild type or Δvib-1 deletion strains.

The ARG-4-GFP construct used to visualize ARG-4-GFP localization is under the regulation of the ccg-1 promoter (BOWMAN et al. 2009), which is commonly used for constitutive gene expression in N. crassa. The wild type and the Δvib-1, Δime-2 and Δvib-1 Δime-2 strains transformed with the pccg-arg-4-gfp construct also contain a native copy of ARG4. Thus, we quantified the transcription of ARG4 in comparison to ccg-1 using Q-RT-PCR in all strains to assess expression levels of ARG4 (Figure 6D). Expression levels for ARG4 (a readout for both the ccg-1-regulated and resident ARG4 genes) were very similar between wild type, Δvib-1 and Δime-2 mutants. These data indicate that the differences observed in ARG4 protein levels between wild type and Δime-2 strains was not due to decreased expression levels of ARG4 in the Δime-2 mutant (Figure 6A-B, D). However, the expression level of ARG4 was significantly elevated in the Δvib-1 Δime-2 strain, consistent with increased ARG4 protein levels in this strain detected via western blot (Figure 6B). Wild type and the Δime-2 mutant also showed similar levels of ccg-1 expression, while the Δvib-1 and Δvib-1 Δime-2 strains showed slightly lower levels of ccg-1 expression (Figure 6D). Because the ccg-1 promoter was
not down regulated in the Δime-2 strain nor upregulated in the Δvib-1 Δime-2, it is likely that differences in proteins levels of ARG-4 are not due to the regulation of the arg-4 transgene. The increased arg-4 transcription in the Δvib-1 Δime-2 strain either originated from the native arg-4 locus or arg-4 transcripts were stabilized in this strain. Thus, ime-2 and vib-1 affect the post-translational modification of ARG-4, and to some degree in the Δvib-1 Δime-2 strains, transcriptional regulation of arg-4.

**Mutations in ime-2 revert the protease secretion phenotype of vib-1 mutants.**

Mutations in the transcription factor vib-1 cause a visible phenotype during vegetative growth, such that Δvib-1 mutants show pinkish (rather than orange) conidial pigmentation, deregulated conidiation, decreased aerial hyphae formation (Figure 2C, 7A) and a slight decrease in growth rate as compared to wild type (DEMENTHON et al. 2006; HUTCHISON et al. 2009; XIANG and GLASS 2002). In contrast, wild type and the Δime-2 mutant show robust aerial hyphae formation and conidiation at the top of the tube or edge of the plate and were nearly indistinguishable, with the exception of the slightly yellow pigmentation observed in the Δime-2 mutant (Figure 7A). In the Δvib-1 Δime-2 mutant, aerial hyphae formation was restored and conidiation only occurred at the top of the slants, a phenotype similar to wild type and the Δime-2 mutant (Figure 7A). However, conidia of the Δime-2 Δvib-1 mutant are pinkish in color, like the Δvib-1 single mutants.

In addition to the vegetative conidiation phenotype, Δvib-1 mutants do not secrete extracellular proteases in response to nitrogen or carbon starvation (DEMENTHON et al. 2006), a phenotype similar to the vib-1 homolog in *A. nidulans*, xprG (KATZ et al. 2006). We therefore evaluated whether mutations in ime-2 restored protease activity in the Δvib-1 mutant. When nitrogen was provided, extracellular proteases were not induced in the
wild type, Δvib-1, Δime-2 or Δvib-1 Δime-2 strains (Figure 7B). When the wild type strain was transferred to nitrogen starvation medium, extracellular protease activity was induced, while no activity was detected in the Δvib-1 mutant. A strain carrying a deletion of Δime-2 produced slightly elevated levels of proteases in response to nitrogen starvation (Figure 7B,C). However, unlike the Δvib-1 mutant, the Δvib-1 Δime-2 mutant showed wild type protease activity in response to nitrogen starvation (Figure 7B,C), indicating that loss-of-function mutations in ime-2 suppressed the defect in protease secretion in Δvib-1 mutants.

Based on the Δvib-1 Δime-2 phenotype, we hypothesized that additional regulators may be functioning redundantly to vib-1 to restore protease production. Two obvious candidate genes that may have redundant functions with vib-1 are the vib-1 paralogs fsd-1 and NCU04729. Previously, we determined that strains containing a deletion of NCU04729 were indistinguishable from wild type under all conditions, while an Δfsd-1 mutants shows defects in protoperithecial formation and ascospore maturation (Hutchison and Glass 2010); neither fsd-1 nor NCU04729 affect cell death due to HI. We therefore tested the ability of a Δvib-1 Δime-2 Δfsd-1 ΔNCU04729 deletion strain (Table 1; strain D49VI.HIS.1) to produce extracellular proteases. As shown in Figure 7, the quadruple mutant (Δvib-1 Δime-2 Δfsd-1 ΔNCU04729) produced near wild type levels of extracellular proteases (Figure 7B), indicating that neither fsd-1 nor NCU04729 were responsible for the restoration of protease secretion in the Δvib-1 Δime-2 mutant.

**VIB-1 is phosphorylated at a predicted IME-2 consensus site.** In *S. cerevisiae*, the consensus sequence for Ime2 phosphorylation Ime2 (R-P-x-S/T-A/R-G) has been well characterized (Holt et al. 2007; Moore et al. 2007). We analyzed the *N. crassa*
genome for matches to the yeast Ime2 phosphorylation consensus sequence using the Scansite program (http://scansite.mit.edu/) (OBENAUER et al. 2003) with a slightly modified phosphorylation consensus identified for N. crassa (R-P-x-S/T-P/A/R-G) (L. Holt Laboratory, unpublished data). There are 30 total predicted phosphorylation targets of IME-2 in the N. crassa genome (Table S2). Consistent the role of ime-2 in protoperithecial formation, one of the predicted IME-2 phosphorylation targets present in the Scansite dataset is NIT-2 (NCU09068), a major regulator of nitrogen utilization. In addition, both AL-1 (albino-1; NCU00552), a phytoene dehydrogenase involved in carotenoid biosynthesis (SCHMIDHAUSER et al. 1990), and NRC-2 (NCU01797), a serine-threonine kinase involved in regulation of entry into the conidiation pathway and conidial development (KOTHE and FREE 1998) were present in the Scansite dataset. Δime-2 strains appear to have a slightly different conidiation phenotype than WT, including less pigmentation and fewer conidia (Figure 2E, Figure 7A). The N. crassa homolog of the yeast protein kinase Ste20 (NCU03894) is also a predicted IME-2 phosphorylation target. Ste20 and its homologs in mammals (Mst1 and Mst2) have been previously shown to have a role in the apoptotic signaling cascade (MADEO et al. 2009; RADU and CHERNOFF 2009). Additionally, the VIB-1 protein contains a match for the Ime2 consensus, while neither of the other two NDT80 homologs in N. crassa (fsd-1 or NCU04729) have an Ime2 consensus site. From a phosphoproteomics study (A. Leeder and N. L. Glass, unpublished results), we identified a phosphopeptide for VIB-1 at the predicted IME-2 consensus site (RPRS*60), as well as four additional phosphorylation sites (MPQS*413; PSKS*537; RHGS*542; HGS*545) (Figure 8A).
To test whether the predicted IME-2 consensus site was necessary for VIB-1 function, we constructed mutant vib-1 alleles such that the IME-2 site was mutated to alanine (predicted to be phospho-null; S60 to A mutation) (1XA; Table 1) or mutated to aspartate (phospho-mimetic; S60 to D mutation) (1XD; Table 1). The growth rate of strains carrying the vib-I<sup>S60A</sup> and vib-I<sup>S60D</sup> alleles were identical to wild type as was nuclear localization of VIB-I<sup>S60A</sup>-GFP and VIB-I<sup>S60D</sup>-GFP (Figure S1, S2). Although identical in phenotype to wild type, both the vib-I<sup>S60A</sup>-gfp and the vib-I<sup>S60D</sup>-gfp strains showed slightly lower protease levels, suggesting that phosphorylation of the S60 site contributes to VIB-1 activity (Figure 7C). Similarly, mutations of the IME-2 consensus sequence in VIB-1 significantly reduced the numbers of protoperithecia produced in vib-I<sup>S60A</sup>-gfp and vib-I<sup>S60D</sup>-gfp strains under conditions of nitrogen starvation (Figure S3). These data indicate that mutations in the Ime2 consensus site on VIB-1 negatively impact protoperithecial development.

To assess the role of the Ime2 phosphorylation sites on HI, the vib-I<sup>S60A</sup>-gfp and vib-I<sup>S60D</sup>-gfp alleles were transformed into Δvib-1 strains of het-c2 pin-c2 haplotype (Table 1). Each strain was then forced in a heterokaryon with a Δvib-1 strain of het-c1 pin-c1 haplotype. The Δvib-1 mutation is recessive, such that a single functional copy of vib-1 in a heterokaryon is sufficient to trigger HI (DEMENTHON et al. 2006; XIANG and GLASS 2002) (Figure 8B, compare panels 3 and 4). When strains containing the vib-I<sup>S60A</sup>-gfp or vib-I<sup>S60D</sup>-gfp were forced in a heterokaryon with a Δvib-1 strain of incompatible het-c pin-c haplotype, a HI response was triggered that was indistinguishable from a wild type HI phenotype (Figure 8B, panels 5 and 6). In addition, the vib-I<sup>S60A</sup>-gfp and vib-
$I^{S60D}$-gfp incompatible heterokaryons displayed wild type levels of cell death (Figure 8C).

We also constructed strains where all five identified phosphorylation sites were mutated to alanine (S60A; S413A; S537A; S542A; S545A; 5XA) or aspartate (S60D; S413D; S537D; S542D; S545D; 5XD) (Table 1). Both the 5XA and 5XD strains grew significantly slower than WT (Figure S2) and were also impaired in cell death during HI (Figure 8C). The 5XA and 5XD mutants also showed a significant decrease in protoperithecial formation as compared to WT (Figure S2), with the 5XD strain showing the most significant reduction, with values similar to the $\Delta$vib-1 and $\Delta$vib-1$\Delta$ime-2 mutants. Consistent with the 5 amino substitutions negatively affecting VIB-1 function, the 5XA and 5XD strains showed less protease activity than WT ($P=0.05$), with values similar to the 1XA and 1XD strains.

**DISCUSSION**

Diversification of kinase cascades may provide a mechanism for eukaryotes to evolve new developmental pathways or adapt to new environments (BHATTACHARYYA et al. 2006). In this study, we showed that *N. crassa* ime-2 regulates cell death due to HI in the absence of *vib-1*, and also regulates post-translational processing of the mitochondrial matrix protein ARG-4. *IME2* homologs have not been previously implicated in programmed cell death, but recent studies in yeast and other fungi have provided evidence that Ime2 and its homologs can function in cellular processes other than meiosis. For instance, Strudwick *et al.* described a role for Ime2 in yeast pseudohyphal formation (STRUDWICK *et al.* 2010), and studies in other fungal species showed that
although Ime2 homologs often function in sexual differentiation or nutrient sensing, they are not generally meiotic regulators (IRNIGER 2011). Our results provide additional evidence that the function of the Ime2 pathway differs among fungal species, and implicate \textit{N. crassa} \textit{ime-2} in nonself recognition and programmed cell death.

Data from this study as well as from a previous study (HUTCHISON and GLASS 2010) indicates that \textit{ime-2} interacts genetically with the transcription factor \textit{vib-1}. For some phenotypes, such as HI, protease production, and conidiation, \textit{ime-2} is epistatic to \textit{vib-1}. However, for other phenotypes, including protoperithecial formation, HET domain gene expression, and ARG-4 localization and protein processing, \textit{vib-1} is epistatic to \textit{ime-2}. These data suggest that the \textit{ime-2/vib-1} signaling pathway is not a simple, linear interaction, but that there are other genetic interactors present depending on the cellular process. We propose that the overall structure of the pathway (with the \textit{IME2} homolog functioning upstream of the \textit{NDT80} homolog) is likely conserved, that IME-2 negatively regulates VIB-1 (likely at the protein level), and that IME-2 regulates a parallel pathway that functions redundantly with VIB-1 (Figure 9) to regulate HI and protease production. In this scenario, protease production and cell death in a \textit{Δvib-1 Δime-2} strain is restored. Mutation of components in the parallel cell death induction pathway in addition to \textit{vib-1} mutations would ameliorate cell death and HI completely, regardless of the presence or absence of \textit{ime-2}. Further experiments will be needed to identify IME-2 targets and additional members of this pathway.

Loss-of-function mutations in \textit{vib-1} result in an inability to secrete proteases in response to nitrogen starvation and presence of extracellular protein, a phenotype that is suppressed in \textit{Δime-2 Δvib-1} mutants. In eukaryotes, including \textit{S. cerevisiae}, caspase
proteases (metacaspases in *S. cerevisiae*) are integral in the activation of the apoptotic cell death cascade (ABDELWAHID et al. 2011; MADEO et al. 2004; TAIT and GREEN 2010). In *N. crassa*, metacaspases are not required for HI-induced cell death (HUTCHISON et al. 2009). However, it is possible that a link between HI-induced cell death and VIB-1/IME-2 regulated proteases occurs *N. crassa* by the utilization of non-metacaspase proteases to induce cell death.

Mitochondria are key players in apoptosis and cell death pathways (TAIT and GREEN 2010). During apoptotic cell death, the pro-apoptotic Bcl-2 proteins BAX and BAK can cause the mitochondrial outer membrane to permeabilize, disrupting mitochondrial function, energy production and redox potential, and promoting the release of pro-apoptotic factors such as cytochrome c (ABDELWAHID et al. 2011; DEGTEREV and YUAN 2008; TAIT and GREEN 2010). In fungi, mitochondria have also been implicated in apoptotic cell death, and have important roles in life span and senescence (MADEO et al. 2004; MAHESHWARI and NAVARAJ 2008; SHARON et al. 2009). Mutations in *ime-2* affected localization and post-transcriptional processing of ARG-4, a phenotype that was restored to a wild type pattern in the Δvib-1 Δime-2 mutant. The observation that IME-2 affects post-translational processing of a mitochondrial matrix protein suggests that the parallel cell death pathway could be acting through the mitochondria or a mitochondria-related pathway. *S. cerevisiae IME2*, and its homolog in *Schizosaccharomyces pombe*, *mde3/pit1*, have a role in meiosis (ABE and SHIMODA 2000; HONIGBERG 2004; KASSIR et al. 2003) as well as pseudohyphal growth (STRUDWICK et al. 2010). Both of these developmental processes are associated with nitrogen starvation, suggesting an additional role for Ime2 in nutrient sensing, similar to that proposed for *N. crassa* (HUTCHISON and
GLASS 2010). It will be of interest to assess whether ime2Δ mutants in S. cerevisiae also have in common a defect in the localization and processing of Arg7 (ortholog of N. crassa arg-4), as observed in N. crassa Δime-2 mutants. Further experiments on the relationship between ime-2/IME2 and mitochondrial function are warranted.

We identified 5 phosphorylation sites on VIB-1, including a site that matches the predicted consensus site for Ime2. However, mutations in the Ime2 consensus site in VIB-1 that are predicted to result in phospho-null mutations (S to A) or activating mutations (S to D) resulted in none to only subtle phenotypic differences from WT. In particular, no role for the Ime2 phosphorylation site was observable for HI. Strains containing vib-1 alleles containing 5 S to A or 5 S to D mutations showed more severe defects, particularly reduced vegetative growth and protoperithecial development, as well as a reduced percentage of cell death during HI. These observations suggest that altering these five sites results in a VIB-1 protein that is not fully functional (hypomorphic allele). Our genetic and phenotypic data show a genetic interaction between ime-2 and vib-1 during protoperithecial development and during HI and protease secretion in response to nitrogen starvation and for the localization and processing of a mitochondrial matrix protein. These observations suggest a complex regulatory interaction between these two proteins in a number of cellular functions. Further experiments will unravel the interconnection of these two proteins and the signaling and transcriptional regulatory pathways they regulate in different cellular contexts.

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FIGURE LEGENDS

FIGURE 1. Schematic for VIB-1 regulation of HI and cell death. VIB-1 is required for HI mediated by genetic differences at mating type, het-6 and het-c/pin-c (LAFONTAINE and SMITH 2012; XIANG and GLASS 2002; XIANG and GLASS 2004) in addition to activating PCD through additional unknown downstream effectors (DEMENTHON et al. 2006). het-6 incompatibility is mediated by un-24 (ribonucleotide reductase) and het-6 interactions (LAFONTAINE and SMITH 2012; MICALI and SMITH 2006), mating type incompatibility is mediated by mating type A-1, mating type a-1 and tol (GLASS et al. 1990; NEWMEYER 1970; PITTENGER 1957; SHIU and GLASS 1999), while het-c incompatibility is mediated by het-c pin-c interactions (GLASS and KANEKO 2003; KANEKO et al. 2006); alternative HET-C polypeptides have been shown to physically interact (SARKAR et al. 2002). Phenotypic consequences of HI in N. crassa include growth inhibition and suppression of conidiation (PERKINS 1988). Hyphal compartments within heterokaryons that carry alternate het haplotypes (such as het-c/pin-c, rnr/het-6 or mat-a/A/tol) undergo compartmentation and rapid cell death (GLASS and KANEKO 2003) and thus stain positive for vital dyes such as methylene blue (arrows).

FIGURE 2. A deletion of ime-2 partially restores HI in Δvib-1 mutants. Heterokaryons of identical het-c pin-c haplotype (compatible=C) show a growth and conidiation pattern indistinguishable from homokaryotic strains, including WT (A; FGSC 4564 + R15-7), Δvib-1 (C; KD13-21 + KD13-51), Δime-2 (E; DI.PYR.4 + DI.HIS.10), Δvib-1 Δime-2 (G; DVI.HIS.48 + DVI.PYR.63) heterokaryons (Table 1). Strains carrying incompatible het-c pin-c haplotypes (incompatible=Inc) in a WT background
show growth inhibition and suppression of conidiation (B; FGSC 4564 + C9-15). Heterokaryons of incompatible *het-c pin-c* haplotypes, but carrying homozygous Δ*vib-1* mutations are similar in phenotype to compatible Δ*vib-1* heterokaryons (D; KD13-33 + KD13-1). Heterokaryons carrying incompatible *het-c pin-c* haplotypes and homozygous Δ*ime-2* mutations show typical HI (F; DI.HIS.10 + DI.A.22), while heterokaryons carrying incompatible *het-c pin-c* haplotypes and homozygous for Δ*vib-1* Δ*ime-2* mutations show partial restoration of HI (decreased growth rate and suppression of conidiation) (H; DVI.PYR.63 + DVI.A.101).

**FIGURE 3. A deletion of ime-2 restores wild type levels of cell death in Δ*vib-1* incompatible heterokaryons.** (A) Heterokaryons of compatible and incompatible *het-c pin-c* haplotype from figure 2 were plated on minimal media overlaid with cellophane and grown for 1-3 days. The percentage of death cell compartments was evaluated using microscopy and methylene blue staining (HUTCHISON et al. 2009; XIANG and GLASS 2002). Heterokaryons of incompatible *het-c pin-c* haplotype but carrying homozygous *vib-1* deletions were suppressed for cell death (Δ*vib-1*), while the addition of Δ*ime-2* mutation in these strains restored wild type levels of cell death (Δ*vib-1* Δ*ime-2*). (B) Expression of *ime-2*, *vib-1*, and two HET domain genes (*pin-c*, *tol*) was assessed using quantitative RT-PCR. Deletion of *vib-1* abolishes expression of HET domain genes during vegetative growth. Expression of *pin-c* and *tol* was not restored in a Δ*vib-1* Δ*ime-2* mutant.
FIGURE 4. Functional category analysis of gene expression differences in wild type versus the Δime-2 mutant and overlap with predicted IME-2 phosphorylation targets. Distribution of significantly enriched MIPS functional categories (http://www.helmholtz-muenchen.de/en/mips/projects/funcat) (Ruepp et al. 2004) for the microarray dataset of wild type compared to an Δime-2 deletion strain. A total of 187 genes showed a reduction in expression in Δime-2 relative to wild type, while 506 genes showed an increase in expression level in the Δime-2 strain relative to wild type.

FIGURE 5. Mutations in ime-2 affect localization of the mitochondrial protein ARG-4. (A) Mitochondria stained with MitoTracker Red FM in FGSC 2489 (WT), FGSC 11308 (Δvib-1), FGSC 17937 (Δime-2), and DVI.4 (Δvib-1 Δime-2) (Table 1). The third column of panels is an enlargement of the region highlighted by a white box in the second column of panels. (B) Localization of ARG-4-GFP to mitochondria in wild type and deletion strains transformed with pccg1-arg-4-gfp (Table 1). Localization of ARG-4-GFP in wild type is identical to MitoTracker Red FM and to that previously reported (Bowman et al. 2009). ARG-4-GFP localization in the Δvib-1 and the Δvib-1 Δime-2 mutants was identical to WT. However, in the Δime-2 strain, mitochondrial tubule structures were not observed and instead ARG-4-GFP localized to either vesicles, punctae, or was diffuse in the cytoplasm. Scale bar = 5 µm.

FIGURE 6. Strains carrying a deletion in ime-2 affect the post-transcriptional regulation of ARG-4. (A) Western blot for ARG-4-GFP in wild type, Δvib-1, Δime-2, and Δvib-1 Δime-2 strains, with molecular weight ladder (kDa) on the left. ARG-4-GFP
is detected as two distinct bands, ~75 kDa and 50 kDa in WT, as previously reported (Bowman et al. 2009). (B) Longer exposure of the blot from (A), more clearly showing the 50 kDa ARG-4-GFP in the Δime-2 strain. (C) Western blot of β-tubulin showing that equal amounts of protein were loaded in each well. (D) Quantitative-RT-PCR of arg-4 and ccg-1 transcript levels in wild type, Δvib-1, Δime-2, and Δvib-1 Δime-2 strains.

**FIGURE 7. An ime-2 deletion restores wild type conidiation patterns and protease production to a Δvib-1 mutant.** (A) wild type (FGSC 2489), Δvib-1 (FGSC 11308), Δime-2 (FGSC 17936), and Δvib-1 Δime-2 (DVI.4) strains grown on minimal media slants. (B) Extracellular protease activity of wild type and deletion strains (from A), as well as the Δvib-1 Δime-2 Δfsd-1 ΔNCU04729 strain (D49VI.HIS.1) was assessed on media with and without nitrogen. Only the vib-1 deletion strain showed inability to secrete proteases. Protease activity units are normalized to WT in nitrogen starvation (-nitrogen) media. (C) Extracellular protease activity of vib-1 phospho mutants (vib-1\textsuperscript{S60A} 1XA; vib-1\textsuperscript{S60D} 1XD; vib-1\textsuperscript{S60A} S413A; S537A; S542A; S545A 5XA and vib-1\textsuperscript{S60D} S413D; S537D; S542D; S545D 5XD) (Table 1). Data are shown as the fold increase of protease secretion compared to the strains grown on media containing nitrogen. Asterisks (*) in both (B) and (C) indicate strains with protease production significantly different than WT grown on media without nitrogen (p<0.05).

**FIGURE 8. Phosphorylation of VIB-1 and phenotype of phospho-site mutants.** (A) VIB-1 is phosphorylated on multiple sites (as determined by mass spectrometry), including a predicted IME-2 consensus site. The asterisk indicates the phosphorylation
site that matches the consensus for yeast Ime2. (B) Mutations of the IME-2 consensus phosphorylation site in VIB-1 do not affect growth inhibition and suppression of conidiation associated with HI. Δ*vib-1* compatible (1; KD13-21 + KD13-51) and incompatible (2; KD13-21 + DV.80) heterokaryons (suppressed for HI). A heterokaryon with only one functional copy of *vib-1* and carrying incompatible *het-c pin-c* haplotypes shows typical HI (Δ*vib-1* mutations are recessive) (4; KD13-51 + JH1) as compared to a heterokaryon carrying one functional copy of *vib-1*, but of compatible *het-c pin-c* haplotype (3; KD13-21 + FGSC 6103). Heterokaryons with a phospho-null mutation (S60 to A) (5; 1XA + KD13-21) or a phospho-mimetic mutation (S60 to D) (6; 1XD + KD13-21) at the predicted IME-2 consensus site in VIB-1 exhibited a typical wild type HI phenotype. (C) Mutations of the IME-2 consensus phosphorylation site in VIB-1 do not affect cell death percentages associated with HI. However, mutations at all five VIB-1 phosphorylation sites reduce cell death percentages, although growth inhibition is still observed. All incompatible (Inc) heterokaryons exhibit significantly higher cell death than compatible (C) heterokaryons. Cell death percentages for incompatible heterokaryons labeled with an asterisk (*) were significantly different (p<0.05) than those in heterokaryons labeled with an arrow (▼).

**FIGURE 9. Model for ime-2 and vib-1 genetic pathway.** (A) VIB-1 positively regulates HI and cell death, as well as HET domain gene expression (via a separate mechanism). IME-2 negatively regulates additional HI cell death mediators that function in parallel to the VIB-1 pathway. Due to the role of IME-2 in the processing of a mitochondrial protein, we reasoned that the alternate cell death effectors and/or pathway
could be acting through the mitochondria. Further, since deletions in *ime-2* do not exhibit increased cell death during HI, we suggest that VIB-1 negatively regulates these alternate death effectors. (B) VIB-1 also positively regulates secreted proteases and, similar to the mechanism for HI cell death regulation, IME-2 negatively regulates a parallel pathway for secreted proteases. Deletions in *ime-2* cause a significant increase in secreted proteases, and thus in this pathway, VIB-1 does not regulate the parallel pathway for secreted proteases. Further, IME-2 appears to negatively regulate VIB-1 with respect to protoperithecial development via a separate pathway. For both pathways, it is likely that IME-2 is regulated by cellular signals of nitrogen availability.
<table>
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<th>Strain Name</th>
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<th>Origin or Reference</th>
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<td>DV.80</td>
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1XA
his-3::pccg1-vib-1(S60A)-gfp; het-c2 pin-c2 Δvib-1::hph; pan-2 A

5XA
his-3::pccg1-vib-1(S60A, S413A, S537A, S542A, S545A)-gfp; het-c2 pin-c2 Δvib-1::hph; pan-2 A

1XD
his-3::pccg1-vib-1(S60D)-gfp; het-c2 pin-c2 Δvib-1::hph; pan-2 A

5XD
his-3::pccg1-vib-1(S60D, S413D, S537D, S542D, S545D)-gfp; het-c2 pin-c2 Δvib-1::hph; pan-2 A

a Strains are of het-c1 pin-c1 genotype unless otherwise indicated.
Figure 2
Figure 3

A

% cell death

WT  Δvib-1  Δime-2  Δvib-1 Δime-2

Compatible

Incompatible

B

Relative Quantification (RQ)

WT  Δvib-1  Δime-2  Δvib-1 Δime-2

ime-2  vib-1  pin-c  tol
Figure 4

Enriched Functional Categories

- 01 Metabolism
- 02 Energy
- 10 Cell cycle and DNA processing
- 11 Transcription
- 12 Protein synthesis
- 14 Protein fate (folding, modification, destination)
- 16 Protein with binding function or cofactor requirement
- 18 Regulation of metabolism and protein function
- 20 Cellular transport, transport facilities, and transport routes
- 30 Cellular communication/signal transduction mechanism
- 32 Cell rescue, defense, and virulence
- 34 Interaction with the environment
- 38 Transposable elements, viral and plasmid proteins
- 40 Cell fate
- 41 Development
- 42 Biogenesis of cellular components
- 43 Cell type differentiation
Figure 6

A  kDa  WT  Δvib-1  Δime-2  Δvib-1  Δime-2
100  75  50  37

B

C

D

RQ (Relative Quantification)

WT  Δime-2  Δvib-1  Δvib-1  Δime-2

arg-4  ccg-1
Figure 8

A

DNA binding domain

a* 55 ...VRPRSR...62
b 390 ...RVSSMDLPRTAFTFTSAKQPQSPMQ...417
c-e 533 ...PSKSPRHGSHGLTNE...550

B

[Images of agar plates]

C

Percent Cell Death

[Bar graph showing cell death and standard deviation]
Figure 9

A

Mitochondria

IME-2

additional mediator(s)

VIB-1

HET domain gene expression

nucleus

Heterokaryon Incompatibility and Cell Death

B

Nitrogen

IME-2

additional mediator(s)

VIB-1

protoperithelial development

nucleus

secreted proteases

secreted proteases