The Role, Interaction and Regulation of the Velvet Regulator VelB in *Aspergillus nidulans*

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**Abstract**

The multifunctional regulator VelB physically interacts with other velvet regulators and the resulting complexes govern development and secondary metabolism in the filamentous fungus *Aspergillus nidulans*. Here, we further characterize VelB’s role in governing asexual development and conidiogenesis in *A. nidulans*. In asexual spore formation, velB deletion strains show reduced number of conidia, and decreased and delayed mRNA accumulation of the key asexual regulatory genes *brlA*, *abaA*, and *vosA*. Overexpression of *velB* induces a two-fold increase of asexual spore production compared to wild type. Furthermore, the *velB* deletion mutant exhibits increased conidial germination rates in the presence of glucose, and rapid germination of conidia in the absence of external carbon sources. In vivo immuno-pull-down analyses reveal that VelB primarily interacts with VosA in both asexual and sexual spores, and VelB and VosA play an inter-dependent role in spore viability, focal trehalose biogenesis and control of conidial germination. Genetic and in vitro studies reveal that AbaA positively regulates *velb* and *vosA* mRNA expression during sporogenesis, and directly binds to the promoters of *velb* and *vosA*. In summary, VelB acts as a positive regulator of asexual development and regulates spore maturation, focal trehalose biogenesis and germination by interacting with VosA in *A. nidulans*.

**Introduction**

Fungal spores are widespread in the environment and have a significant impact on daily human life. Asexual spores are the main reproductive form of many fungi and the primary agent for infecting hosts for many pathogenic fungi [1]. In some *Aspergillus*, asexual spore formation is closely correlated with the production of toxic secondary metabolites called mycotoxins [2,3]. Among *Aspergillus* species, *Aspergillus nidulans* is an excellent model system for studying the regulation of gene expression and the mechanisms of asexual development [4,5]. Asexual development (conidiation) in *A. nidulans* involves the formation of specialized multicellular structures called conidiophores. Conidiophore formation starts from hyphal cells, which form the mycelium, followed by the sequential formation of vesicles, metulae, phialides and conidia [4,6]. The process of conidiation is genetically regulated and the three genes *brlA*, *abaA* and *vosA* have been proposed to define a central regulatory pathway activating conidiophores formation [4,7–10]. After the spore is physically formed, it must undergo the maturation process. During conidiophore maturation, a key process is the focal biogenesis of large amounts (up to 15% of dry weight) of trehalose, α-D-glucopyranosyl-β-D-glucopyranoside, within the spore thereby conferring long term viability [11]. Trehalose, found in a wide variety of bacteria, fungi, plant, and invertebrates, serves as a vital protectant against desiccation and various environmental stresses and as an energy source [12]. Recent studies have identified the novel regulator VosA, which functions in trehalose biosynthesis and conidia maturation in *A. nidulans*. VosA also exerts negative feedback control of conidia by down-regulating *brlA* expression. Moreover, VosA is mainly localized in the nucleus of mature conidia and it contains a potential transcriptional activation domain at the C-terminus. These findings led to the hypothesis that VosA is a transcription factor regulating conidia maturation and the completion of conidiogenesis [13].

The *velvet* family proteins, including VeA, VelB, VelC and VosA, have defined a novel protein family. These four proteins all contain the *velvet* domain, and are highly conserved in dimorphic and filamentous fungi [13,14]. A series of recent studies have revealed that the *velet* proteins form the multimeric *velvet* complexes such as VelB/VeA/LaeA, VelB/VosA, and VelB/VelC [15,16]. The heterotrimeric VelB/VeA/LaeA complex controls sexual development and secondary metabolism in response to light [15]. The nuclear VelB-VosA heterodimer has been proposed to regulate trehalose biogenesis and spore maturation [16].
VelB, a primary component of the velB complexes, plays a crucial role in sexual development and secondary metabolism in _A. nidulans_ [15,16]. However, additional role and interaction of VelB in the life cycle of conidigenesis, and temporal regulation of _velB_ expression in the late phase of conidiation in _A. nidulans_ have not been studied. In this study, we further characterize _velB_ by genetic and biochemical approaches. The deletion of _velB_ results in elevated accumulation of brown pigment(s) and decreased production of conidia. Conversely, overexpression of _velB_ results in increased conidiation in air-exposed conditions, suggesting an activating role of VelB in conidiation. In addition, VelB plays a negative role in regulating conidial germination regardless of the presence or absence of an external carbon source. VelB predominantly interacts with VosA in asexual and sexual spores presence or absence of an external carbon source. VelB negative role in regulating conidial germination regardless of the activating role of VelB in conidiation. In addition, VelB plays an increased conidiation in air-exposed conditions, suggesting an elevated accumulation of brown pigment(s) and decreased production of conidia.

### Materials and Methods

#### Strains, Media and Culture Conditions

_A. nidulans_ strains used in this study are listed in Table 1. The fungal strains were grown on solid or liquid minimal medium with supplements (simplified as MM) as described previously [13,17] and incubated at 37°C. To determine the number of conidia, wild type (WT) and mutant strains were point inoculated and grown on solid MM at 37°C for 3 to 5 days. The conidia were collected in ddH2O from the entire colony and counted using a hemocytometer. For liquid submerged cultures, conidia of WT and mutant strains were inoculated in 50 ml of liquid MM (5×10⁵ conidia/ml) and incubated at 37°C, 250 rpm. To examine the effects of overexpression (OE) of _velB_ by an ectopic copy of _velB_ under the alcA promoter, all strains were inoculated on solid MM with 1% glucose (MMG) or MM with 100 mM threonine (MMT) to induce overexpression of _velB_ and 0.1% yeast extract at 37°C for 4 days. For Northern blot analysis, samples were collected as described [13,18,19]. Briefly, for vegetative growth phases, conidia (5×10⁵ conidia/ml) of WT and mutant strains were inoculated in 100 ml liquid MM in 500 ml flask cultures and incubated at 37°C. Samples were collected at designated time points of liquid submerged culture, squeezed-dried and stored at −80°C. For sexual and asexual developmental induction, 18 h vegetatively grown mycelia were filtered, washed and transferred to solid MM and the plates were air exposed for asexual developmental induction, or tightly sealed and blocked from light for sexual developmental induction. To examine _velB_ mRNA levels in _velB_ strains, _alcA_ strains were grown in liquid glucose medium (G) at 37°C, 250 rpm for 14 h (designated as time point “0”) and then transferred into liquid glucose medium (G; non-inducing) or liquid threonine medium (T; inducing). Samples were collected at the designated time points after transfer. *Escherichia coli*

#### Table 1. _Aspergillus_ strains used in this study.

| Strain name | Relevant genotype | References |
|-------------|------------------|------------|
| FGSC4       | _A. nidulans_ wild type, veA⁺ | FGSC a |
| FGSC26      | biA¹; veA¹ | FGSC a |
| FGSC33      | pyrA⁴; pyroA⁴; veA⁺ | N. P. Keller |
| RUM0.59     | pyrG⁸⁸; pyroG⁸⁸; veA⁺ | [52] |
| FGSC583     | biA¹; pyrA⁴; veA⁺ | [52] |
| FGSC590     | biA¹; veA⁺ | [52] |
| FGSC580     | biA¹; wetA; veA⁺ | [52] |
| TTA292-1    | biA¹; argB::alcA(p)::brlA; metG¹; veA⁺ | [48] |
| SJA7        | pabaA¹; pyrA⁴; veA⁺ | J. Aguirre |
| TTA021      | biA¹; pabaA¹; alcA(p)::brlA; abA¹; veA⁺ | [48] |
| RN14.1      | biA¹; ∆vosA:argB⁶⁺; veA⁺ | [13] |
| RN18.3      | pyrA⁴; ∆velB::AfupyrG⁺; veA⁺ | [15] |
| TNJ140.1    | pyrG⁸⁸; pyroA⁴; ∆ganB::AfupyrG⁺; veA⁺ | N.-J. Kwon & J.-H. Yu |
| THS13.1     | pyrA⁴; ∆ganB::pyroA⁴; ∆velB::AfupyrG⁺; veA⁺ | This Study |
| THS14.1     | pyrA⁴; ∆ganB::pyroA⁴; ∆velB::AfupyrG⁺; veA⁺ | This Study |
| THS16.1     | pyrG⁸⁸; pyroA⁴; ∆velB::AfupyrG⁺; veA⁺ | This Study |
| THS20.1     | pyrG⁸⁸; pyroA⁴::velB::FLAG⁺::pyroA⁴; ∆velB::AfupyrG⁺; veA⁺ | This Study |
| THS28.1     | pyrG⁸⁸; pyroA⁴::velB::FLAG⁺::pyroA⁴; ∆ganB::AfupyrG⁺; veA⁺ | This Study |
| THS7.1      | biA¹; pyroA::alcA(p)::velB::FLAG⁺::pyroA⁴; veA⁺ | This Study |
| THS18.1     | pyrG⁸⁸; pyroA::alcA(p)::velB::FLAG⁺::pyroA⁴; veA⁺ | This Study |
| RHS2.1,2    | pyroA::alcA(p)::velB::FLAG⁺::pyroA⁴; veA⁺ | This Study |
| RHS2.3,4    | pyroA::alcA(p)::velB::FLAG⁺::pyroA⁴; veA⁺ | This Study |

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The 3/4 _pyroA_ marker selects for the targeted integration at the _pyroA_ locus.

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### Table 2. Oligonucleotides used in this study.

| Name     | Sequence (5’—3’)                                                                 | Purpose                      |
|----------|----------------------------------------------------------------------------------|-----------------------------|
| OJA142   | CTGGCAGTGGAACAAGTC                                                              | 5' brlA probe               |
| OJA143   | AGAAGTTAACACCGTGA                                                              | 3' brlA probe               |
| OJA154   | AGCTCTCAGAATACTGCT                                                            | 5' abaA probe               |
| OJA155   | GTTGGAGATGGCTCCTCAT                                                          | 3' abaA probe               |
| OMN66    | TTTCCAGATCTTCCGAG                                                             | 5' vosA probe               |
| OMN63    | ATAGAAACACCCCAAGCAG                                                           | 3' vosA probe               |
| OMN125   | TATGCACTGGCACTCACAAGCAACCCG                                                    | 5' velB probe               |
| OMN126   | GTGATGGCGAGTCTATCTGGTCC                                                       | 3' velB probe               |
| OMN176   | CCATCACCATAAGCGTACG                                                          | 5' tpiA probe               |
| OMN177   | CAGTTGGAAGTGAAGGACCC                                                         | 3' tpiA probe               |
| OMN338   | CATGGCCAGATACAACTTGA                                                         | 5' rosA probe               |
| OMN339   | AAGATTGCCATCTTCCCGAAGCC                                                       | 3' rosA probe               |
| OHS490   | ATGCCGGCAGGAGGACCGAGAAG                                                       | 5' nodA probe               |
| OHS491   | TCAAAGAAGTAAGTGTTCAACCG                                                      | 3' nodA probe               |
| OMN340   | ATGGGATCACTAGAGGCTG                                                          | 5' nsdD probe               |
| OMN341   | TTAGAAGATCCGAGGACCC                                                         | 3' nsdD probe               |
| OJA246   | CAGCCTCAGAAGCTAAGCTG                                                         | 5' mutA probe               |
| QJA247   | CTAGTTGCAATACTGCTTGG                                                          | 3' mutA probe               |
| OJH84    | GCTGAACTGATGACGACGCCAA                                                      | 5' AfupyrG marker           |
| OJH85    | ATCTCGGGGAGTTGTTCACTC                                                       | 3' AfupyrG marker           |
| OMN131   | GAAAGTGGATGTTGTTGATG                                                         | 5' flanking region of velB  |
| OMN132   | CTAGAAATGACGACGACGAG                                                         | 3' flanking region of velB  |
| OMN133   | CTGATGCTGAACTGACGAG                                                         | 5' nested of velB           |
| OMN134   | TCTTCTGACAGATGACGCTG                                                        | 3' nested of velB           |
| OMN135   | GGTTGAAGGCTGACGGGACGAGG                                                       | 5' velB with AfupyrG tail   |
| OMN136   | AGTGCTCCCTCAGAAGCAAGAAT GAATAAAGGAATACACTAAAGGAGCAGG                          | 3' velB with AfupyrG tail   |
| OMN54    | TTTTGGCGTGCGAGTTGAGGTAG                                                       | 5' flanking region of vosA  |
| OMN55    | AAGAGGCTTTGGGTTGCTG                                                          | 3' flanking region of vosA  |
| OMN58    | GCATCAAAGAGAGAGAGAGG                                                        | 5' nested of vosA           |
| OMN59    | TTTGAAAATATGCCGGGGCCG                                                         | 3' nested of nosA           |
| OHS184   | ACTTCTGACCTGAAATGGGCTG GAGCCATATGAGAGACGACTG                                    | 5' vosA with pyroA tail     |
| OHS185   | TGGTGAACACATGACCAACATTTGGAAGTGGTTCAACGAGCAGG                                  | 3' vosA with pyroA tail     |
| OHS170   | ACCACGCTTCACTGAGAACATG                                                        | 5' flanking region of ganB  |
| OHS171   | TCCGGGAGAGCAGTGTGAGT                                                          | 3' flanking region of ganB  |
| OHS172   | AGCTGCTGTAGATTGGGTGCGG                                                        | 5' nested of ganB           |
| OHS173   | GGAGAGCAAGTCCAGATGACGCTG                                                      | 3' nested of ganB           |
| OHS174   | ACTTCTGACGTGAATGGGCTG AGATAGCTGTTCCGAGGAGG                                  | 5' ganB with pyroA tail     |
| OHS175   | TGGTGAACACATGACCAACATTTGGAAGTGGTTCAACGAGCAGG                                  | 3' ganB with pyroA tail     |
| ONK395   | ATCTCAGGGGTGTTGGAGAAAG                                                       | 5' pyroA marker             |
| ONK396   | TTGATCGAGCATGATGACGTC                                                         | 3' pyroA marker             |
| OMN196   | CGGGATCCATGAGTCTGGTGGAGGATGG                                              | 5' velB with BamHI          |
| OHS140   | AATTGAAATCCGTGATGGTGCTGAGGAGAGG                                            | 5' velB with EcoRI          |
| OHS141   | AATTAAAGCTTGTTATGCTGAAGCTACAGTGGTCC                                          | 3' velB with HindIII        |
| OHS258   | AATTGAATCCCGAGTGTAGCTGCTATCAAGCTTACGTC                                       | 5' abaA with EcoRI          |
| OHS259   | AATTGGCCGGCTCTTATGGATGAGTTGATGAGGAGACCAG                                    | 5' abaA with NotI           |
| OMN364   | CAGCTCAGCCCAGCGCCCTTCCC                                                       | 5' velB EMSA probe 1        |
| OMN365   | GGAAAGCAGGGAAGTGGAGGCT                                                        | 3' velB EMSA probe 1        |
| OHS408   | AGCTCAGCTTCCAGGCTAGGCTC                                                       | 5' velB EMSA probe 2        |
| OHS409   | CGCGAGAAGGAGAGAAGCTGAGGAGGCTAGG                                             | 3' velB EMSA probe 2        |
| OHS395   | GTGATGCTCAGTCTTCCGAGCTTTTTGCCC                                               | 5' vosA EMSA probe 3        |
strains, DH5α and BL21 (DE3), were grown in Luria–Bertani medium with ampicillin (50 mg/ml) for plasmid amplification.

**Generation of velB Mutants**

The oligonucleotides used in this study are listed in Table 2. For the deletion of *velB*, DJ-PCR method was used [20]. Both flanking regions of *velB* were amplified using the primer pairs OMN131;OMN135 and OMN132;OMN136 using *A. nidulans* FGSC4 genomic DNA as a template. The *A. fumigatus* pyroG marker was PCR-amplified from *A. fumigatus* AF293 genomic DNA with the primer pair OJH64;OJH65. The final PCR constructs for the *velB* deletion were amplified with OMN133;OMN134. The deletion cassettes were introduced into RJMP1.59 protoplasts generated by deletion were amplified with OMN133;OMN134. The deletion genomic DNA as a template. The *ganB* deletion constructs were amplified using OMN58;OMN59 and OHS172;OHS173, respectively, and introduced into RNI LpHS13. This cloning vector contains the BamHI and HindIII and cloned into *A. nidulans* alcA promoter and the *trpC* terminator (this study). The resulting plasmid pHSN6 was then introduced into RJMP1.59 or FGSC4 genomic DNA as a template (Table 2).

**Spore Viability Test**

Spore viability was determined as described [13]. Two-day old conidia (10^7 per plate) of WT and mutant strains were spread onto solid MM and incubated at 37°C for 24 h. The mycelial mat was collected and centrifuged, and genomic DNA was isolated as described [20]. Total RNA isolation and Northern blot analyses were carried out as previously described [18,20,24]. The DNA probes were prepared by PCR-amplification of the coding regions of individual genes with appropriate oligonucleotide pairs using FGSC4 genomic DNA as a template (Table 2).

**Spore Trehalase Assay**

Trehalose was extracted from conidia and analyzed as described [13,25]. Two-day old conidia (2x10^8) were collected and washed with ddH2O. Conidia were resuspended in 200 μl of ddH2O and incubated at 95°C for 20 min, and then the supernatant was collected, centrifuged, and genomic DNA was isolated as described [20]. Total RNA isolation and Northern blot analyses were carried out as previously described [18,20,24]. The DNA probes were prepared by PCR-amplification of the coding regions of individual genes with appropriate oligonucleotide pairs using FGSC4 genomic DNA as a template (Table 2).

**Germination of Conidia**

Germination rates were measured as previously described [26]. The conidia of WT and mutants were spread onto solid MM and incubated at 37°C for 24 h. The mycelial mat was collected and centrifuged, and genomic DNA was isolated as described [20]. Total RNA isolation and Northern blot analyses were carried out as previously described [18,20,24]. The DNA probes were prepared by PCR-amplification of the coding regions of individual genes with appropriate oligonucleotide pairs using FGSC4 genomic DNA as a template (Table 2).

**Eletrophoretic Mobility Shift Assay (EMSA)**

The cDNA fragment of *abaA* encoding amino acids 1–588 with the ATTA domain was cloned into pGEX-5X-1 (GE Healthcare).
Figure 1. Phenotypes of the ΔvelB mutant. (A) Colony photographs of WT (FGSC4), ΔvelB (THS16.1) and complemented (THS20.1) strains point-inoculated on solid MM and grown for 4 days (Top and bottom panels). The bottom panel shows the underside of the plates. (B) Quantitative analysis of conidiation of strains shown in (A) (** P<0.05). (C) About 10^5 conidia of WT (FGSC4), ΔvelB (THS16.1) and complemented (THS20.1) strains were spread onto solid MM and grown for 2 days. The bottom panel shows close-up views of the middle of the plates. (D) Quantitative analysis of...
The resulting plasmid (pHSE3) was introduced into *E. coli* BL21 (DE3) (Stratagene). The GST fusion protein expression and purification was carried out following the manufacturer’s instruction. For concentration and buffer exchange, Amicon Ultra Centrifilter Unit (Millipore) was used. BCA Protein Assay Kit (Pierce) was used to estimate protein concentration. EMSA was carried out as described [27–29]. Probes for EMSA were generated by annealing two single-stranded reverse-complementary oligonucleotides. The ds oligonucleotides were labeled with γ-32P-ATP (PerkinElmer) using T4 polynucleotide kinase (Promega). After labeling, the probes were purified by Sephadex G-25 spin columns. Binding reactions were performed in a 24 μl reaction volume containing 6% glycerol, 12 mM HEPES-KOH [pH 7.6], 4 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 0.5 mM DTT, 0–12 μg poly(dI-dC), ~10 ng DNA probe and appropriate amounts of the purified GST::AbaA protein. The reactions were done at RT for 20 min. The complexes were resolved on a 6% polyacrylamide gel (29:1 crosslinking) with 0.5%

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**Figure 2. Effects of overexpression of velB.** (A) WT (FGSC4: veA+ and FGSC26: veA1) and velB overexpression (RHS 2.1 and RHS 2.3) strains were point inoculated on solid MMG (non-inducing) or MMT (100 mM threonine, inducing) and photographed at day 4. (B) Effects of overexpression of velB in conidiospore formation. Quantification was done as described in the experimental procedures (** P<0.05). (C) Northern blot for mRNA levels of brlA in WT (FGSC4) and OEvelB (RHS 2.1) strains in liquid submerged culture. Numbers indicate the time (h) post transfer to liquid MMG (non-inducing) or liquid MMT (inducing). Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA.

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Figure 3. Roles of velB in conidial germination. (A) Kinetics of germ tube formation in inoculated WT (FGSC4), ΔvelB (THS16.1) and complemented (THS20.1) strains inoculated on solid MM at 37°C in the presence of glucose (1%). The number of conidia showing a germ tube protrusion was recorded at different times and is presented as a percentage of the total number of conidia in these fields. Data were obtained from two independent experiments. (B) Conidia of the designated strains were inoculated on solid MM (1% glucose) with or without carbon source and incubated at 37°C for 4.5 h (bar = 0.02 mm). (C) Colonies of WT (FGSC4), ΔvelB (THS16.1), ΔganB (TNJ140.1) and ΔvelB ΔganB (THS13.1) strains grown on solid MM for 4 day (Top panels). Status of conidial germination of the same strains on solid MMG, 37°C for 4 h (Bottom panels, bar = 0.02 mm). (D) Proposed model of GanB and VelB in controlling pigmentation and conidial germination.

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TBE running buffer at 100 V for 1 h. The gel was dried down under vacuum to three layers Whatman 3 MM filter paper. Autoradiography was performed at 280°C with Kodak XAR film.

Purification of in vivo VelB Interacting Proteins

For sample preparation, conidia and ascospores from two and seven day grown colonies, respectively, of WT and THS 20.1 (ΔvelB; velB:3xFLAG) strains were collected and suspended in cold PBS with 0.02% Triton X-100. The spores were sonicated for 300 sec (50% pulse) on ice to remove rodlets, and centrifuged for 10 min at 3,000 rpm and the supernatants were removed. For preparation of the protein samples from the mycelium, conidia (5×10^7 conidia/ml) of WT and THS 20.1 strains were inoculated in 500 ml of liquid MM and incubated for 18 h at 37°C. Mycelial samples were collected, squeeze dried and stored at −80°C. Prepared spores or mycelial samples were broken by a mini-bead beater for 2 cycles (1 min homogenization with 10 min sitting on ice) and centrifuged in a microcentrifuge for 15 min at 15,000 rpm at 4°C. The supernatant was incubated with anti-FLAG M2 affinity gel (A2220, Sigma). The agarose beads were collected by centrifugation and washed three times. The sample was boiled for 5 min and separated by SDS–PAGE and visualized by Coomassie Blue. Each stained band was cut out and subjected to mass spectrometry.

Nano-LC-ESI-MS/MS and Protein Identifications

All MS/MS experiments for peptide identification were performed using a nano-LC-MS system consisting of an Ultimate HPLC system (Dionex) and Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nano-ESI source, as described previously [30]. Briefly, for each sample, 10 μl was loaded by the autosampler onto a C18 trap column (id 300 mm,
Figure 5. *In vivo* interacting proteins of VelB in conidia, ascospores and hyphae. (A)–(C) SDS-polyacrylamide (10%) gel electrophoresis of total and VelB-FLAG enriched proteins stained with brilliant blue G. Lanes 1 & 4: Molecular mass standard; lane 2: soluble lysates from WT strain; lane 3: soluble lysates from THS 20.1 (*velB*; *velB*::3xFLAG) strain; lane 5: Eluted proteins from WT strain; lane 6: Eluted proteins from THS 20.1 (*velB*; *velB*::3xFLAG) strain. Arrows indicate the antibody fragments. After staining, the indicated regions were excised and proteins were identified using *A. nidulans* VelB peptides.
length 5 mm, particle size 5 μm; LC Packings) for desalting and concentrating at a flow rate of 20 μl/min. Then the trapped peptides were separated on a 100-mm homemade microcapillary column composed of C18 (Aqua; particle size, 5 μm) packed into 75-μm silica tubing. The mobile phases, A and B, were composed of 0 and 50% acetonitrile, respectively, each containing 0.1% formic acid. The gradient began with 5% B for 15 min; ramped to 20% B over 3 min, to 60% over 45 min, and to 95% over 2 min; and remained at 95% B for another 7 min. The column was equilibrated with 5% B for 10 min before the next run. The voltage applied to produce an electrospray was 2.5 kV. Argon was introduced as a collision gas at a pressure of 10 psi. The three most abundant MS ions were selected by data-dependent peak selection. The previously fragmented ions were excluded for 60 s. The proteins were identified by searching fungi subset (219981 entries) of the National Center for Biotechnology Information (NCBI) protein databases (20100312) using the MASCOT 2.0 search algorithm (Matrix Science). The general parameters for a search were considered to allow a maximum of one missed cleavage, the modifications of N-terminal Glu to pyroGlu, oxidation of methionine, acetylation of protein N terminus, carbamidomethylation of cysteine, and acylamide modified cysteine. A peptide charge state of +2 or 3, and peptide/fragment mass tolerance of ±0.5 Da were used for the MS/MS ion search. Probability based MASCOT scores were estimated by comparison of search results against estimated random match population and reported as −10*Log(P) where P is the absolute probability. The significance threshold was set at P<0.05.

Microscopy
The colony photographs were taken by using a Sony digital camera (DSC-F828). Photomicrographs were taken using a Zeiss M² BIO microscope equipped with AxioCam and AxioVision digital imaging software (Zeiss).

Statistical Analysis
Statistical differences between WT and mutant strains were evaluated with student’s unpaired t-test (2-tailed). Mean ± SD are shown. P values <0.05 were considered significant.

Results
Developmental Defects Caused by the Lack of VelB
To further investigate the roles of velB, we generated velB deletion (∆velB) and complemented strains and compared their phenotypes. As shown in Fig. 1A, ∆velB strains produced highly elevated levels of brown pigment(s). In the submerged culture, dark brown pigment(s) accumulated in the ∆velB hyphae and was detected in liquid medium (data not shown). The velB deletion mutant also exhibited reduced and delayed conidiation when point inoculated on solid medium (Fig. 1A). When 10⁷ conidia were inoculated onto solid medium and incubated for 2 days, the ∆velB mutant produced a reduced number of conidia (Fig. 1C); about a tenfold decrease in the yield of conidia compared to WT (Fig. 1B, 1D). Essentially, the same results were observed in the velB deletion strains with the velA allele (data not shown).

To correlate phenotypic changes caused by deletion of velB with the molecular events, we examined the mRNA levels of brlA, abad and rosA in WT and ∆velB strains grown under conditions that induce asexual development (Fig. 1E). In WT, accumulation of brlA mRNA was detectable at 6 h post developmental induction and decreased after 24 h. In the ∆velB mutant, however, brlA mRNA started to accumulate at 12 h and stayed at high levels even after 24 h. Levels of abad mRNA decreased and delayed in the ∆velB mutant compared to WT, too. While the short transcript of rosA started to accumulate at 24 h and the long transcript of rosA accumulated at 48 h in WT, neither long or short transcripts accumulated at 24 h and 48 h after induction of development in the ∆velB mutant (Fig. 1F). These results indicate that VelB is necessary for the proper progression of conidiation.

Previous study reported that the ∆velB mutant failed to form sexual fruiting bodies in the light and dark conditions [31]. We examined whether the deletion of velB affected the expression patterns of key sexual developmental genes including mutA, nosA, veA and nosD in WT and ∆velB strains grown under the conditions that preferentially induce sexual development (Fig. 1G, 1H) [32–35]. As shown in Fig. 1H, the deletion of velB caused about 24 h delayed (and reduced) accumulation of mutA (α-L-3-glucanase) that is associated with Hülle cells [32]. For nosA (repressor of sexual development; [33]) transcript, WT showed low level accumulation of its mRNA at 0~12 h, which then decreased after 24 h post developmental induction. However, the ∆velB mutant continued to exhibit high level accumulation of nosA mRNA even at 24 h post developmental induction, i.e., ~12 h delay in turning off nosA expression compared to WT. During early sexual developmental induction (0~12 h), levels of nosD (number of sexual spores; [34]) mRNA slightly decreased in the ∆velB mutant compared to WT. Levels of nosD (never in sexual development; [35]) mRNA were not distinctly different between WT and ∆velB strains. These results indicate that VelB is required for proper and timely expression of mutA/nosA/nosD in A. nidulans, which is critical for the development of Hülle cells and cleistothecia.

VelB is Conditionally Sufficient to Enhance Conidiation
We then generated the velB overexpression mutant by fusing the velB ORF (open reading frame) with the inducible alcA promoter [36]. Under non-inducing conditions, there were no differences between WT and overexpression of velB. When point inoculated under inducing conditions, overexpression of velB with the velA or veA allele induced a two-fold increase of asexual spore production compared to WT (Fig. 2A, 2B). We then tested whether VelB has a direct activating potential on brlA by examining mRNA levels of brlA in OEvelB strains grown in liquid submerged culture, where neither WT nor OEvelB strains developed conidiophores (not shown). As shown in Fig. 2C, OEvelB was not sufficient to induce the activation of brlA, indicating that VelB is necessary, and conditionally sufficient for activating conidiation in A. nidulans.

VelB Negatively Controls Germination of Conidia
To test the role of velB in spore germination, we examined germination rates of conidia of WT, ∆velB and complemented strains in the presence of 1% glucose. At 4 h of incubation, germination rates of WT, ∆velB and complemented strains were about 0%, 60% and 0%, respectively. All the ∆velB conidia formed germ tubes by 7 h, whereas all conidia of WT and complemented strains germinated by 8 h (Fig. 3A). In further tests, conidia of WT, ∆velB and complemented strains were inoculated onto solid media without an external carbon source. At 5 h after inoculation, some of the ∆velB mutant conidia began to form germ tubes but...
Figure 6. AbaA regulates velB and vosA expression. (A) Northern blot analyses for the levels of velB, brlA, abaA and vosA transcripts in WT (FGSC26), brlA42 (FGSC A83), abaA14 (FGSC A590), and wetA6 (FGSC A580) strains at 0, 12, 24, 48 h of asexual development (A0–A48). brlA42, abaA14 and wetA6 are temperature sensitive alleles that exhibit loss of function at 37°C. (B) Northern blot analyses for the levels of velB mRNA in WT (FGSC26), alcA(p)::brlA (TTA292-1), alcA(p)::abaA (SJA7) and alcA(p)::brlA abaA14 (TTA021) strains. Strains were grown in liquid MMG at 37°C for 14 h (designated as time point "0") and transferred into liquid MMG (G; non-inducing) or liquid MMT (T; inducing). Samples were collected at designated time points after the transfer. (C) Positions of putative AREs (5′-CATTCY-3′, indicated by the black oval-shape) in the promoter regions of velB and vosA. Black lines show the probes that cover ARE. (D) EMSA using the GST-AbaA fusion protein and the radiolabeled probes. Non-radioactive WT or mutant probes were used as competitors and added in 5 to 25 fold molar excess. f.p. = free probe. doi:10.1371/journal.pone.0045935.g006
these were not detectable in WT and complemented strains (Fig. 3B). These results indicate that VelB is negatively associated with conidial germination.

Previous studies suggested that GanB-mediated signal transduction plays a crucial role in controlling germination and pigmentation via cAMP/PKA signaling pathway, and is attenuated by the regulator of G-protein signaling (RGS) protein RgsA [24,37–39]. Similar to DrgsA, a dominant activating GanB mutant allele caused enhanced accumulation of brown pigment(s) and increased conidial germination rates in the absence of a carbon source [24,37]. To study a potential relationship between VelB and the GanB signaling pathway, we generated the DvelB DganB double mutant and compared its phenotypes including brown pigment production and conidial germination with the DvelB mutant. The DvelB DganB double mutant showed reduced accumulation of brown pigment compared to the DvelB single mutant. Similar to DvelB, however, the DvelB DganB double mutant exhibited reduced radial growth. As shown in Fig. 3C, although no WT and DganB conidia germinated, about 60% of the DvelB DganB double mutant conidia could germinate at 4 h of incubation. Taken together, these results suggest that VelB may play an independent role to GanB in brown-pigment(s) production, but function downstream of GanB-mediated signaling in regulating conidial germination (Fig. 3D).

Inter-dependence of VelB and VosA

Previous studies showed that both VelB and VosA are needed for sporogenesis and trehalose biogenesis in A. nidulans [16]. To examine the potential inter-dependent relationship between VelB and VosA, we generated the vosA and velB double deletion mutant and checked the spore viability, trehalose amount and mRNA levels of tpsA in WT and mutants. When point inoculated on solid medium (Fig. 4A), the DvelB DvosA double mutant showed reduced conidiation compared to the WT and DvosA single mutant, but increased the number of conidia compared to the DvelB single mutant. These results suggest that VelB (activator) and VosA (repressor) provide independent inputs to the final levels of asexual development and the double deletion results in an inter-mediate phenotype (Fig. 4B). The conidia of both single and double mutants displayed severe viability defects (Fig. 4C). We measured trehalose amounts in 2 day old fresh conidia and found that trehalose was undetectable in the double deletion mutant (Fig. 4D). Northern blot of tpsA, which is associated with the synthesis of trehalose, revealed that both single and double mutants displayed severe viability defects (Fig. 4C). We measured trehalose amounts in 2 day old fresh conidia and found that trehalose was undetectable in the double deletion mutant (Fig. 4C).

Figure 7. Proposed model for VelB. The cell-type specific roles of the velvet complexes in A. nidulans are indicated. In vegetative cells (hyphae), VelB functions as a positive regulator of asexual development, whereas VosA represses conidiation. The VelB-VeA heterodimer controls sexual development and forms the VelB-VeA-LaeA trimeric complex that affects secondary metabolism. During conidiogenesis (in phialide) AbaA directly binds to AREs present in the velB/ vosA promoters and regulates velB/vosA mRNA expression. VosA may bind to the promoter region of brlA and negatively controls of brlA expression. In conidium, the VelB-VosA complex localizes in the nucleus and plays an essential role in the completion of conidiogenesis, and controlling spore maturation, dormancy and germination.

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caused increased conidial germination rates in the presence or absence of carbon source (not shown). We also observed that germination rates of the ΔvelB ΔvosA mutant conidia were similar to the ΔvelB or ΔvosA single mutant conidia regardless of the presence or absence of an external carbon source. Taken together, these results suggest that VelB and VosA play an inter-dependent (not additive) role in sporogenesis, trehalose biogenesis and controlling conidial germination.

**VelB Primarily Interacts with VosA in Asexual and Sexual Spores**

VelB has been shown to form not only the VelB-VeA-LaeA heterotrimeric complex, which coordinates secondary metabolism and development, but also the VelB-VosA heterodimer, which may be the functional unit of trehalose biosynthesis and spore maturation [15,16]. The C-terminal region of VelB interacts with VeA or VosA [15]. Genetic data for the inter-dependent role of VelB and VosA in spores, and high accumulation of both vosA and velB transcripts in asexual and sexual spores [15], led us to hypothesize that VelB mainly interacts with VosA and forms the functional hetero-dimeric complex in the spores. To test this, we first generated the strains expressing the VelB protein fused with a 3xFLAG C-terminal tag in the ΔvelB background in A. nidulans and examined the levels of the VelB protein throughout the life cycle. Through Western blot with FLAG antibody, we found that the VelB::3XFLAG protein is clearly detectable during vegetative growth and early asexual development, and accumulates at high levels in conidia (Fig. S1). We confirmed that the VosA protein highly accumulates in conidia, too (Fig. S1). We then collected conidia and ascospores from 2 and 7 day, respectively, grown colonies of the strain expressing a functional VelB::3XFLAG fusion driven by the velB native promoter and identified the VelB interacting protein(s). We found that the VelB::3XFLAG immuno-pull-down predominantly isolated VosA and VelB, but not VeA or LaeA, in both asexual and sexual spores (Fig. 5A&B). We further examined the VelB interacting protein(s) in hyphae and found that VelB::3XFLAG isolates VeA, but not VosA (Fig. 5C). These results suggest that VelB interacts with VeA (hyphae) or VosA (spores), and plays a differential role in controlling various biological processes.

**AbaA Regulates velB and vosA Expression**

During asexual development, mRNA levels of velB and vosA increase during the formation and maturation of conidia [13,15]. Through sequence analysis, we found that three and one AbaA response elements (AREs, 5'-CATTTCY3') are located at the promoter and at N-terminal ORF regions, respectively, of velB, and hypothesized that AbaA directly binds and regulates velB (and vosA) expression in the late asexual development phases. To test this hypothesis, levels of velB mRNA were examined in various mutants carrying the brlA142, abaA14, wetA6. In the brlA142 and abaA14 mutants, velB mRNA accumulation decreased compared to WT, but in the wetA6 mutant, levels of velB mRNA were comparable to WT, suggesting that brlA and/or abaA are required for velB mRNA accumulation (Fig. 6A). To further test a potential direct activating role of BrlA and/or AbaA in velB expression, we examined levels of velB mRNA in the alcA:p::brlA, alcA:p::brlA abaA14 and alcA:p::abaA14 mutants. As shown in Fig. 6B, velB mRNA highly accumulated at 6 h upon abaA overexpression. Levels of velB mRNA slightly increased at 24 h after brlA overexpression, which was dependent on the WT abaA allele, as velB mRNA was not induced in the alcA:p::brlA abaA14 mutant. These results indicate that AbaA, not BrlA, is necessary and sufficient for velB mRNA expression.

We then investigated whether AbaA directly binds to the velB and vosA promoter regions by EMSA. We designed and used two probes covering AREs in the velB promoter and one probe covering ARE in the vosA promoter (Fig. 6C). As shown in Fig. 6D, the GST-AbaA fusion protein binds to all three probes containing ARE whereas the GST protein alone failed to bind to these probes. To further corroborate these AbaA-DNA interactions, we designed another probe with the mutated ARE motif. The GST-AbaA fusion protein could not bind to these mutated probes (data not shown). Moreover, only the unlabeled WT probes, but not mutated probes, competed during the complex formation with the labeled WT ARE probes (Fig. 6C). These results demonstrate that AbaA directly binds to the promoter regions of velB/vosA and regulates velB/vosA mRNA expression.

**Discussion**

The *velet* proteins are fungi-specific multifunctional regulators that control development and secondary metabolism in various filamentous and dimorphic fungi [14,40]. In *A. nidulans*, VelB plays an essential role in sexual development, the mycotoxin sterigmatocystin production and trehalose biosynthesis [15,16]. VelB homologues have been characterized in many fungi and are reported to regulate: sporulation and secondary metabolite production in *Fusarium fujikuroi* [41]; production of the trichothecene and zearalenone mycotoxins, pathogenicity, and sexual reproduction in *Fusarium graminearum* [42]; cell morphology and spore formation in *Histoplasma capsulatum* [43]; trehalose biosynthesis and conidiation in *Aspergillus fumigatus* (Park and Yu, unpublished); and production of the carcinogenic mycotoxin aflatoxin and formation of sclerotia in *Aspergillus flavus* (Park, Lovendahl and Yu, unpublished).

Our characterization of VelB in asexual development and sporogenesis indicates that VelB acts as a positive regulator of asexual development, and an essential component for trehalose biogenesis in spores. Previous studies suggested that the VosA-VelB heterodimer represses asexual development during vegetative growth in liquid culture and early asexual development in the dark [16]. Unlike ΔvosA strains, however, the ΔvelB mutant (i) cannot produce conidiophores in liquid submerged culture; (ii) produces a lower number of asexual spores than WT; and (iii) exhibits lowered brlA mRNA accumulation in early phase of asexual development. In addition, unlike the ΔvosA single mutant, the ΔvelB ΔvosA double mutant does not develop conidiophores in liquid submerged culture, i.e., VelB is epistatic to VosA. These results suggest that VelB is required for the hyper-conidiation in the absence of vosA, further supporting the proposed positive role of VelB in asexual development. Taken together, we propose that the VelB monomer or homo-dimer functions as a positive regulator of asexual development, whereas the VosA homo-dimer plays a negative regulatory role in conidiation during vegetative growth and the early phase of conidiophore formation (see Fig. 7).

VelB participates in the formation of multiple *velet* complexes including VelB-VeA and VelB-VeA-LaeA that play differential roles in sexual development and secondary metabolism [14,13]. Our studies suggest that in hyphae VelB predominantly interacts with VeA and may form a VeA-VelB complex (Fig. 5C). As shown in Figs 1&2, however, VelB’s function in asexual spore formation does not require the physical interaction with VeA, as VeA cannot physically interact with VelB (Bayram et al., 2008). These data suggest that VelB and VeA may play a redundant role in controlling asexual spore formation, but an inter-dependent role in sexual development by forming a VelB-VeA functional unit [15]. Similar to veA, moreover, the deletion of velB resulted in reduced...
colony size and elevated accumulation of brown pigment(s). The phenotypes, accumulation of brown pigment(s) and reduced colony growth, of the ΔvelA or ΔvelB single mutant were similar to those of the ΔvelA ΔvelB double mutant, suggesting that VeA and VelB also function inter-dependently to regulate brown pigmentation and colony growth. In addition, VeA and VelB control secondary metabolism by forming trimeric complexes with the master regulator of secondary metabolism LacA [15].

It appears that, in addition to activating conidiation, VelB is required for proper control of conidial germination. Trehalose is not only required for the acquisition of tolerance to various stresses but also utilized in glycolysis, spore maturation and germination [11,44]. Trehalose is rapidly degraded during conidial germination and the absence of the trehalose-6-phosphate synthase gene caused delayed conidial germination in aspergilli [11,45–47]. However, exogenous trehalose supplemented in the medium had no effects on rescuing the delayed conidial germination caused by the deletion of trehalose-6-phosphate synthase in A. fumigatus, suggesting that trehalose-6-phosphate synthase genes, but not trehalose, may play an additional role in conidial germination [47]. In our present study, despite the lack of trehalose in the velB deletion mutant conidia, germination rates of ΔvelB conidia were increased (Fig. 3). These suggest the possibility of VelB playing a more complex role in governing spore germination, which may be associated with the GanB(N)-mediated signal transduction. We propose that VelB functions as a negative regulator of conidial germination acting downstream of GanB-mediated signaling (Fig. 3D).

Previous studies demonstrated that the absence of velB or vosA resulted in the lack of trehalose in conidia and shortened viability of spores, and that the VosA-VelB complex is formed in the nucleus. These suggest that the VelB-VosA complex is the functional unit governing conidial maturation [13,16]. Results of our double mutant analyses are generally in agreement with the proposed inter-dependent role of VelB and VosA in spore viability, trehalose biosynthesis and conidial germination. Our data further demonstrate that VelB predominantly interacts with VosA in the asexual and sexual spores (Fig. 5). Taken together, we confirmed that the VelB-VosA complex is a major unit playing a vital role in controlling spore maturation (trehalose biogenesis and cell wall completion), long-term viability, dormancy and germination.

BrlA is mainly localized in vesicles metulae, and phialides, and young spores and regulates conidio genesis during the early phase of conidiation [48,49]. However, brlA mRNA has not been detected in mature spores [50]. To complete sporogenesis turning off the expression of brlA is crucial for fungal spores. Uncontrolled production of brlA may inhibit growth of A. nidulans, likely causing a generalized metabolic shutdown, leading to an inability of cells to acquire nutrients from the growth medium. [51]. Previous study demonstrated that VosA exerts negative feedback regulation of conidiation, i.e., turns off brlA expression after spore formation, and the deletion of vosA resulted in high level accumulation of brlA mRNA in conidia [13]. Our preliminary genome wide analyses of in vivo VosA-DNA interactions indicate that the promoter regions of brlA are specifically enriched, suggesting that VosA may directly bind to the promoter of brlA and repress brlA expression in phialides and/or conidia.

Based on these data, we propose a genetic model depicting the roles and regulation of VelB and VosA during conidiogenesis. VelB is a multifunctional developmental regulator playing a pivotal role throughout the life cycle of A. nidulans. The interaction between VelB and its partner proteins (VelB, VeA or VosA) is time and/or cell-type specific and the multiple VelB-related complexes play differential roles in controlling fungal development and sporogenesis (see model in Fig. 7). During conidiogenesis, both velB and vosA are activated by AbaA in the vesicle and phialide [13]. High level production of VelB and VosA in developing cells would induce the formation of the VosA-VosA and VelB-VosA complexes. Genetic data in conjunction with VosA's localization in metulae, phialides and conidia suggest that the VosA monomer or homo-dimer turns off brlA expression in conidia [13]. The VelB-VosA hetero-dimer activates focal trehalose biogenesis in spores, controls spore wall integrity, and negatively regulates precocious conidial germination (Fig. 7). Further in-depth analyses of the roles of the individual velvet proteins and complexes, and the mechanisms of velvet-mediated regulation are in progress and will provide new insights into fungal development and secondary metabolism.

**Supporting Information**

**Figure S1 Levels of the VelB and VosA proteins throughout the lifecycle of A. nidulans.** Western blot for the VelB::3xFLAG and VosA::3xFLAG fusion proteins in velB(p)::VelB::3xFLAG (THS28.1) and vosA(p)::VosA::3xFLAG (THS28.1) strains, respectively. These fusion proteins were detected by anti-FLAG antibody. Protein crude extracts (10 μg) were loaded in each lane. Conidia (asexual spores) were indicated as C. The numbers indicate the time (hours) after incubation in liquid MMG (Vegetative) and solid MMG inducing asexual development (Asexual). (TIF)

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**Author Contributions**

Conceived and designed the experiments: HSP MN KCJ JHY. Performed the experiments: HSP MN KCJ YHK. Analyzed the data: HSP MN KCJ YHK JHY. Contributed reagents/materials/analysis tools: KCJ YHK.

Wrote the paper: HSP MN JHY.

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