Supplementary information

Supplementary Figure 1 | Medium WC-2 is a C-terminally shortened proteolytic fragment of WC-2
Supplementary Figure 2 | Antagonistic expression of *wc-2* and *swc-2*

A

Exon 1 Probe

Exon 2 Probe

wc-2 RNA levels (a.u.)

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\[\Delta_{wc-1} \quad fqr\]

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\[\Delta_{wc-1} \quad fqr\]

B

wc-2 RNA levels (a.u.)

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\[\Delta_{wc-2} \quad \Delta_{wc-2 \ fqr} \quad \Delta_{wc}\]

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\[wc-2 \text{ ORFi}\]
Supplementary Figure 3 | Alignment of WC-2 protein sequences from fungi that harbor frequency genes
Supplementary Figure 1 | Medium WC-2 is a C-terminally shortened proteolytic fragment of WC-2. A) Upper part: The second intron of the wc-2 gene is located close to the 3’ end of the coding region. If intron 2 were not spliced, wc-2 would encode a C-terminally truncated protein of 52 kDa. To analyze whether the medium isoform of WC-2 results from translation of mRNA that contains intron 2 we constructed a wc-2 gene that lacks the intron (P 2.2 wc-2 ΔIntron2). In addition, we constructed a wc-2 gene with a C-terminally fused GFP moiety that is expressed under control of the ccg-1 promoter. Lower part: Western blot analysis showing that Δwcc strains transformed with P 2.2 wc-2 and P 2.2 wc-2 ΔIntron2 both express medium WC-2 in addition to full size and short WC-2. The data demonstrate that medium WC-2 is not translated from partially unspliced wc-2 RNA containing intron 2. Medium WC-2 is also detected in the ccg-1 wc-2-GFP strain, demonstrating that it is a C-terminally truncated proteolytic cleavage product. B) Diagram of WC-2 domains. N-termini of WC-2 and sWC-2 are indicated by arrows. Dashed line and scissors mark the putative proteolytic processing site of mWC-2.

Supplementary Figure 2 | Antagonistic expression of wc-2 and swc-2. A) wc-2 RNA measured with E1 and E2 probes in Δwc-1 and Δfrq at DD24. RNA levels in Δwc-1 were set to 1. Levels of E2 containing RNA (P_{wc-2} + P_{int-wc-2}) are less affected by FRQ and WC-1 than levels of E1 containing RNA (P_{wc-2}). This suggests that P_{wc-2} transcription interferes with transcription initiation at the downstream located P_{int-wc-2}. B) wc-2 RNA measured with E2 probe at DD24 in Δwc-2, Δwc-2 Δfrq and Δwcc transformed with the wc-2 coding sequence including introns (ORFi) and expressing only sWC-2 (not shown). RNA levels in wt (not shown) were set to 1. swc-2 RNA levels are identical in these strains indicating that FRQ and WC-1 do not regulate swc-2 in the absence of wc-2 transcription (data are means of 4 experiments ± SEM).

Supplementary Figure 3 | Alignment of WC-2 protein sequences from fungi that harbor frequency genes. Species: Neurospora crassa, Fusarium graminearum, Magnaporthe grisea, Phaeosphaeria nodorum and Sclerotinia sclerotiorum. The
alignement was performed with Multalin. Red: identical, blue similar amino acid residues.

**Strains and Culture Conditions**

All strains used in this study carried the *bd* mutation (Loros et al, 1986). For transformations, Δfrq; *bd*; *his*-3, Δwc-1; *bd*; *his*-3, Δwcc; *bd*; *his*-3 and Δfrq; Δwc-2; *bd*; *his*-3 were used. Δwcc; *bd*; *his*-3 was obtained by crossing Δwc-1; *bd*; *his*-3; *mat a* with Δwc-2; *bd*; *his*-3; *mat A* and Δfrq; Δwc-2; *bd*; *his*-3 was made by crossing Δfrq; *bd*; *his*-3; *mat a* with Δwc-2; *bd*; *his*-3; *mat A.

Standard growth medium contained 2% glucose, 0.5% L-arginine, 1x Vogel’s and 10 ng/ml biotin. For liquid cultures containing QA, 0.3% QA was added into liquid medium containing 1x Vogel’s, 0.2% sucrose and 0.17% arginine. Race tube medium contained 1xVogel’s medium, 0.17% L-arginine, 10 ng biotin and 2.2% agar.

**Plasmid constructs and Neurospora transformation**

**Construction of pBM60 tub-frq**

The β-tubulin promoter was amplified from pMYX2 using the following primers:

Tubulinpr_Fw: TTTTTTTACGTATCGACAGGGGGCCTTCC

FRQ-Tubulinpr_Rv: TTTTGCATGCCCTGGGATTTATCCCATCCGCCCATGTT

CACCTTGACTAGTCTTTGACGG

The resulting PCR fragment was inserted into the SnaBI / SphI sites of the *frq* gene in pBM60 Cla-frq generating pBM60 tub-frq.

**Construction of pBM60 cpc1-sWC-2**

The *swc*-2 gene was amplified from genomic DNA using the following primers:

swc-2 Fw: AAAAAAAAGCTTCAACATGGCCATGTCCAGCGCCGGACC

WC-2 Rv: AAAAAATCTAGACTATCCCATATGATCGCCC
The resulting PCR fragment was inserted into the HindIII / XbaI sites of a pBM60 derivative harboring the cpc1 promoter (Heise and Kumar Behera, unpublished) generating pBM60 cpc1-sWC-2.

**Construction of pBM61 WC-2 ORFi**

The wc-2 gene was amplified from genomic DNA using the following primers:

WC-2 ORF SpeI Fw: AAAAAACTAGTATGTCTCAGGACAGCCTCC
WC-2 ORF XmaI Rv: AAAAAACCGGGGTATCCCATATGATCGCCCATG

The resulting PCR fragment was inserted into the SpeI / AgeI sites of pBM61-wc-1.ORF (Káldi *et al*, 2006) generating pBM61 WC-2 ORFi.

**Construction of pBM61 WC-2 ORFi M127/133L**

pBM61 WC-2 ORFi M127/133L was constructed by mutagenesis of both possible sWC-2 start codons (figure 3D) in pBM61 WC-2 ORFi using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions and the following oligonucleotides:

sWC-2 Mut Fw: GCGACCAGCCTGTCCAGCGCCGGACCGCTGATCGCCACG
sWC-2 Mut Rv: CGTGGCGATACAGCGGTCCGGCGCTGGACAGGCTGGTCGC

**Construction of pBM61 wc-2 P 1.2 WC-2**

The wc-2 promoter fragment including 440 bp of the wc-2 gene was amplified from genomic DNA using the following primers:

WC-2 P1.2 Fw: AAAAAACCCGGGAATAAAGGCTGTTGTGTGTGACG
WC-2 E2 Rv: AAAAAACCTGCAGGACCAGGGGTGGGGATCGCCACC

The resulting PCR fragment was inserted into the XmaI / SbfI sites of pBM61 WC-2 ORFi generating pBM61 wc-2 P 1.2 WC-2.

**Construction of pBM61 wc-2 P 1.5 WC-2**

pBM61 wc-2 P 2.2 WC-2 was used as a template in a PCR using the following 5’ phosphorylated primers:

P-P1.53 Fw: CAGGTAGAGAGAGCATGCTAGGAACATGG
Following PCR the template plasmid was digested with DpnI and the PCR product was circularized by ligation resulting in pBM61 wc-2 P 1.5 WC-2.

**Construction of pBM61 wc-2 P 1.9 WC-2**

pBM61 wc-2 P 2.2 WC-2 was used as a template in a PCR using the following 5’ phosphorylated primers:

P-P1.86 Fw: CAGGCGTGCTCGCGAGGTGTTTTGTTTGG
P-Pr-WC-2 600 up DEL Rv: CAGGCCCCGCTGCGAGGAATTGCATATCAA

Following PCR the template plasmid was digested with DpnI and the PCR product was circularized by ligation resulting in pBM61 wc-2 P 1.9 WC-2.

**Construction of pBM61 wc-2 P 2.2 WC-2**

The wc-2 promoter fragment including 440 bp of the wc-2 gene was amplified from genomic DNA using the following primers:

WC-2 P2.2 Fw: AAAAAACCGGGCATGAGTGGCTCCATCAGTGACC
WC-2 E2 Rv: AAAAAACCTGCAGGAGGGGTGGCGGGACC

The resulting PCR fragment was inserted into the XmaI / SbfI sites of pBM61 WC-2 ORFi generating pBM61 wc-2 P 2.2 WC-2.

**Construction of pBM61 wc-2 P 2.2 WC-2 M127/133L**

pBM61 wc-2 P 2.2 WC-2 M127/133L was constructed by mutagenesis of both possible sWC-2 start codons (figure 3D) in pBM61 wc-2 P 2.2 WC-2 using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions and the following oligonucleotides:

sWC-2 Mut Fw: GCGACCAGCCTGTCCAGCGCCGGACCGCTGATCGCCACG
sWC-2 Mut Rv: CGTGGCGATCAGCGGTCCGGCGCTGGACAGGCTGGTCGC

**Construction of pBM61 wc-2 P 3.2 WC-2**

The wc-2 promoter fragment including 440 bp of the wc-2 gene was amplified from genomic DNA using the following primers:
The resulting PCR fragment was inserted into the XmaI / SbfI sites of pBM61 WC-2 ORFi generating pBM61 wc-2 P 3.2 WC-2.

**Construction of pBM61 wc-2 P 0.6 WC-2**

pBM61 wc-2 P 1.2 WC-2 was used as a template in a PCR using the following 5’ phosphorylated primers:

- P-Pr-WC-2 600 up DEL Fw: CAGGCGTCTCTTACTGGGCTTGTTCTCTTTG
- P-Pr-WC-2 600 up DEL Rv: CAGGCCCGGGCTGCAGGAATTCGATA

Following PCR the template plasmid was digested with DpnI and the PCR product was circularized by ligation resulting in pBM61 wc-2 P 0.6 WC-2.

**Construction of pBM61 wc-2 P 1.2-0.6 WC-2**

pBM61 wc-2 P 1.2 WC-2 was used as a template in a PCR using the following 5’ phosphorylated primers:

- P-Pr-WC-2 600 down DEL Fw: TACAACCGCCAGAACAATGTCTCACGGACAGCCTCC
- P-Pr-WC-2 600 down DEL Rv: CAGTAAGGACGGGAGCGGGGGGTGGGCGTG

Following PCR the reaction was treated with DpnI to remove the template plasmid and the PCR product was circularized by ligation resulting in pBM61 wc-2 P 1.2-0.6 WC-2.

**Construction of pBM61 wc-2 P 2.2 mut dist+prox Box WC-2**

First, pBM61 wc-2 P 2.2 mut dist Box WC-2 was constructed using fusion PCR. For this purpose two overlapping fragments containing the mutation of the distal Box were amplified using pBM61 wc-2 P 2.2 WC-2 as template and the following primers:

**Fragment mut dist a:**

- WC-2 P2.2 Fw: AAAAAACCCGGGCGCATGAGTGCTCCATCATCAGTGACC
- distBox mut EcoRI Rv: GAATATCAAAAACGCTTCTGGGACAAGGACACACACAG

**Fragment mut dist b:**

- AATTCCATGTAAGTGGAGTTTTTCTGCTTCACCTG
The two PCR fragments were fused in a second PCR using primers WC-2 P2.2 Fw and WC-2 E2 Rv. The resulting PCR fragment was inserted into the XmaI / SbfI sites of pBM61 wc-2 P 2.2 WC-2 generating pBM61 wc-2 P 2.2 mut dist Box WC-2.

pBM61 wc-2 P 2.2 mut dist+prox Box WC-2 was constructed using fusion PCR. For this purpose two overlapping fragments containing the mutation of the proximal Box were amplified using pBM61 wc-2 P 2.2 mut dist Box WC-2 as template and the following primers:

**Fragment mut dist a:**

WC-2 P2.2 Fw: AAAAAACCCGGGCGTGGAGTGCTCCATCAGTGACC
proxBox mut EcoRI Rv: CTTTATTTATTTGTCGAAGGTATTGCTGTCAAG
GAATTCAAGCTGTGGAAGATAC TAACCGAAATCTGCTCAAG

**Fragment mut dist b:**

proxBox mut EcoRI Fw: CTTGAGCAGATTTCGTTTAGATCTTTCCACACAG
CTTCGAATTCGCGAGTACTGCTGGACCAAAATAAAGG
WC-2 E2 Rv: AAAAAACCTGCAAGGGAGGTTGGCGGGACC

The two PCR fragments were fused in a second PCR using primers WC-2 P2.2 Fw and WC-2 E2 Rv. The resulting PCR fragment was inserted into the XmaI / SbfI sites of pBM61 wc-2 P 2.2 mut dist Box WC-2 generating pBM61 wc-2 P 2.2 mut dist+prox Box WC-2.

**Construction of pBM60 wc-2 P 2.2 WC-2-DHFR**

The WC-2-DHFR fragment was amplified from pqa-2-WC-2-DHFR as a template using the following primers:

XmaI WC-2 Fw: AAAAAACCCGGGGAACAATGTCTACGGACAGCGCG
MluI DHFR Ec Rv: AAAAAACCGGTTTACCGCGCTCCA GAATCTCAAGGC
The resulting PCR fragment was inserted into the XmaI / MluI sites of pBM60 wc-2 P 2.2-FLAG-DHFR generating pBM60 wc-2 P 2.2 WC-2-DHFR.

**Construction of pBM61 wc-2 P 2.2 WC-2 ΔIntron2**

pBM61 wc-2 P 2.2 WC-2 was used as a template in a PCR using the following 5’phosphorylated primers:

P-WC-2 Intr2 Del Fw: GTACG CTGATTCCC CCGAATGGCG AAAAG

P-WC-2 Intr2 Del Rv: CGCAGTCGGTGACACGTATTCCCTCGCAAC

Following PCR the reaction was treated with DpnI to remove the template plasmid and the PCR product was circularized by ligation resulting in pBM61 wc-2 P 2.2 WC-2 ΔIntron2.

pBM60 qa-2-TAP-WC-2 and pBM60 ccg1-WC-2-GFP were gifts from Andrea Haase and Nadine Wolf, respectively (unpublished).

For **transformation**, *Neurospora* cells were grown on solid medium for 5-7 d, and conidia were harvested by shaking in 1 M sorbitol. Conidia were washed in 1 M sorbitol three times and concentrated to a suspension with an optical density of 300-600/ml. 70 µl of the suspension were incubated with linearized plasmid DNA (derivatives of pBM60/61 for targeted insertion into the *his-3* locus) for 5 min on ice. Cells were transformed by electroporation in a 0.2-cm cuvette (voltage gradient: 1.5 kV/cm; capacitance: 25 µfd; resistance: 600 Ω; Gene Pulser; Bio-Rad). 1 ml of 1 M sorbitol was added to each cuvette, the suspension was mixed by gently pipetting up and down, and 200-800 µl were added to 10 ml of top-agar, and plated on medium. Transformants were picked after 2-3 d of incubation at 30°C and transferred into standard liquid medium. Insertion of the target gene was verified by PCR. Homocaryotic strains were generated by purification by several plating steps and checked by quantitative PCR (Schafmeier *et al*, 2006).
RNA analysis

RNA was prepared using peqGOLD TriFAST™ (peqLab, Erlangen, Germany). cDNA was synthesized from 1 µg RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). wc-2 and actin cDNA was detected by real-time PCR (ABI-Prism 7000, Applied Biosystems) using TaqMan, probes (Görl et al, 2001). Triplicate reactions (20 µl) containing cDNA equivalent to 0.1 µg RNA were analyzed. The following primers and TaqMan probes were used.

actinII:
Forward primer: GATGACACAGATCGTTTTTCGAGACT
Reverse primer: CGGAGGCGTAGAGAGAAAGGA
Probe: FAM-CCGCCCTTCTACGTCTCCATCCA-TAMRA

lwc-2 (exon1):
Forward primer: ACAACCGCCAGAAACATGTCT
Reverse primer: CCCCATGGCTCCAAAGC
Probe: FAM-ACGGACAGCCTCCCCCTGGATC-TAMRA

lwc-2 (across intron 1):
Forward primer: CATGTCAGCCTCGCAGATGA
Reverse primer: TCAAACCAGGAAACAATGTAAGT
Probe: FAM-CCACAGGACATGATCGCTTTCTGGGA-TAMRA

total wc-2:
Forward primer: AGTTTGACACCAATCCACAGA
Reverse primer: AGGGTCGAGCCATCATGAAC
Probe: FAM-AGTGCCTTTCTGACGGCCG-TAMRA

5' RLM-RACE was carried out according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA; Invitrogen, Carlsbad, CA, USA).
Primers used for the PCR steps in 5'RLM-RACE were the following:

**lwc-2**
First Choice RLM-RACE Kit, Ambion
Outer Primer: GCAGAGAGTAAAGGTGAAGTG
Nested Primer: AGAGAACAAGCCCAAGTAAGGAC

GeneRacer Kit, Invitrogen
Outer Primer: CGCAATAAGGTCGTCGGGGGAATAG
Nested Primer: GGAGGCTGTCCGTGAGACATTGTTCCTG

**swc-2**
First Choice RLM-RACE Kit, Ambion
Outer Primer: AGGGTCGAGCCATCATGAAC
Nested Primer: CAGTCTCGAAAAATGGTCCAG

**uvs-6**
GeneRacer Kit, Invitrogen
Outer Primer: GATGAGGGTCAATGGGGTGTTGAAAGC
Nested Primer: GCACGCCCAAGATGGACATTTTCTCG