β-Carbonic Anhydrases Play a Role in Fruiting Body Development and Ascospore Germination in the Filamentous Fungus *Sordaria macrospora*

Skander Elleuche1,2, Stefanie Pöggeler1*

1 Institute of Microbiology and Genetics, Department of Genetics of Eukaryotic Microorganisms, Georg-August University, Göttingen, Germany, 2 Faculty of Biology and Biotechnology, Ruhr-University of Bochum, Bochum, Germany

Abstract

Carbon dioxide (CO2) is among the most important gases for all organisms. Its reversible interconversion to bicarbonate (HCO3−) reaches equilibrium spontaneously, but slowly, and can be accelerated by a ubiquitous group of enzymes called carbonic anhydrases (CAs). These enzymes are grouped by their distinct structural features into α-, β-, γ-, δ- and ζ-classes. While physiological functions of mammalian, prokaryotic, plant and algal CAs have been extensively studied over the past years, the role of β-CAs in yeasts and the human pathogen Cryptococcus neoformans has been elucidated only recently, and the function of CAs in multicellular filamentous ascomycetes is mostly unknown. To assess the role of CAs in the development of filamentous ascomycetes, the function of three genes, *cas1*, *cas2* and *cas3* (carbonic anhydrase of *Sordaria*) encoding β-class carbonic anhydrases was characterized in the filamentous ascomycetous fungus *Sordaria macrospora*. Fluorescence microscopy was used to determine the localization of GFP- and DsRED-tagged CAs. While *cas1* and *cas3* are cytoplasmic enzymes, *CAS2* is localized to the mitochondria. To assess the function of the three isoenzymes, we generated knock-out strains for all three genes (Δcas1, Δcas2, and Δcas3) as well as all combinations of double mutants. No effect on vegetative growth, fruiting-body and ascospore development was seen in the single mutant strains lacking *cas1* or *cas3*, while single mutant Δcas2 was affected in vegetative growth, fruiting-body development and ascospore germination, and the double mutant strain Δcas1/2 was completely sterile. Defects caused by the lack of *cas2* could be partially complemented by elevated CO2 levels or overexpression of *cas1*, *cas3*, or a non-mitochondrial *cas2* variant. The results suggest that CAs are required for sexual reproduction in filamentous ascomycetes and that the multiplicity of isoforms results in redundancy of specific and non-specific functions.

Introduction

Carbon dioxide (CO2) and its hydration product bicarbonate (HCO3−) are essential molecules in various physiological processes. In all domains of life, from microorganisms to mammals, CO2 is the end-product of respiration. Plants, algae and cyanobacteria are able to fix CO2 during photosynthesis. Although it represents only 0.036% of the atmosphere, CO2 is found at concentrations of roughly 5% in the human bloodstream and in tissues, where respiration takes place. CO2 is able to diffuse through lipid-containing cell membranes, but bicarbonate is negatively charged and not permeable to lipid bilayers [1,2]. Since the reversible interconversion of CO2 and HCO3− is spontaneously balanced, CO2 is in equilibrium with HCO3− and carbonic acid. The reaction CO2+H2O ↔ HCO3−+H+ is slow, but can be accelerated by the ubiquitous enzyme carbonic anhydrase (CA; EC 4.2.1.1). CAs are typically Zn2+-metalloenzymes. Based on their amino acid sequence and structure they can be divided into five distinct classes, α-, β-, γ-, δ- and ζ-, that share no sequence similarity, and appear to have evolved independently [3–5]. At least 16 different α-CA isoforms have been isolated in mammals, while plants and fungi encode α- and β-class carbonic anhydrases [3,6,7]. The γ-class is predominantly distributed in archaebacteria and eubacteria, but has also been identified in the mitochondria of plants [8,9]. The ζ-class, which has a cadmium center and the δ-class have been discovered in marine diatoms [10,11].

To date, only β-CAs have been characterized in fungi. The function of a β-CA in the hemiascomycetous yeasts *Saccharomyces cerevisiae* and in the human pathogen *Candida albicans* has been elucidated. In both, deletion of the β-CA-encoding *NCE103* gene resulted in a mutant that required elevated CO2 levels for growth [12–14]. The *S. cerevisiae* mutant could be functionally complemented with a plant β-CA from *Medicago sativa* [14]. In addition, expression of the *S. cerevisiae NCE103* gene was dramatically up-regulated under ambient air conditions [12]. In *C. albicans* the β-CA functions as a CO2 scavenger essential for pathogenicity in niches where the available CO2 is limited [13]. In contrast to *S. cerevisiae* and *C. albicans*, the basidiomycetous human pathogen *Cryptococcus neoformans* encodes two β-CAs whose functions have recently been elucidated [15,16]. Similar to *C. albicans*, growth and morphogenesis of *C. neoformans* is strongly influenced by CO2, which is directly sensed by the bicarbonate-regulated adenyl cyclases [13,15]. The *CA2* gene of *C. neoformans* was shown to...
encode its major CA. Its activity is essential to survival and proliferation as well as for basidial and basidiospore formation, but is dispensable for lethality during infection [16].

Similar to C. neoformans, filamentous ascomycetes seem to contain multiple β-CAs: three β-CA homologues have been identified in the genome of the ascomycete Aspergillus fumigatus [7]. However, the functional role of β-CAs in filamentous fungi has not yet been assessed and it is still not known if the multiplicity of isoforms is related to organelles or cell- or tissue- specific expression and function during development. Hence, we were interested in assessing the functional role of CAs in filamentous ascomycetes.

Here, we present a genetic analysis of three β-CAs from the model organism Sordaria macrospora, which has been intensively used to study sexual reproduction and fruiting-body (perithecium) differentiation [17–22]. S. macrospora is a coprophilous fungus that lives on the dung of herbivores. It is a close relative of Neurospora crassa, but is in contrast to the heterothallic (self-sterile) N. crassa, homothallic (self-fertile) [23,24]. Under laboratory conditions, S. macrospora completes its life cycle within seven days and produces pear-shaped fruiting-bodies with asci and ascospores. A genetic advantage of S. macrospora is that recessive mutations can directly be tested for impairment in fruiting-body development, without the need of crossing strains of opposite mating-types [25].

In this study we identified three genes encoding β-CAs (cas1, cas2 and cas3) in S. macrospora. Localization studies revealed that CAS2 was translocated into the mitochondria, while CAS1 and CAS3 are cytoplasmic. A genetic analysis of knock-out strains, Δcas1, Δcas2, and Δcas3, demonstrate that CAS2 may be the major CA in S. macrospora, involved in vegetative growth and ascospore germination.

Developmental defects were severely increased in a Δcas1/2 double deletion strain, which was completely sterile and produced a reduced number of empty fruiting-bodies only after 21 days of growth. The defect in fruiting-body development, but not the decrease in germination efficiency, could be complemented by elevated CO2 (5%) concentrations. Interestingly, we were also able to demonstrate that a decrease in germination efficiency, could be complemented by carbonic anhydrase activity. The defect in fruiting-body development, but not the Δcas1/2 strain, which was completely sterile and produced pear-shaped fruiting-bodies with asci and ascospores. A genetic advantage of S. macrospora is that recessive mutations can directly be tested for impairment in fruiting-body development, without the need of crossing strains of opposite mating-types [25].

In this study we identified three genes encoding β-CAs (cas1, cas2 and cas3) in S. macrospora. Localization studies revealed that CAS2 was translocated into the mitochondria, while CAS1 and CAS3 are cytoplasmic. A genetic analysis of knock-out strains, Δcas1, Δcas2, and Δcas3, demonstrate that CAS2 may be the major CA in S. macrospora, involved in vegetative growth and ascospore germination.

Developmental defects were severely increased in a Δcas1/2 double deletion strain, which was completely sterile and produced a reduced number of empty fruiting-bodies only after 21 days of growth. The defect in fruiting-body development, but not the decrease in germination efficiency, could be complemented by elevated CO2 (5%) concentrations. Interestingly, we were also able to complement the delay in fruiting-body formation in the Δcas1/2 strain by overexpressing either cas1, cas3, or a cytoplasmically-located cas2 variant, indicating that the function of CAs is redundant for fruiting-body formation in S. macrospora.

Results

Identification of three carbonic anhydrase genes in S. macrospora

S. macrospora and N. crassa are closely related and share a high degree of nucleic acid identity within open reading frames. Therefore, the N. crassa database is often used to identify homologous genes in S. macrospora, whose genome is not yet sequenced [24]. Database searches with the S. cerevisiae CA NCE103p revealed the presence of two putative CA genes in the genome of N. crassa. The enzymes are encoded by the ORFs NCU04778.3 and NCU08133.3. A BLASTP search using the catalytic region of the putative CA NCU08133.3 resulted in the identification of a third CA encoded by NCU91103.3. Using heterologous primers designed from the N. crassa CA ORFs, we succeeded in isolating three putative CA genes from S. macrospora. Sequence alignments with several fungal CAs assigned the three S. macrospora CAs to the β-class. Three highly conserved residues function as zinc-binding ligands (Figure 1A). Based on these homologies, the three S. macrospora genes were termed cas1 (carbonic anhydrase of Sordaria macrospora; NCU04778.3 homologue, accession number FM878639), cas2 (NCU08133.3 homologue, accession number FM878640) and cas3 (NCU01103.3 homologue, accession number FM878641). The conserved β-CA domain is located in the central part of CAS1 and CAS3, and in the C terminus of CAS2 (Figure 1A). In cas3, the coding region of the conserved domain is interrupted by an intron. To confirm the splicing of predicted introns, cDNAs of the three cas genes were generated by RT-PCR with primer pairs cynT1-pQF/cynT1-r; cynT2-pQF/cynT2-r and cynT3-pQF/cynT3-r, respectively. We confirmed the presence of one predicted intron within the 770 bp coding region of the cas3 gene (65 bp), and two predicted introns within the 733 bp coding region of the cas3 gene (103 and 105 bp). Sequence analysis of the 855 bp cas2 coding region revealed that this gene is not interrupted by an intron. The deduced peptide sequences of the putative S. macrospora cas1, cas2 and cas3 cDNAs encode for 234, 284 and 174 aa proteins with predicted molecular masses of 25.1, 32.1 and 19.1 kDa, respectively, and theoretical isoelectric points of 6.16, 8.15 and 5.16. cDNAs of the three S. macrospora cas genes fused to either RGS-His-tag or GST-tag were heterologously expressed in E. coli, SDS-PAGE and Western blot analyses with anti-RGS-His and anti-GST antibodies revealed protein bands with apparent molecular weights consistent with the calculated molecular weights of the S. macrospora CAs (data not shown).

When comparing the entire amino acid (aa) sequence of the β-CAs of S. macrospora and N. crassa, all enzymes shared a high degree of aa sequence similarities. BLASTP analysis of the identified proteins also revealed high degrees of sequence similarities to a recently characterized fungal β-CA from the pathogenic yeast C. albicans, two CAs of the pathogenic basidiomycete Cryptococcus neoformans as well as to non-characterized homologues from other ascomycetes and the zygomycete Rhizopus oryzae (Figure 1A+B).

Expression of cas genes in S. macrospora

The CA Nce103p of S. cerevisiae is induced by low CO2 levels, whereas C. albicans Nce103p is constitutively expressed [12,13]. To understand the functions of CAs in the filamentous fungus S. macrospora, we investigated the transcriptional expression of cas1, cas2 and cas3 in the wild type grown in ambient air or with exposure to 5% CO2, by real time PCR. In all experiments, cas1 mRNA levels were significantly lower under ambient air conditions after three days of development and up-regulated after five days whereas cas2 transcript was not significantly regulated under the conditions investigated. Expression of cas3 was significantly down-regulated under ambient air conditions after three days of growth and not regulated by different CO2 conditions after five and seven days (Figure 2).

Subcellular distribution of carbonic anhydrases

To gain a better understanding of the cellular localization of CAs in S. macrospora, we performed fluorescence microscopy experiments and transformed plasmids pCAS1-GFP, pCAS2-GFP and pCAS3-GFP carrying cas-egfp fusion genes under control of the strong constitutive gpd promoter of A. nidulans, into the S. macrospora wild type. According to an in silico TargetP and MitoProt II - v1.101 analysis, CAS2 was predicted to be located in the mitochondria, whereas CAS1 and CAS3 were predicted to be cytoplasmic. Microscopic analysis of the transformants revealed that CAS1-EGFP and CAS3-EGFP proteins were uniformly distributed throughout the cytoplasm of fast-growing, young hyphae, as well as old hyphae. Fluorescence was not concentrated in any organelle and was absent from vacuoles (Figure 3). The CAS2-EGFP fusion protein clearly localized to tubular structures throughout young hyphae and hyphal tips (Figure 4A). Tubular or round-shaped structures are typical for mitochondria in fungal...
hyphae and a high density of mitochondria can be often found at hyphal tips [26].

To confirm the mitochondrial localization of CAS2-EGFP, we stained the hyphae with Mito-Tracker or, as a negative control; we performed a co-transformation of pCAS2-GFP and pHsRED-SKL. Plasmid pHsRED-SKL encodes a DsRED with a classical C-terminal PTS1 signal for peroxisomal matrix import [27,28].

The CAS2-EGFP fusion protein clearly co-localized with the

Figure 1. Multiple sequence alignment of the zinc coordinating region from fungal β-class CAs. (A) ClustalX alignment was created using the following sequences: Sma1 [S. macrospora, Accession No. FM878639], Sma2 [FM878640], Sma3 [FM878641], Ncr1 [Neurospora crassa, Q75631], Ncr2 [Q75438], Ncr3 [Q8XOH0], Afu1 [Aspergillus fumigatus, Q4WQ18], Afu2 [Q4WQ18], Afu3 [Q4WQ18], Cne1 [Cryptococcus neoformans, Q34V7], Cne2 [Q30E79], Sce [Saccharomyces cerevisiae, P5615], Spo [Schizosaccharomyces pombe, O94255] and Ror [Rhizopus oryzae, RO8G_10751.1]. Conserved amino acids important for Zn2+ coordination are marked by an asterisk. Identical amino acids, which are conserved in all proteins, are shaded in black; residues conserved in at least 13 of 15 sequences are shaded in dark grey and residues conserved in at least ten sequences are shaded in light grey. Arrows indicate S. macrospora cas genes with introns are given as grey boxes. The dashed box marks the region encoding the part of the protein which was used for the alignment at the top. The coding region for the mitochondrial target sequence of CAS2 is indicated as black box. (B) Amino acid identity in % is given for all sequences in pair-wise comparisons. Percentages given are based on amino acid comparison of the conserved region shown in (A).

doi:10.1371/journal.pone.0005177.g001
Mito-Tracker, indicating that CAS2 is a mitochondrial protein (Figure 4A). No co-localization was observed with DsRED-SKL. Co-transformants carrying pDsRED-SKL and pCAS2-GFP displayed a punctuated red fluorescence pattern of the peroxisomes that did not overlap with the green fluorescent tubular structures of mitochondria (Figure 4B). Some yellow patches in the merge might be because the fluorescent proteins, which were present in the specific organelles, were located in different planes. Overall, the subcellular distribution of CAS2-EGFP was clearly different from that of the DsRED-SKL marker protein.

To address the question of whether the predicted mitochondrial target sequence is sufficient for translocation into mitochondria, we constructed plasmids pMito-DsRED and pMito-CAS2-DsRED. According to MitoProt II-v1.101 prediction program [29], CAS2 should be targeted to mitochondria with a probability of 99%. The N-terminus is predicted to be cleaved between residue His59 and Ser60. Hence, we fused the coding region of the 60 N-terminal residues of CAS2 to DsRed and analyzed the subcellular distribution of the fusion protein after transformation of pMito-DsRED into the S. macrospora wild type. As expected, this protein also localized to tubular or punctate structures, indicating its transport into mitochondria (Figure 4C). Furthermore the expression of cas2 without its mitochondrial target sequence, encoded by plasmid pAMito-CAS2-DsRED, led to fluorescence appearing uniformly distributed throughout the cytoplasm of the hyphae (Figure 4C). This result showed that the N-terminal 60 amino acid residues of CAS2 were essential and sufficient for correct localization of the CA to mitochondria.

Generation of cas knock-out strains

For a functional characterization of the S. macrospora CAs, we generated Δcas1, Δcas2 and Δcas3 deletion strains by gene replacement. Linear fragments containing flanking regions of the desired S. macrospora cas gene interrupted by the hph-cassette were amplified by PCR from plasmids pCAS1-KO, pCAS2-KO and pCAS3-KO. In the Δcas1 and Δcas2 knock-out constructs, the selectable marker cassette replaced the central part of the gene, including all functional domains, while in the Δcas3 knock-out construct, the complete 5’ region of the gene including all regions encoding putative functional domains was deleted (Figure S1A). Linear knock-out fragments were transformed into the S. macrospora

![Figure 2. Expression analyses of cas genes. Real time PCR was performed with total RNA isolated from S. macrospora wild type grown at 27°C in liquid BMM for three, five and seven days under ambient air conditions or 5% CO2. Comparisons are given as logarithmic values of the ambient air/5% CO2 ratios and are mean expression ratios from two independent biological replicates, each done in triplicate. For normalization, transcript levels of the SSUrRNA were calculated as described in Material and Methods. Asterisk indicate significance according to REST [70]. doi:10.1371/journal.pone.0005177.g002](image)

![Figure 3. Fluorescence microscopic analyses of S. macrospora wild type strains expressing cas1-egfp or cas3-egfp. The images illustrate the fluorescence of CAS1-EGFP and CAS3-EGFP caused by the transformation of plasmids pGFP-CAS1 or pGFP-CAS3, respectively. Transformants were analyzed after growth for two days on solid SWG medium supplemented with hygromycin. DIC: differential interference contrast. Scale bar indicates 20 μm. doi:10.1371/journal.pone.0005177.g003](image)
Figure 4. Localization of the carbonic anhydrase CAS2. (A) Fluorescence microscopic analysis of a *S. macrospora* wild type strain carrying plasmid pGFP-CAS2. Expression of full-length cas2-egfp leads to EGFP import into mitochondria, visible as tubular structures. CAS2-EGFP co-localizes to mitochondria stained with Mito-Tracker (Invitrogen, Germany) (B) Co-transformation of pGFP-CAS2 and pDsRED-SKL results in clearly distinguishable signals from green fluorescent mitochondria and red peroxisomes. (C) Fluorescence microscopic analysis of a *S. macrospora* wild type strain carrying either pMito-DsRED (N-terminal mitochondrial signal sequence of cas2 fused to DsRed) or pΔMito-CAS-DsRED (cas2 gene without the signal sequence fused to DsRed). DIC: differential interference contrast. Scale bar indicates 20 μm.
doi:10.1371/journal.pone.0005177.g004
Δku70 strain, which is impaired in the repair of DNA double-strand breaks and has been shown to be an ideal recipient strain for gene targeting experiments [30]. Since S. macrospora primary transformants are often heterokaryotic, we isolated single spore isolates to segregate the wild-type and mutant alleles of cas genes in a Δku70 background. Strains that were homokaryotic for the desired cas deletion and carried the Δku70-deletion were further crossed against color spore mutant fus1-1. By conventional genetic analyses, we succeeded in isolating hygromycin resistant spores without the Δku70 (nourseothricin-resistant) background, indicating that cas genes are not coupled to ku70 and that none of the cas genes is an essential gene. PCR amplification with primer pairs specific for external flanking regions in combination with primers specific for the integrated hph-cassette confirmed the integration of the hygromycin-resistant cassette at the desired cas-locus (Figure S1). By conventional crossing of the single mutant strains, we generated all three possible double knock-out mutants. The genotype of double knock-outs was confirmed by PCR (Figure S1B). In addition, using primer pairs cynT1-GFP-f/cynT1-r, cynT2-GFP-f/cynT2-r and cynT3-GFP-f/cynT3-r, we verified by RT-PCR that transcripts of the deleted genes were absent in all single and double knock-out mutant strains (Figure 5).

**Phenotypic characterization of carbonic anhydrase mutants**

The characterization of Δcas1 and Δcas3 single knock-out mutants revealed no obvious phenotypic changes compared to the wild type. Almost no alterations in vegetative growth behavior were detectable, when measuring the growth rate under ambient air conditions (Figure 6). Only Δcas1 showed a 14% elevated growth velocity at 5% CO2. Furthermore, neither Δcas1 nor Δcas3 displayed irregularities in mycelial morphology or the formation of sexual reproductive structures (Figure 7). When compared to the wild type or single knock-out strains, the Δcas1/3 double deletion strain also showed no changes in vegetative morphology or reproductive structures. Interestingly, similar to Δcas1, Δcas3 and Δcas1/3 sexual reproduction of the S. macrospora wild type was not affected by high CO2 concentrations, as recently reported for the basidiomycete C. neoformans [16].

**Figure 5. RT-PCR analyses of wild type, single and double knock-out strains to confirm gene deletions.** The coding sequence of the cas genes were amplified with primer combinations cynT1-GFP-f/cynT1-r, cynT2-GFP-f/cynT2-r and cynT3-GFP-f/cynT3-r, respectively. Equal concentrations of DNA were loaded in each lane. Sizes of amplicons from genomic DNA control (gDNA) and cDNA is indicated aside. Control: negative control, without DNA template. doi:10.1371/journal.pone.0005177.g005

**Figure 6. Vegetative growth rate of S. macrospora wild type and cas mutant strains in ambient air and at elevated CO2.** Strains were grown for 3 days on solid BMM either in ambient air (black bars) or at 5% CO2 (white bars). Growth rate of wild type in ambient air was defined as 100%. Growth rates shown are averages from nine measurements of three independent experiments. Error bars are given as indicated. doi:10.1371/journal.pone.0005177.g006
In contrast to Δcas1 and Δcas3, the mycelial growth rate of Δcas2 was reduced by 37% under ambient air conditions. This growth defect was fully restored by incubating the mutant strain under elevated CO2 conditions (Figure 6). The vegetative growth defect of Δcas2 is severely increased when mycelium is grown from germinated single spores (Δcas2_ssi: single spore isolate). BMM plate supplied with 0.5% sodium acetate was either inoculated with Δcas2 mutant strain or with an isolated single ascospore of Δcas2. Plates were incubated with or without CO2 for three days and images were taken from young mycelia. After prolonged incubation, Δcas2_ssi develops less fruiting-bodies under ambient air than a consecutively transferred mutant strain after nine days of growth. The defect is complemented at 5% CO2. Scale bar indicates 1 mm (C) Double knock-out mutant strain Δcas1/2 produced only few perithecia after 21 days of growth. Fruiting-bodies exhibit a wild-type phenotype, but are accumulated at several places on the plate. No mature ascospores were discharged from perithecia developed from Δcas1/2 strain.

doi:10.1371/journal.pone.0005177.g007

Figure 7. Fruiting-body developmental of wild type, single and double knock-out strains. (A) Strains were grown on BMM medium for 7 days under natural environmental conditions (low CO2) or at high CO2 concentrations (5%). No morphological changes were observed in single and double Δcas1/3 and Δcas2/3 knock-out strains, respectively. Mature perithecia of Δcas1/2 strain in ambient air were not detectable after 7 days. Elevated CO2 concentrations complemented this phenotype. Scale bar indicates 500 μm (B) CO2/HCO3− accumulates in the fungal cell. Growth defect of Δcas2 is severely increased when mycelium is grown from germinated single spores (Δcas2_ssi: single spore isolate). BMM plate supplied with 0.5% sodium acetate was either inoculated with Δcas2 mutant strain or with an isolated single ascospore of Δcas2. Plates were incubated with or without CO2 for three days and images were taken from young mycelia. After prolonged incubation, Δcas2_ssi develops less fruiting-bodies under ambient air than a consecutively transferred mutant strain after nine days of growth. The defect is complemented at 5% CO2. Scale bar indicates a 1 mm (C) Double knock-out mutant strain Δcas1/2 produced only few perithecia after 21 days of growth. Fruiting-bodies exhibit a wild-type phenotype, but are accumulated at several places on the plate. No mature ascospores were discharged from perithecia developed from Δcas1/2 strain.
number of mature ascospores. While perithecia produced by S. macrospora wild type contain 105.2 ± 19.9 asci, only 21.1 ± 12.0 asci per perithegium were produced in the Δcas1/2 mutant strain.

RT-PCR analyses of cas gene expression in single and double knock-out strains revealed that cas1 was significantly down-regulated in Δcas3 after five days of growth under ambient air conditions, but not in Δcas2. A slight down-regulation of cas1 was also observed in Δcas2/3. The cas2 transcript was also significantly reduced in the Δcas3 knockout strain, whereas a de-regulation of the transcriptional cas2 expression was measured in the Δcas1 and Δcas1/3 strains. In contrast, the cas3 transcript showed no up- or down-regulation in Δcas1, Δcas2 or Δcas1/2 after five days of growth (data not shown).

Since CA mutants of S. cerevisiae are more sensitive to several stress-inducing conditions [14], we tested growth and sexual development of wild type and mutant strains on stress inducing media. Neither hypersomotic media, nor H2O2, which induces stress-inducing conditions [14], we tested growth and sexual growth (data not shown).

D. melanogaster are observed when spores were germinated at 5% CO2 (data not shown). Decreased growth velocity and hyphal density under ambient air conditions, and to 53% when spores were germinated under ambient air conditions, but not in Δcas2. A slight down-regulation of cas1 was also observed in Δcas2/3. The cas2 transcript was also significantly reduced in the Δcas3 knockout strain, whereas a de-regulation of the transcriptional cas2 expression was measured in the Δcas1 and Δcas1/3 strains. In contrast, the cas3 transcript showed no up- or down-regulation in Δcas1, Δcas2 or Δcas1/2 after five days of growth (data not shown).

Since CA mutants of S. cerevisiae are more sensitive to several stress-inducing conditions [14], we tested growth and sexual development of wild type and mutant strains on stress inducing media. Neither hypersomotic media, nor H2O2, which induces oxidative stress, nor sodium dodecylsulphate, which induces cell-wall damage, led to marked phenotypic differences between wild type and mutant strains (data not shown).

**CAS2 is needed for proper ascospore germination**

To assess the role of β-CAs during ascospore germination, we investigated the ascospore-germination rate of single and double mutants (Figure 8a). Under ambient air conditions, the germination efficiency of Δcas2 and Δcas2/3 decreased by about 50% while all other mutants exhibited germination efficiencies similar to wild type. Interestingly, the spore germination defect of Δcas2 and Δcas2/3 was not complemented by 5% CO2 concentration. The Δcas1/2 strain produced ascospores only when grown at 5% CO2, therefore we isolated ascospores from fruiting-bodies developed under this condition. These exhibited a germination defect similar to the Δcas2 single and the Δcas2/3 double knock-out strain. Compared to wild type, the germination efficiency of Δcas1/2 decreased to 58% when spores were germinated under ambient air conditions, and to 53% when spores were germinated under elevated CO2 (Figure 8a). As described for Δcas2 and Δcas2/3, mycelia derived from ascospores of Δcas1/2 showed a decreased growth velocity and hyphal density under ambient air conditions (Figure 8b). In contrast, no defects in mycelial growth were observed when spores were germinated at 5% CO2 (data not shown). The severe growth defect of Δcas2 single and double knockout mutant strains was also seen when ascospore germination was investigated under the microscope (Figure 8c). While wild type, Δcas1 and Δcas3 ascospores form non-vacuolated hyphae, germings of the Δcas2 mutant exhibited multiple vacuoles.

**Complementation of Δcas1/2-fus strain by overexpression of cas1, cas2 and cas3**

To address the question whether the overexpression of one of the β-CAs of S. macrospora can complement the severe developmental defects in the Δcas1/2 double mutant, we transformed this strain with overexpression constructs of cas1, cas2, cas3 and cas2-CTG. The latter construct contains a variant of cas2 that lacks the mitochondrial target sequence and starts with a CTG codon instead of an ATG. This construct was made because the N. crassa homologue of cas2, ORF NCU08133.3, is annotated with a CTG start codon. The CTG is conserved in the predicted S. macrospora cas2 ORF, but if the homologous CTG was used as a start codon, the CAS2 protein would lack 35 N-terminal residues and have a truncated mitochondrial target sequence.

In all transformants, the cas gene was ectopically integrated and under control of the strong constitutive gpd promoter of A. nidulans. Overexpression of an ectopic cas2 did almost fully complement the mutant phenotype of Δcas1/2 (strain Δcas1/2-gpd-cas2<sup>ext</sup>). Mature perithecia with a wild type-like number of asci developed, and mycelial colonies exhibited a wild type-like hyphal morphology as well as a wild type-like colony density. However, the number of germinated ascospores (63%) was only slightly increased in comparison to the mutant strain (53%) (Figure 9). Thus, it seems that only transcription from the endogenous cas2 promoter led to a complementation of all developmental defects.

Overexpression of cas2-CTG complemented the defects of Δcas1/2 even worse. Transformants carrying pGPD-CAS2-CTG develop fruiting-bodies, but discharge no mature ascospores. Cracking of the fruiting-bodies revealed the presence of only undifferentiated asci (Figure 9B). This result indicated that S. macrospora is able to translate a cas2-CTG ORF, but that the truncated CAS2 is not able to fully complement sterility of Δcas1/2. To investigate whether full complementation depends on translocation of CAS2 into the mitochondria, we tested whether the truncated mitochondrial target sequence encoded by pCTG-CAS2-DSRED can target DSRED into mitochondria. For this experiment, plasmid pCTG-CAS2-DSRED was transformed into a S. macrospora wild type strain. As shown in Figure S2, the CTG-CAS2-DSRED fusion construct was mainly distributed throughout the cytoplasm of the hyphae, and in only a few hyphal tips were fluorescent tubular structures detectable, indicating that the construct was inefficiently targeted to mitochondria. This result shows that both CAS2 variants may be produced in vivo, but that only the full length version of the cas2 is able to fulfill its complete function.

In contrast to the truncated version of cas2, overexpression of cas1 lead to the formation of fertile fruiting-bodies with asci and mature ascospores under ambient air, whereas overexpression of cas3 resulted in a reduced number of asci and ascospores. Moreover, these generated only after a prolonged incubation of 18 days (Figure 9B). Determination of the ascospore germination rate of Δcas1/2 strains overexpressing cas1 or cas3 resulted in a decreased germination efficiency, which showed that the germination defect was neither complemented by cas1 nor by cas3 (Figure 9C).

**Complementation of Δcas1/2-fus strain by the addition of fatty acids**

Since HCO<sub>3</sub><sup>-</sup> is an important key substrate for decarboxylating enzymes involved in various biosynthetic pathways, it has been hypothesized that the lack of CA activity might affect the acetyl-CoA carboxylase dependent biosynthesis of fatty acids [16,31]. Therefore, we tried to complement the sterile mutant strain Δcas1/2-fus by the addition of a mixture of fatty acids (palmitic, myristic and stearic acid and 1% Tween 80 as surfactant) in different concentrations. Corn meal medium supplemented with 15 or 30 mg/l of fatty acids completely restored the vegetative growth defect of Δcas1/2-fus, whereas 60 mg/l leads to an increased production of aerial hyphae. Sexual development was slightly reduced, because perithecia were formed earlier in contrast to ambient air conditions without fatty acids and they were equally distributed all over the plate (data not shown). Nevertheless, no mature ascospores were produced by the Δcas1/2-fus deletion strain after 14 days of growth under the conditions tested. The Δcas1/2-fus-gpd-cas2<sup>ext</sup> and wt were not influenced by the addition of fatty acids.

**Discussion**

Although the main function of CAs in all organisms is to accomplish the regulation of CO2/HCO<sub>3</sub><sup>-</sup> homeostasis little is known about how CO2 is metabolized and transported in mycelial
Figure 8. Germination rate of S. macrospora wild type and cas mutant strains. (A) Ascospores were isolated from strains grown in ambient air. Only strain Δcas1/2 grew at 5% CO₂ (marked by an asterisk); because it develops mature perithecia with asci and ascospores only at elevated CO₂ levels (Figure 7). 100 single spores from 10 different perithecia of wild type and mutant strains were isolated and plated on BMM medium with 0.5% sodium acetate. Ascospores were incubated in ambient air or at 5% CO₂. Germinated spores were counted after 2–5 days of incubation. The percentage of germination efficiency was determined for each strain. (B) Mycelia from germinated spores of Δcas2, Δcas1/2 and Δcas2/3 are affected in growth velocity and hyphal density. Ten spores were plated from each strain and images were taken after two days of growth. (C) Germlings of Δcas2 mutants are highly vacuolated. Spores were plated on glass slides overlaid with a thin layer of BMM supplied with 0.5% sodium acetate and incubated for one day. Vacuoles in hyphae of Δcas2 are marked by white arrows. Scale bar indicates 50 μm.

doi:10.1371/journal.pone.0005177.g008
fungi. Despite being well-characterized in prokaryotes, plants and animals, CAs have been analyzed in only three fungal species [12,13,16,32,33]. The data presented here demonstrate that three β-CAs are encoded within the genome of the filamentous ascomycete *S. macrospora*. To the best of our knowledge, this is the first genetic analysis on the function of β-CAs in filamentous ascomycetes. *S. macrospora* has three β-CAs The proteins encoded by *cas1*, *cas2* and *cas3* showed a significant degree of sequence identity when compared to known β-CAs of bacterial and plant origin. The crystal structures of β-CAs from plants, bacteria, the archaeon *Methanobacterium thermoautotrophicum* and *C. neoformans* revealed that the active site zinc is coordinated by two conserved cysteines and one conserved histidine [32,34–38]. In addition to these three residues, an aspartic acid and an arginine are also structurally conserved in every β-CA sequence known [4]. These two residues presumably assist in a number of catalytic steps, including substrate binding, proton shuffling and product release, or act as the fourth zinc ligand [32,35,39]. Sequence alignment demonstrated that these five residues are also conserved in CAS1 and CAS2 (Figure 1A). Based on other key active site residues, β-CAs can be further divided into “plant”-type β-CAs, including all known sequences of plants and of some bacteria and into “cab”-type enzymes of *M. thermoautotrophicum*, *Mycobacterium tuberculosis* and *Bacillus subtilis* and other bacteria and archaea [36,40]. Active site residues corresponding to key active site residues Gln-151, Ser-161, Ser-163, Phe-179, and Tyr-205 of the *Pisum sativum* β-CA [36] are conserved in CAS1 and CAS2. Only Val-184 is replaced by Ile in CAS1 as well as in CAS2. However, a survey of other plant β-CA sequences indicated that either Ile or Val is found at this position [36]. Thus, CAS1 and

**Figure 9. Complementation of the mutant phenotype of Δcas1/2 by ectopically integrated cas1, cas2 and cas3, respectively.** (A) Morphological characterization of Δcas1/2 strain complemented with different *cas* genes. The images show vegetative hyphae in case of Δcas1/2 strain grown in ambient air and fruiting-body development when grown at 5% CO₂ or complemented with *cas1*, *cas2* or *cas3*. The gene *cas2* starts either with an ATG or CTG start codon; all constructs are driven by the constitutive gpd promoter of *A. nidulans*. Scale bar indicates 1 mm (B) At ambient air condition, mature ascospores were produced and discharged, only when Δcas1/2 is grown at elevated CO₂-levels or complemented either with *cas1* or ATG-*cas2*. After a prolonged incubation time of 18 days, complementation with *cas3* also results in discharge of few mature ascospores. Scale bar indicates 50 μm (C) Germination rate and restoration of vegetative growth defects is partially complemented by constitutively expressed *cas2* starting with ATG (+), but not by *cas1*, *cas2*-CTG and *cas3* (—). Asterisk indicates that spores were isolated from a strain grown at 5% CO₂, because no ascospores were developed in ambient air. doi:10.1371/journal.pone.0005177.g009
CAS2 belong to "plant"-type β-CAs. Similar to "cab"-type β-CAs, in CAS3, the amino acid residues corresponding to P. sativum Gln-151 are substituted with Pro, Ser-161 with Met, Ser-163 with Ala, Phe-179 with His, Val-184 with Ala and Tyr-205 with Val. Therefore, CAS3 can be classified as a "cab"-type β-CA [36].

A further key difference between CAS1, CAS2 and CAS3 is the N-terminal extension of CAS2, which encodes for a mitochondrion target sequence. This is not a special feature of CAS2 from S. macrospora. CAs located in the mitochondria have been identified in algae, plants and animals [6,8,41–43]. A careful analysis of the complete genome sequences of other filamentous ascomycetes revealed that many of them encode one isoform with a mitochondrial target sequence. These sequences differ in length and exhibit very low sequence identity to the target sequence of S. macrospora [44]. The CAS2 homologue of N. crassa NCU08133.3 has been annotated without a mitochondrial target sequence and has been predicted to start with a CTG codon, a codon also conserved in S. macrospora CAS2. TargetP reliability indicated a lower likelihood of transport of the predicted protein NCU08133.3 into mitochondria. Since expression of a S. macrospora CTG variant of cas2 is not able to fully complement the phenotype of a Δcas1/2 deletion strain, we speculate that the genomic annotation of the N. crassa homologue is not correct. Re-annotation of the N. crassa NCU08133.3 by extending the first exon would lead to a protein with a mitochondrial target sequence [44].

Transcriptional regulation of β-CA genes in response to CO2 differs

Three β-CA genes are simultaneously expressed in S. macrospora. Cas1 and cas3 are significantly regulated by CO2 on day three and five of development, whereas cas2 seems to be constitutively expressed during development (Figure 2). After three days of growth mRNA levels of cas1 and cas3 are significantly lower under ambient air conditions than under elevated CO2. While cas1 becomes up-regulated on day five under ambient air conditions, cas3 is no longer regulated by CO2. This result might implicate a specific function for CAS1 under CO2-limited conditions with regard to the production of sexual structures. Examination of the transcriptional regulation of fungal β-CA genes revealed that Ne103 of S. cerevisiae is up-regulated at low CO2 levels, whereas expression of C. albicans Ne103 and C. neoformans CA11, encoding the major CA of C. neoformans, are not regulated by the CO2 concentration [12,16,45,46].

Evidence for a possible dependency between cas1 and cas2 is their transcriptional regulation in different cas knockout backgrounds. Whereas cas1 is not significantly regulated in a Δcas2 background, cas2 is completely de-regulated in Δcas1 and Δcas1/3 deletion strains. Although the Δcas1 strains do not exhibit any morphological phenotype, it seems that cas2 must be antagonistic to cas1.

Similar to cas1, cas3 is only down-regulated at day three of sexual development but constitutively expressed under both conditions at later stages of development. However, in contrast to Δcas1 and Δcas2, growth of a Δcas3 strain is not influenced by elevated CO2 conditions (Figure 6). Interestingly, the transcriptional regulation is also not influenced by the presence or absence of the other two CA genes, indicating that cas3 might be involved in another bicarbonate-dependent pathway that is independent from CO2 concentrations under the conditions tested.

CAS2 is targeted to mitochondria and is required for germination and vegetative growth

In many eukaryotes encoding multiple CAs, different isoforms of the enzyme have been shown to be distributed in different tissues and cell compartments [3]. When fused to eGFP, the product of cas2 was exclusively targeted to the mitochondria, thus confirming the in silico prediction of a classical mitochondrial import sequence by TargetP and MitoProt II. While β- and γ-CAs have been reported to become targeted to mitochondria in mammals, plants and algae [3,6,8,42], this is the first time that a fungal β-CA has been shown to be located exclusively to mitochondria.

No rigorous phenotypic changes related to sexual development were observed in CA single knockout strains. Only the Δcas2 mutant displayed a weak delay in vegetative growth, and this defect was complemented by the elevation of the CO2 concentration. Deletion of the cas2 gene may lead to a decrease in the mitochondrial bicarbonate concentration under ambient air conditions.

In algae mitochondrial CAs are involved in the inorganic carbon-concentrating mechanism, supporting anaerobic β-carboxylation and they are proposed to act as pH stabilizers [42,47,48]. Raven [49] hypothesized that if there is a HCO3− channel in the inner mitochondrial membrane, almost all of the inorganic carbon would leave the mitochondria as HCO3−, thus limiting the potential for CO2 leakage through the plasmalemma. Similarly, mitochondrial β-CA CAS2 of S. macrospora may contribute to the cytoplasmic bicarbonate pool required for carboxylating enzymes relevant to many metabolic processes. Among these, acetyl-CoA carboxylase uses bicarbonate to produce malonyl-CoA for fatty acid synthesis. Indeed, it was shown recently that biosynthesis of fatty acids is affected by deletion of fungal CA genes. In the basidiomycete C. neoformans, the phenotype of a Δcas2 knockout was partially complemented by the addition of the fatty acid palmitate [16]. The growth defect of a S. cerevisiae Δne103 strain under atmospheric pressure can be complemented only when end-products of bicarbonate-dependent metabolic pathways are supplied to the growth medium [31]. Complementation assays with a mixture of fatty acids also restored the vegetative growth defect of CA-deletion mutants in S. macrospora.

In addition to the vegetative growth defect, the Δcas2 strain also displays a considerable decrease in germination efficiency, which was also obvious in Δcas2/3 and Δcas1/2 double deletion strains. S. macrospora appears to need a certain HCO3− concentration in the mitochondria for efficient ascospore germination. In contrast to vegetative growth defects, this defect of the Δcas2 mutant is not complemented by an elevated CO2 concentration. Therefore, we conclude that CAS2-mediated CO2/HCO3− homeostasis in mitochondria plays an important role in spore germination and in vegetative growth. Interestingly, the vegetative growth defect increased when colonies were derived from germinated spores.

The mycelium derived from a germinating Δcas2 ascospore was composed of thin vacuolated hyphae indicating that CAS2 is responsible for the HCO3− accumulation effect in the Δcas2 mutant. Δcas3 exhibited no obvious morphological phenotype under any conditions tested. Furthermore, a Δcas2/3 double knock-out strain behaved similarly to the Δcas2 mutant. HCO3− produced by CAS3 might be the co-factor for a reaction that is not linked to sexual reproduction, vegetative growth or
ascospore germination. In this context, it would be interesting to investigate a possible connection between CA mediated HCO_3\(^-\) production and cyanate decomposition, a reaction that in S. macrospora, has been recently shown to be catalyzed by a bicarbonate-dependent cyanase. Deletion of the cyanase-encoding gene cyn1 does not lead to any defects in vegetative growth or fruiting-body development [50]. In E. coli the β-class CA CynT is encoded in an operon together with the cyanase-encoding gene cynX, and the function of the E. coli CA appears to be to prevent HCO_3\(^-\) depletion during cyanate decomposition [51].

Deletion of cas1 and cas2 blocks sexual reproduction

The Δcas1/2 double deletion strain displays a more severe phenotype than the Δcas2 single knockout strain and the Δcas2/5 double knockout strain, indicating that in addition to CAS2, CAS1 is also an active CA. While perithecia produced by S. macrospora wild type contain about 100 asci in ambient air [52], the production of fruiting-bodies and differentiated ascus rosettes is drastically impared in the Δcas1/2 double deletion strain. A small number of fruiting-bodies with only immature asci was produced after 21 days of growth. The defect could be partially complemented by incubation at elevated CO_2 levels, which resulted in the production of fruiting-bodies containing significantly fewer mature ascospores than the wild type (Figure 7 and Figure 9). The phenotype of the Δcas1/2 mutant resembles the S. macrospora developmental mutant per5, which produces only ascus precursors without any mature spores. The per5 mutant has been shown to encode a defective ATP-citrate lyase that is involved in the formation of acetyl-CoA used for the biosynthesis of fatty acids [53]. In contrast to the complementation of the vegetative growth defect, the impaired production of mature asci in the Δcas1/2-fus was not reversed by fatty acids. This result indicates that specific CA activity impacts further biochemical pathways than fatty acid biosynthesis during sexual development. Thus, in 5% CO_2, the small amount of bicarbonate generated through nonenzymatic, spontaneous hydration of CO_2 and/or through the activity of the remaining CAS3 is sufficient to support fruiting-body and ascospore formation. Similarly a Δcas2 mutant of C. neoformans was shown to exhibit a severe sporulation defect [16]. However, it should be taken into account that complementation of Δcas1/2-fus strain by overexpression of cas1, cas2 and cas3 due to the use of a strong heterologous gpdA promoter of A. nidulans could increase some paths and decrease others, consequently affecting the overall phenotype.

These results demonstrated that the protein encoded by cas3 has CA activity, albeit it is probably less active than CAS1 and CAS2. Growth defects caused by the deletion of CA genes in bacteria, have been shown to be rescued by introducing heterologous CA genes [54,55]. The growth defect of a S. cerevisiae CA-mutant strain was complemented by plant, plant chloroplast, human, prokaryotic and fungal CAs [14,32,56,57]. Apparently, in these unicellular organisms, only a small amount of CA activity is sufficient for growth at ambient air conditions, irrespective of its origin, whereas in S. macrospora, mitochondrial targeting of CAS2 seems to also be essential for growth and sexual reproduction.

In summary, complementation assays in combination with phenotypic observations indicate that in S. macrospora cas2 and possibly also cas1 may encode for a major CA involved in fruiting-body and ascospore development, as well as in ascospore germination, while cas3 encodes for a minor CA. This must be confirmed by in vitro activity measurements, however, as recently shown for the C. neoformans and the C. albicans CAs [58]. Furthermore, analysis in C. albicans and C. neoformans revealed a functional link between cAMP signaling and CO_2/HCO_3\(^-\) sensing and showed that bicarbonate directly activates adenyl cyclase [7,13,15]. Similar to the phenotypes presented here, deletion of adenyl cyclase in S. macrospora has recently been shown to affect vegetative growth and sexual development in S. macrospora [19]. In this respect, further examination of the functional connection between CAs and adenyl cyclase in fruiting-body and ascospore formation of filamentous ascomycetes will be of great interest.

Materials and Methods

Strains, media and culture conditions

Cloning and propagation of recombinant plasmids was done under standard conditions with Escherichia coli strain SURE used as a host for plasmid amplification [59]. Genotypes of S. macrospora strains used and generated in this study are provided in Table 1. All strains were cultivated either on solid or in liquid corn meal medium (BMM) or in liquid complete medium (CM) or on solid SWG medium [23], incubated in ambient air or at 5% carbon dioxide in the Incubator C42 (Labotec, Germany). Transformation of S. macrospora was performed as described previously with 20 mg/ml Glucanex 200G (Schliessmann, Germany) to degrade fungal cell walls [53]. The determination of germination rates were conducted by isolating 100 single ascospores. After inoculation for 2–5 days on BMM supplied with 0.5% of sodium acetate, the number of germinated spores was determined. Dry cell weight was measured according to Nolting and Poggeler [60] and growth velocities were either determined according to Nowrousian and Cebula [61], or by measuring the colony radius on agar plates after 1 to 10 days. For complementation assays with fatty acids, 30 mg/l of palmitic, myristic and stearic acid and 1% Tween 80 were added to BMM medium according to Aguilera et al. [31]. Genomic DNA was isolated from cultures grown for 3 days in liquid CM medium; RNA samples for qRT-PCR were isolated from cultures grown for 5 days in liquid CM or BMM.

Sequence analysis

Primers were synthesized at MWG Biotech AG (Ebersberg, Germany). DNA sequencing was performed by the sequencing service of the Department of Biochemistry (Ruhr-University of Bochum, Germany) or by the G2L-sequencing service of the Goettinger Genom Labor (Georg-August University of Gottingen, Germany) or by MWG Biotech Customer Service (Ebersberg, Germany).

Molecular weights and isoelectric points of proteins were calculated with programs from the ExPaSy Proteomics Server (http://www.expasy.org). Protein sequence alignments were performed using the ClustalX program [62]. Protein and nucleotide sequence data of β-CAs from other organisms were obtained from the public databases at NCBI [http://www.ncbi.nlm.nih.gov/entrez/] or by BLAST searches of the complete sequenced genomes at the Broad Institute [http://www.broad.mit.edu/annotation/fungi/fgi/]. Subcellular localization of carbonic anhydrases was predicted with the TargetP program provided on the CBS Prediction Server [http://www.cbs.dtu.dk/services/] and with MitoProt II - v1.101 [29].

Preparation of nucleic acids

Isolation of genomic DNA from S. macrospora was done as described previously [22]. DNA was isolated from mycelial samples, which were three times incubated in liquid nitrogen for 5 sec. Afterwards the frozen sample was supplemented with 1 ml Trizol (Invitrogen) and homogenized two times (2 min, 1000 Hz) using the TissueLyser (Qiagen), followed by centrifugation...
(10 min, 20°C, 12,000 rpm). Then, 1 ml from the supernatant was mixed with 0.2 ml chloroform and centrifuged again. One volume of isopropanol was then incubated at −80°C supplemented with 500 µl from the supernatant. After precipitation (10 min, 20°C, 12,000 rpm), the pellet was washed with 500 µl 75% ethanol (10 min, 20°C, 12,000 rpm) and dried at room temperature. The precipitated RNA was resolved in 100 µl of sterile DNA free water and tested on a gel, before it was used for cDNA synthesis and qPCR analysis.

Quantitative real-time PCR
Reverse transcription was done using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) as described by the manufacturers. Quantitative RT-PCR was performed as described previously [63]. The qPCR MasterMix Plus for SYBR green Kit (Eurogentec, Belgium) was used for real-time PCR performance previously [63]. The qPCR MasterMix Plus for SYBR green Kit (Eurogentec, Belgium) was used for real-time PCR performance previously [63]. The qPCR analysis was done using the Mastercycler eP realplex (Eppendorf, Germany). Primer pair CynT1-RT_f/CynT1-RT_r was used to amplify a 133 bp part of the cas1 gene. Combinations of primers CynT2-RT_f/CynT2-RT_r and CynT3-RT_f/CynT3-RT_r resulted in amplification of 134 bp in case of cas2 and 146 bp in case of cas3. Primers used are given in Table S1. All real-time experiments were performed at least two times in triplicates with independent biological samples. An amplicon derived from the small-subunit rRNA was used as a reference for normalization (primer pair SSU-f/SSU-r) according to Poggeler et al. [63].

Identification and isolation of three genes encoding for carbonic anhydrases
Two different strategies were used to isolate cas genes encoding for putative carbonic anhydrases in S. macrospora. The genes cas1 and cas3 and their flanking sequences were in parts or directly amplified from S. macrospora genomic DNA using heterologous oligonucleotides derived from sequences of homologous N. crassa genes, while an inverse PCR strategy was used in case of the cas2 gene according to Nowroussian et al. [21]. Oligonucleotides used for cloning are given in Table S1. Firstly, primer pair cynT1-a/cynT1-b, whose nucleotide sequences are based on the sequence of the appropriate N. crassa homologue, was used to amplify an internal 593-bp cas1 fragment, followed by the design of S. macrospora specific primers cynT1-c1 and cynT1-d. Primer cynT1-c1 was used in combination with the heterologous primer cynT1-79 (based on the sequence of ACU04779.3 from N. crassa) to amplify a fragment of approximately 2800 bp. Only 689 bp downstream of the cas1 gene were sequenced and used for further analysis. With primer cynT1-d and the heterologous primer

| Strain    | Genotype and Phenotype     | Source                  |
|-----------|---------------------------|-------------------------|
| S17736    | Wild type                 | Lab Collection          |
| S23442    | fus1-1, spore color mutant| Lab Collection          |
| S66001    | Δku70:nat1:hph1           | [30]                    |
| Δcas1     | Δcas1:hph1, single spore isolate, fertile | This study            |
| Δcas1-fus | Δcas1::hph1::fus1-1, single spore isolate, fertile | This study            |
| Δcas2     | Δcas2::hph1, single spore isolate, fertile | This study            |
| Δcas2-fus | Δcas2::hph1::fus1-1, single spore isolate, fertile | This study            |
| Δcas3     | Δcas3::hph1, single spore isolate, fertile | This study            |
| Δcas3-fus | Δcas3::hph1::fus1-1, single spore isolate, fertile | This study            |
| Δcas1/2   | Δcas1::hph1::Δcas2::hph1 , single spore isolate, sterile | This study            |
| Δcas1/2-fus | Δcas1::hph1::Δcas2::hph1::fus1-1, single spore isolate, sterile | This study            |
| Δcas2/3   | Δcas2::hph1::Δcas3::hph1, single spore isolate, fertile | This study            |
| Δcas1/3   | Δcas1::hph1::Δcas3::hph1, single spore isolate, fertile | This study            |
| Δcas1/2-fus::gpd-cas1<sup>txt</sup> | Δcas1::hph1::Δcas2::hph1::fus1-1, ectopic copy of cas1 under control of A. nidulans gpd promoter, nat<sup>1</sup>, single spore isolate, fertile | This study            |
| Δcas1/2-fus::gpd-cas2<sup>txt</sup> | Δcas1::hph1::Δcas2::hph1::fus1-1, ectopic copy of cas2 under control of A. nidulans gpd promoter, nat<sup>1</sup>, single spore isolate, fertile | This study            |
| Δcas1/2-fus::gpd-cas3<sup>txt</sup> | Δcas1::hph1::Δcas2::hph1::fus1-1, ectopic copy of cas3 under control of A. nidulans gpd promoter, nat<sup>1</sup>, single spore isolate, fertile | This study            |
| Δcas1/2-fus::gpd-cas2<sup>txt</sup> | Δcas1::hph1::Δcas2::hph1::fus1-1, ectopic copy of cas2 (deletion of nucleotides 1–108) under control of A. nidulans gpd promoter, nat<sup>1</sup>, primary transformant, blocked in ascosporogenesis | This study            |
| wt-CAS1-GFP | Primary transformant of S17736 with pGFP-CAS1 | This study            |
| wt-CAS2-GFP | Primary transformant of S17736 with pGFP-CAS2 | This study            |
| wt-CAS3-GFP | Primary transformant of S17736 with pGFP-CAS3 | This study            |
| wt-CAS2-GFP::DsRED-SKL | Primary transformant of S17736 with pGFP-CAS2 and pDsRED-SKL | This study            |
| wt-Mito-CAS2-DsRED | Primary transformant of S17736 with pMito-CAS2::DsRED | This study            |
| wt-Mito-DsRED | S17736 transformed with pMito-DsRED, single spore isolate | This study            |
| wt-CTG-Mito-DsRED | S17736 transformed with pCTG-Mito-DsRED, single spore isolate | This study            |

<sup>1</sup> hph<sup>1</sup> – hygromycin resistant, nat<sup>1</sup> – nourseothricin resistant
<sup>2</sup> Department of General and Molecular Botany, Ruhr-University Bochum

*PLOS ONE* | www.plosone.org 13 April 2009 | Volume 4 | Issue 4 | e5177
cytT1-77 (NCU04777.3), a 2,271-bp fragment located upstream of the cas1 gene was amplified and sequenced. All together, a 3,558 bp part of the genomic sequence including the 770-bp cas1 coding region with 5' and 3' regions was sequenced.

The 586-bp sequence of an internal part of cas2 was amplified with the heterologous primer pair cytT2-a/cytT2-b. Since, heterologous oligonucleotides designed according to N. crassa genes flanking the cas2 open reading frame in N. crassa failed to amplify sequences adjacent to cas2 of S. macrospora, we performed an inverse PCR strategy as described previously [21]. In brief: genomic DNA from S. macrospora was digested with restriction endonuclease BspI210I. Afterwards, all fragments were self-ligated using T4-Ligase (Fermentas, Germany) and precipitated. The DNA was then used for inverse PCR with primer pair cytT2-c1/cytT2-d1 with Thermozym-Hotstart Taq-Polymerase (Molzyme, Germany). The resulting PCR fragment was cloned into pDrive cloning vector (Qiagen, Germany) and sequenced by means of primer walking, resulting in a 2,418-bp fragment including the complete 855-bp cas2 open reading frame and adjacent 5' and 3' regions.

In case of cas3, a 3,242-bp fragment with the entire protein coding sequence and 5' and 3' flanking regions were amplified with the heterologous primer pair T3-1102/T3-1104, based on the sequence of the adjacent gene homologues NCU01104.3 and NCU01102.3 of N. crassa. The 2,342-bp fragment was cloned into pDrive cloning vector (Qiagen) and sequenced.

**Construction of fluorescence plasmids**

For construction of cas-egfp fusion constructs under the control of the constitutive A. nidulans gpd promoter, the coding regions of cas2 and cas3 were amplified by PCR with primer pairs cytT2-GFP/I-cytT2-I and cytT3-GFP/I-cytT3-I (Table S1) from S. macrospora genomic DNA. With this PCR, flanking NcoI restriction sites were generated on each end. The fragments were subcloned into pDrive vector (Qiagen, Germany). Since the cas1 gene contains an internal NcoI restriction site, a site directed point mutation was introduced by overlap extension PCR with primer pairs cynT1-GFP/I-cytT2-I/Mut-t and 11-Mut-t/cynT1-r (Table S1). All PCR reactions were accomplished, using HotStarTaq DNA Polymerase (Qiagen, Germany). After sequencing, NcoI restriction resulted in fragments of 773-, 838- and 736-bp, which were cloned into the single NcoI site of egfp containing vector p1783.1 to create plasmids pGFP-CAS1, pGFP-CAS2 and pGFP-CAS3, respectively. Plasmid p1783.1 [64] contains the egfp gene downstream of the NcoI restriction site, resulting in CAs fused to the N terminus of EGFP.

Furthermore, a truncated cas2-DsRed fusion gene was constructed by PCR using primers cytT2-56f/NcoI and cytT2-r (Table S1). The amplification resulted in the deletion of the first 165 bp encoding for 55 amino acids. The resulting sequence was verified by sequencing.

The generation and complementation of S. macrospora knock-out strains

Knock-out constructs for homologous recombination were produced utilizing homologous recombination in yeast as described previously [67]. The 5’- and 3’-regions of the genes cas1, cas2 and cas3 were amplified from genomic DNA of S. macrospora wild type strain using primer pairs cynT1-5f/cynT1-3r, cynT1-3f/cynT1-3r, cynT2-5f/cynT2-3r, cynT2-3f/cynT2-3r, cynT3-5f/cynT3-3r and cynT3-3f/cynT3-3r (Table S1). The PCR reactions were conducted to add specific overhangs to the 5'- and 3'-regions of the respective cas gene, homologous to the hph-cassette and the yeast plasmid pRS426, respectively. The hph-cassette was generated with primers hph-f and hph-r using pCYN1-KO as a template [50]. In each case, three PCR fragments together with the EcoRI/Xhol linearized vector pRS426 [68] were co-transformed into yeast strain Pj69-4A [69], where homologous recombination took place. Transformants were selected on SD plates lacking uracil, afterwards plasmid DNA was isolated with the NucleoSpin® Plasmid Kit (Macherey-Nagel,according to Nolting and Poggeler [20]). To isolate the resulting plasmids pCAS1-KO, pCAS2-KO and pCAS3-KO (Table S2), total DNA was subsequently transformed into E. coli strain SURE and plasmid DNA isolation was carried out. The KO-plasmids were used as templates to amplify the CA-hph cassettes with oligonucleotide pairs cynT1-5f/cynT1-3r, cynT2-5f/cynT2-3r or cynT3-5f/cynT3-3r (Table S2 and Figure S1). To generate the Δcas1, Δcas2 and Δcas3 mutant strains, the resulting PCR amplicons (cas1: 3,619 bp; cas2: 3,071 bp; cas3: 3,258 bp) were transferred into S. macrospora Δku70 which is impaired in heterologous recombination [30]. In each strain the major part of the CA coding region including its putative catalytic domain is substituted against the hph gene (Figure S1). Transformants were screened by PCR with primer pairs which are specific for external flanking regions and the integrated hph-cassette (Table S1, Figure S1), and with a primer pair specific to the external flanking 5’ region (Table S1, Figure S1). Additionally, to prove that mutant strains do not contain the respective wild type cas gene, primer pairs matching the substituted coding region of the cas gene were used, and resulted in amplicons only in the wild type and not in the deletion mutants (data not shown). Furthermore, RT-PCR experiments were performed to confirm the knock-out mutants on their transcriptional levels using primer pairs cynT1-GFP/I-cynT1-r, cynT2-GFP/I-cynT2-r and cynT3-GFP/I-cynT3-r.

To complement the phenotypes, rescue plasmids pGPD-CAS1, pGPD-CAS2 and pGPD-CAS3 were constructed by amplifying the entire coding regions with primer pairs cynT1-GFP/I-cynT1-3r, cynT2-GFP/I-cynT2-3r and cynT3-GFP/I-cynT3-3r. Oligonucleotides used generated NcoI and BamHI restriction sites. Genomic DNA was used as template and in case of cas1 (because of its internal NcoI restriction site) with plasmid pGFP-CAS1 as template. After subcloning into pDrive (Qiagen, Germany) and sequencing, specific NcoI/BamHI fragments were cloned into pEHN-nat1 (Table S2), containing the nourseothricin resistance cassette. In all constructs, the CA gene is under control the constitutive A. nidulans gpd promoter and trpC terminator. Plasmid pGPD-CAS2-Ctg was constructed, by using primers cytT2-Ctg-f and cytT2-BamHI-r to generate an amplicon with NcoI and BamHI restriction sites. After sequencing, a specific NcoI/BamHI fragment was cloned into pEHN-nat1.
this case, the cas2 open reading frame starts with a CTG, encoding for Leu37 in the original gene, instead of the ATG start codon.

Construction of double knock-out strains
Conventional genetic analyses were used to generate double knock-out strains as described previously [23]. Two single knock-out strains were crossed on BMM medium plates and ascii from recombinant perithecia were isolated after 7–10 days. For the identification of hybrid perithecia, mutant strains producing black spores were crossed against mutant strains carrying in addition the spore color marker fus1-1. Single spore isolates of selected ascii were analyzed by PCR to verify double knock-out strains.

Microscopic investigations
S. macrospora strains were inoculated on glass slides for 1 to 7 days. Slides were overlaid with BMM or SWG minimal medium as described earlier [18]. Light and fluorescence microscopic investigations were carried out according to Elleuche and Pöggeler [28]. Co-localization of mitochondrial localized CAS2-constructs was carried out using Mito-Tracker (Invitrogen, Germany).

Supporting Information
Figure S1  Knock-out of cas genes

References
1. Misner A, Kugler P, Saparov SM, Sommer K, Mathai JC, et al. (2008) Carbon dioxide transport through membranes. J Biol Chem 283: 25340–25347.
2. Casey JR (2006) Why bicarbonate? Biochem Cell Biol 84: 930–939.
3. Supuran CT (2008) Carbonic anhydrases-an overview. Curr Pharm Des 14: 603–614.
4. Tripp BC, Smith K, Ferry JG (2000) Carbonic anhydrase: new insights for an ancient enzyme. J Biol Chem 275: 48615–48618.
5. Hewett-Emmett D, Tashian RE (1996) Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. Mol Phylogenet Evol 5: 50–77.
6. Faber N, Reiter IM, Becuwe-Linka N, Genty B, Rumeau D (2007) Characterization and expression analysis of genes encoding alpha and beta carbonic anhydrases in Arabidopsis. Plant Cell Environ 30: 617–629.
7. Baln YS, Midichlelegel FA (2006) CO2 sensing in fungi and beyond. Curr Opin Microbiol 9: 572–578.
8. Parisi G, Perales M, Fornarisi MS, Colaneri A, Gonzalez-Schain N, et al. (2004) Gamma carbonic anhydrase in plant mitochondria. Plant Mol Biol 55: 193–207.
9. Smith KS, Jakubuck C, Whitman TS, Ferry JG (1999) Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. Proc Natl Acad Sci U S A 96: 13184–13189.
10. McGinn PJ, Morel FM (2008) Expression and regulation of carbonic anhydrases in the marine diatom Thalassiosira pseudonana and in natural phytoplankton assemblages from Great Bay, New Jersey. Physiol Plant 133: 78–91.
11. Xu Y, Feng L, Jeffrey PD, Shi Y, Morel FM (2008) Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. Nature 452: 56–61.
12. Amoroso G, Morell-Avrahov L, Müller D, Klug K, Sultemeyer D (2005) The Saccharomyces cerevisiae Leu37 gene shows a growth defect. Yeast 144: 1316–1327.
13. Kamerewerd J, Jansson M, Nowrousian M, Pöggeler S, Kück U (2008) Three α-Subunits of Heterotrophic G Proteins and an Adenylyl Cyclase Have Distinct Roles in Fruiting Body Development in the Homothallic Fungus Sordaria macrospora. Genetics 190: 191–206.
14. Nolting N, Pöggeler S (2006) A STE12 homologue of the homothallic ascomycete Sordaria macrospora interacts with the MAD2 box protein MCM1 and is required for ascosporeogenesis. Mol Microbiol 62: 853–860.
15. Nowrousian M, Piotrowski M, Kück U (2007) Multiple layers of temporal and spatial control regulate accumulation of the fruiting body-specific protein APP in Sordaria macrospora and Nannospora cinerea. Fungal Genet Biol 44: 602–614.
16. Pöggeler S, Risch S, Kück U, Ovaracz HD (1997) Matring-type genes from the homothallic fungus Sordaria macrospora are functionally expressed in a heterothallic ascomycete. Genet Res 70: 367–380.
17. Esser K (1982) Cryptogams-Cyanobacteria, Fungi, Algae and Lichens. London: Cambridge University Press.
18. Nowrousian M, Wurz C, Pöggeler S, Kück U (2004) Comparative sequence analysis of Sordaria macrospora and Nannospora cinerea as a means to improve genome annotation. Fungal Genet Biol 41: 283–292.
19. Kamerewerd J, Jansson M, Nowrousian M, Pöggeler S, Kück U (2008) Three α-Subunits of Heterotrophic G Proteins and an Adenylyl Cyclase Have Distinct Roles in Fruiting Body Development in the Homothallic Fungus Sordaria macrospora. Genetics 190: 191–206.
20. Nolting N, Pöggeler S (2006) A STE12 homologue of the homothallic ascomycete Sordaria macrospora interacts with the MAD2 box protein MCM1 and is required for ascosporeogenesis. Mol Microbiol 62: 853–860.
21. Nowrousian M, Piotrowski M, Kück U (2007) Multiple layers of temporal and spatial control regulate accumulation of the fruiting body-specific protein APP in Sordaria macrospora and Nannospora cinerea. Fungal Genet Biol 44: 602–614.
22. Pöggeler S, Risch S, Kück U, Ovaracz HD (1997) Matring-type genes from the homothallic fungus Sordaria macrospora are functionally expressed in a heterothallic ascomycete. Genet Res 70: 367–380.
23. Esser K (1982) Cryptogams-Cyanobacteria, Fungi, Algae and Lichens. London: Cambridge University Press.
24. Nowrousian M, Wurz C, Pöggeler S, Kück U (2004) Comparative sequence analysis of Sordaria macrospora and Nannospora cinerea as a means to improve genome annotation. Fungal Genet Biol 41: 283–292.
25. Pöggeler S, Nowrousian M, Kück U (2006) Fruiting body development in ascomycetes. In: Kües U, Fischer R, eds. The Mycota I. Berlin, Heidelberg: Springer. pp 325–355.
26. Levine NN, Lew RR (2006) The role of tip-localized mitochondria in hyphal growth. Fungal Genet Biol 43: 65–74.
27. Subramani S (1998) Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. Physiol Rev 78: 171–188.
28. Elleuche S, Pöggeler S (2000) Visualization of peroxisomes via SKL-tagged DsRed protein in Sordaria macrospora. Fungal Genet Rep 55: 9–12.
29. Claros MG, Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241: 779–786.
30. Pöggeler S, Kück U (2006) Highly efficient generation of signal transduction knockout mutants using a fungal strain deficient in the mammalian Iк7 ortholog. Gene 378: 1–10.
31. Aguirre J, Van Dijken JP, De Winde JH, Pronk JT (2005) Carbonic anhydrase (Nce103p): an essential biosynthetic enzyme for growth of Saccharomyces cerevisiae at atmospheric carbon dioxide pressure. Biochem J 391: 311–316.
32. Cronk JD, Endrizzi JA, Cronk MR, O’Neill JW, Zhang KY (2001) Crystal structure of E. coli beta-carbonic anhydrase, an enzyme with an unusual pH-dependent activity. Protein Sci 10: 911–922.
33. Tette SG, Tanu SK, Vella N, Burnett JW, Ludwig M (2007) The Flaviviridae bunteni beta-carbonic anhydrase gene family encodes cytosolic and chloroplastic isoforms demonstrating distinct organ-specific expression patterns. Plant Physiol 144: 1316–1327.
34. Schlicker C, Hall RA, Vullo D, Middelhauwe S, Gertz M, et al. (2008) Structure and Inhibition of the CO2-Sensing Carbonic Anhydrase Cas2 from the Pathogenic Fungus Cryptococcus neoformans. J Mol Biol.
45. Mogensen EG, Mühlschlegel FA (2008) CO2 sensing and virulence of
43. Nagao Y, Srinivasan M, Platero JS, Svendrowski M, Waheed A, et al. (1994)
49. Raven JA (2004) A role for mitochondrial carbonic anhydrase in limiting CO2
48. Giordano M, Norici A, Forssen M, Eriksson M, Raven JA (2003) An anaplerotic
47. Henry RP (1996) Multiple roles of carbonic anhydrase in cellular transport and
41. Sunderhaus S, Duškina NV, Jansch L, Kleshmann J, Heinemeyer J, et al. (2006)
36. Kimber MS, Pai EF (2000) The active site architecture of
38. Suarez Covarrubias A, Larsson AM, Högbom M, Lindberg J, Bergfors T, et al. (2006)
37. Strop P, Smith KS, Ferry JG (1999) A plant-type (beta-class) carbonic anhydrase in the
39. Smith KS, Ingram-Smith C, Ferry JG (2002) Roles of the conserved aspartate
35. Schabtach E, Pogel MA, Schwarzenbach OP, Kreutz H, von Wettstein D, et al. (2000)
34. Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M (2004) A gene homologous to
33. Daniels SC, Shoubridge EA, Koshland DE Jr (1990) In: Brakhage A, Zipfel PF, eds. The Mycota IV. Berlin, Heidelberg: Springer. pp 61–73.
32. van den Hondel CAJM, Marchesi MR, de Vries RW, Verrips CT, van der Maarel SMH, et al. (1998) Eukaryotic Protein Expression Systems. Weinheim: WILEY-VCH pp 215–231.
31. Nowrousian M, Masloff S, Hoff B, Maysifer S, Kuck U (2005) Versatile EGFP reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. Curr Genet 43: 54–61.
30. Christianson TW, Sikorski RS, Danse M, Sheri JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 110: 119–122.
29. James P, Halliday J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144: 1425–1436.
28. Pfaffl MW, Hergen GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.