A p53-like transcription factor similar to Ndt80 controls the response to nutrient stress in the filamentous fungus, *Aspergillus nidulans* [version 1; peer review: 2 approved]

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**Abstract**

The *Aspergillus nidulans* xprG gene encodes a putative transcriptional activator that is a member of the Ndt80 family in the p53-like superfamily of proteins. Previous studies have shown that XprG controls the production of extracellular proteases in response to starvation. We undertook transcriptional profiling to investigate whether XprG has a wider role as a global regulator of the carbon nutrient stress response. Our microarray data showed that the expression of a large number of genes, including genes involved in secondary metabolism, development, high-affinity glucose uptake and autolysis, were altered in an *xprG*Δ null mutant. Many of these genes are known to be regulated in response to carbon starvation. We confirmed that sterigmatocystin and penicillin production is reduced in *xprG*− mutants. The loss of fungal mass and secretion of pigments that accompanies fungal autolysis in response to nutrient depletion was accelerated in an *xprG*T gain-of-function mutant and decreased or absent in an *xprG*− mutant. The results support the hypothesis that XprG plays a major role in the response to carbon limitation and that nutrient sensing may represent one of the ancestral roles for the p53-like superfamily. Disruption of the AN6015 gene, which encodes a second Ndt80-like protein, showed that it is required for sexual reproduction in *A. nidulans*.

**Keywords**
xprG, Ndt80, Aspergillus nidulans, nutrient stress
Introduction

XprG and two non-catalytic hexokinase-like proteins (HxkC and HxkD) were first identified as regulators of extracellular protease production in *Aspergillus nidulans* through genetic analysis7-3. In *A. nidulans*, extracellular proteases are produced in response to carbon, nitrogen or sulfur starvation1. Genetic evidence indicates that XprG activates expression of extracellular protease genes in response to nutrient stress and that HxkC and HxkD are negative regulators of XprG2-3,5,6. The hskCAΔ and hskDΔ null mutations and the xprG1 gain-of-function mutation increase production of extracellular proteases3,5,8. In contrast, loss-of-function mutations in xprG abolish carbon-starvation-induced production of extracellular proteases and are epistatic to the hskCAΔ and hskDΔ null mutations3,5,8. The production of an acid phosphatase in response to phosphate limitation and of extracellular proteases in response to nitrogen- and sulfur-starvation is also reduced in xprG mutants1. Thus, there is evidence that XprG could be involved in a general response to starvation.

XprG is similar to VIB-1 of *Neurospora crassa*, and both are members of the Ndt80 family of p53-like, Ig fold transcriptional activators (Pfam PF005224). VIB-1 is required for expression of genes involved in heterokaryon incompatibility, a type of programmed cell death (PCD)4. XprG is also similar to the *Sacharomyces cerevisiae* meiosis-specific transcriptional activator, Ndt807. Ndt80 activates the transcription of more than 150 genes during the middle phase of meiosis and is required for progression through meiosis22. It has recently been shown that Ndt80 is also involved in resetting lifespan during meiosis and that transient expression of *NDT80* extends the lifespan of aging yeast cells23.

HxkC and HxkD are similar to sequence in catalytic hexokinases but lack some of the conserved residues found in the sugar-binding and ATP-binding domains1. In addition, both possess an extra stretch of amino acids within the adenosine-binding domain. Several plant hexokinase-like proteins that lack catalytic activity also possess an insertion in this same position15,13. The hskC− and hskD− mutants have similar phenotypic effects on extracellular protease production but the proteins encoded by these genes are located in different subcellular compartments1. HxkD is a nuclear protein and HxkC is the first fungal hexokinase shown to be associated with mitochondria. Binding of hexokinase to mitochondria blocks apoptosis in human cells and PCD in plants14-16.

As meiosis in *S. cerevisiae* requires nutrient deprivation and genes expressed during heterokaryon incompatibility are also expressed in response to starvation, we have suggested that nutrient sensing may be a feature of all Ndt80 family members. Previous studies have shown that XprG regulates production of extracellular proteases and an acid phosphatase in response to starvation15,16. In this report, we show that XprG has a wider role as a global regulator of the carbon nutrient stress response and is involved in triggering autolysis, a form of fungal programmed cell death induced by starvation.

Materials and methods

*Aspergillus* media, growth conditions, and genetic techniques

*A. nidulans* was cultured at 37°C in *Aspergillus* complete or minimal medium17 except that glucose was omitted from media that contained other carbon sources. For media that contained 1% skim milk as a carbon source, sodium deoxycholate (0.08%) was used to induce compact colony formation. For RNA extraction, mycelia were grown for 24 h in minimal medium containing glucose and then transferred to minimal medium containing glucose or no carbon source for 16 h. To monitor autolysis, six flasks containing 50 mL of minimal medium, 10 mM ammonium tartrate and vitamin supplements were each inoculated with 3 × 10⁷ conidia and placed on an orbital shaker. Flasks were removed at 24 or 48 h intervals, the submerged mycelia harvested using Miracloth (Calbiochem/Merck) and samples of filtered culture medium collected. To observe conidiophore development on solid medium, strains were inoculated into 1 cm² blocks of complete medium on microscope slides as described by Larone41. The techniques used for genetic analysis of *A. nidulans* have been described17. The *Aspergillus* strains used in this study are listed in Table 1.

RNA extraction and qRT-PCR

Total RNA was prepared using a procedure developed by Reinert *et al.*20. mRNA was prepared from total RNA using the PolyATtract® mRNA Isolation System IV as described by the manufacturer (Promega Corp.). DNA was removed from total RNA or polyA+ RNA with the Ambion Turbo DNA-free Kit® (Applied Biosystems) prior to quantification with a NanoDrop® spectrophotometer. The primers (Supplementary Table 1) used in qRT-PCR experiments were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3). Each primer pair was first tested with serial dilutions of MH2 RNA to determine the linear range of the qRT-PCR assays using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kits (Invitrogen). The experiments were performed using a Corbett CAS1200 liquid handling robot and Corbett Rotor-Gene 3000 real-time thermal cycler (QIAGEN). In the assays to determine relative transcript levels, 1 ng of total RNA was added to each reaction. Each reaction was performed in duplicate or triplicate and the *actA* control reactions were included in each run.

cDNA labeling, microarray hybridization and scanning

cDNAs labeled with Alexa Fluor® 555 and Alexa Fluor® 647 were prepared from mRNA using the SuperScript™ Plus Indirect cDNA Labeling System according to the instructions of the manufacturer (Invitrogen). *A. nidulans* DNA microarrays, supplied by the Pathogen Functional Genomics Resource Center (PFGRC) at The Institute for Genomic Research (TIGR) were hybridized with the labeled cDNAs using the TIGR protocol1. The *A. nidulans* microarrays consisted of 11,481 unique 70-mer oligonucleotides spotted in duplicate on the array plus an additional 1,000 control probes from *Arabidopsis thaliana* and 1,430 empty features (negative controls). The hybridized slides were scanned immediately in an Axon 4200AL scanner (Molecular Devices). The intensity values for the two channels for each spot were acquired by automatic photomultiplier tube gains to obtain the highest intensity with 0.05 saturated pixels. The resulting images were analyzed by measuring the fluorescence of all features on the slides using GenePix Pro 6.1 software (Molecular Devices). The median fluorescence intensity of these pixels within each feature was taken as the intensity value for the feature.

Microarray data analysis

The NCBI Gene Expression Omnibus (GEO) accession number for the microarray data reported in this paper is GSE36235 and the
Table 1. List of *Aspergillus nidulans* strains used in this study.

| Strain   | Genotype                                                                                                           | Source          |
|----------|--------------------------------------------------------------------------------------------------------------------|-----------------|
| MH2      | *bia*1; *niiA4*                                                                                                     | M.J. Hynes      |
| MH97     | *pabaA1* *yA1* *acuE215*                                                                                            | M.J. Hynes      |
| MK85     | *bia*1; *xprG1*; *niiA4*                                                                                            | Katz et al. [2] |
| MK86     | *suA-adE20* *yA1* *adE20*; *xprG1*; *niiA4* *rboB2*                                                                | Katz et al. [2] |
| MK186    | *yA1* *acuE215*; *prnA309* *xprG2*; *niiA4* *rboB2*                                                               | Katz et al. [3] |
| MK198    | *pabaA1*; *prnA309* *xprG2*; *niiA4*                                                                             | Katz et al. [3] |
| MK320    | *pabaA1* *yA2*; *argB2*; *hxkDΔ3* (*hxkD::argB*)                                                                  | Bernardo et al. [1] |
| MK388    | *pabaA1* *yA2*; *hxkCΔ1* (*hxkC::argB*); *argB2* *amds::lacZ*; *xprG2*                                            | Bernardo et al. [1] |
| MK408    | *pabaA1* *yA2*; *argB2*; *xprGΔ2* (*xprG::argB*)                                                                  | Katz et al. [7] |
| MK413    | *pabaA1* *yA2*; *argB2*; *xprGΔ1* (*xprG::argB*)                                                                  | Katz et al. [5] |
| MK414    | *pabaA1* *yA2*; *argB2*; *pyroA4*                                                                                   | This study      |
| MK481    | *hdxAΔ* (*ndtA::A. fumigatus pyroA*); *pyroA4* *nkuA::argB*; *rboB2*                                               | This study      |
| MK505    | *hdxAΔ* (*ndtA::A. fumigatus pyroA*); *pyroA4* *nkuA::argB*; *prnA309* *xprG2*; *niiA4*                          | This study      |
| MK531    | *hdxAΔ* (*ndtA::A. fumigatus pyroA*); *pyroA4* *nkuA::argB*; *prnA309* *xprG2*; *niiA4*                          | This study      |
| MK532    | *hdxAΔ* (*ndtA::A. fumigatus pyroA*); *pabaA1* *yA2*; *argB2*; *pyroA4* *nkuA::argB*; *hxkDΔ3* (*hxkD::argB*)     | This study      |
| MK563    | *bia1*; *xprG1*; *veA*                                                                                             | This study      |
| MK565    | *pabaA1*; *xprG2*; *veA*                                                                                            | This study      |
| MK592    | *bia1*; *fluG701*                                                                                                   | This study      |
| MK593    | *pabaA1* *yA2*; *fluG701*                                                                                            | This study      |
| MK594    | *bia1*; *fluG701* *xprG1*                                                                                           | This study      |
| MK595    | *pabaA1* *yA2*; *fluG701* *xprG1*                                                                                   | This study      |
| WIM-126  | *pabaA1* *yA2*; *veA*                                                                                               | Butnick et al. [68] |

*The gene symbols are described in the *Aspergillus Genome Database*. Data are available at [http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/). Also available for download from this GEO accession is a Supplementary Analysis File containing all pre-processing analyses, annotated lists of differentially expressed genes with links to NCBI as well as gene ontology, pathway analyses and other relevant images and diagrams ([http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36235&submit.x=15&submit.y=14](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36235&submit.x=15&submit.y=14)).

Quality control measures, pre-processing and analyses were performed using the statistical computing language R and Bioconductor. All microarray images and quality control measurements were within recommended limits. The quality of the arrays was assessed through standard quality control measures: pseudo-images of the arrays (to detect spatial effects), MA (M is the intensity ratio and A is the average intensity) scatter plots of the arrays versus a pseudo-median reference chip, and other summary statistics including histogram and boxplots of raw log intensities, signal-to-noise ratios on both channels, boxplots of plates and print tips, boxplots of normalized log ratios, among others. Transcription intensities in adjusted log2 were estimated after normalization within arrays using maximum likelihood followed by between array variance stabilization. Briefly, the data were adjusted by an affine transformation and then all slides were log2 transformed to stabilize the variance. Prior to testing for differential expression, the data were filtered to remove control (n=1,000 from *Arabidopsis thaliana*) and empty spots (n=1,430) and spots flagged as bad in over 90% of the slides (n=4,754), thus leaving 9,104 unique features to be tested.

Differential expression was tested on a gene by gene basis using a moderated t-test with intensities adjusted using an Empirical Bayes approach. A covariance structure to account for the duplicate probes and within array variability was also fitted to the model. Features were considered significantly differentially expressed for a false discovery rate adjusted p-value of 0.05 using the Benjamini-Hochberg correction.

### Annotation and functional analysis of differentially expressed probes

The annotation of the array features was derived from the AspGD – *Aspergillus Genome Database* and identifiers were annotated to
gene ontology terms and pathway information for testing gene set enrichment in GO and KEGG (Kyoto Encyclopedia of Gene and Genomes). In subsequent text the term probe is replaced by gene. The differentially expressed genes were analyzed in the context of their Gene Ontology (GO) and involvement in KEGG biological pathways.

Functional profiles for the differentially expressed genes were derived for each of the GO categories: cellular component, molecular function and biological process. Differentially expressed genes were mapped from their Entrez identifier to their most specific GO term and these were used to span the tree structure and test for gene enriched terms. Profiles for each category were also constructed for the differentially expressed genes for different tree depths (Supplementary Analysis File). To avoid over-inflated p-values, the background for both GO and KEGG pathway analyses consisted exclusively of the array probes used in the analyses after the removal of control probes, unexpressed probes and unannotated probes. Gene ontologies and KEGG pathways reported in this manuscript include those with a significance value of p < 0.05.

**Extraction and detection of sterigmatocystin**

For sterigmatocystin assays, flasks containing 50 mL of Aspergillus minimal medium were inoculated with 3 × 10³ conidia scraped from cultures grown on complete medium containing 2.2% agar. After 24 h, the growth medium was collected and the mycelia were transferred to carbon-free medium for 24 h. Sterigmatocystin was extracted from 10 mL aliquots of filtered growth medium using the method described by Keller et al. with the following modifications. An equal volume of chloroform was added to each sample, mixed vigorously and agitated on a shaking platform for 15 min. After centrifugation at 1600 x g for 5 min, the aqueous phase was transferred to a fresh tube and the chloroform extraction was repeated. The chloroform from the first and second extractions was pooled, dried in a rotary evaporator and the residue resuspended in 50 µL chloroform. A 5 µL sample of each extract was applied to aluminum-backed, silica thin layer chromatography sheets (Merck) and separated using a mixture of benzene and glacial acetic acid (95:5). After drying, the plate was sprayed with 15% ACI, dissolved in 95% ethanol, baked at 65°C for 15 min and photographed under 365 nm UV illumination. Sterigmatocystin (Sigma) was used as a standard.

Sterigmatocystin was also extracted from three 16 mm plugs taken from conidiating colonies grown on solid minimal medium with the method described by Keller et al. with the following modifications. Chloroform (1 mL) was added to the agar plugs and mixed vigorously. After centrifugation at 1000 x g for 5 min, the chloroform containing the extracted sterigmatocystin was transferred to a fresh tube, washed twice with 0.5 mL Milli-Q water (QPAK 2 purification pack, Millipore) and then evaporated. The residue was resuspended in 0.1 mL chloroform.

**Penicillin bioassays**

Penicillin levels in filtered penicillin production broth containing 3% lactose or 3% glucose were assayed as described by Espeso and Peñalva. 5 mL aliquots of filter-sterilized culture medium were lyophilised and resuspended in 300 µL of 10 mM sodium phosphate buffer pH 6.8. The volume (35–50 µL) corresponding to the penicillin produced by 9.3 mg mycelium (dry weight) was applied to 6 mm wells in Luria Broth plates seeded with Micrococcus luteus (UNE014). Penicillin G (Sigma) dissolved in 10 mM sodium phosphate buffer pH 6.8 was applied as a control. The filtrates were left to diffuse for 18 h at 4°C and then incubated at 30°C for 32 h. For samples treated with penicillinase (Sigma Aldrich), 1 µL containing 1 U of enzyme in 100 mM Tris-HCl pH 7.0 with 0.1% BSA was added and the samples were incubated at 25°C for 15 min before they were applied to the plates. The samples that were not treated with penicillinase were treated in an identical manner except that the 1 µL of 100 mM Tris-HCl pH 7.0 1% BSA did not contain any enzyme.

**Glucose uptake assays**

The uptake of D-[U-¹⁴C] glucose (10.6 GBq/mmol, Amersham) was measured in germinating conidia as described previously. Conidia were germinated in minimal medium containing 1% glucose, 0.1% yeast extract, 10 mM ammonium tartrate and vitamins and then washed five times with carbon-free minimal medium containing 10 mM NH₄Cl and vitamins. Glucose uptake was measured in aliquots of 2.5 × 10⁷ germinating conidia 5, 30, 60 and 90 s after transfer to media containing 0.025, 0.125, 0.5 or 2 mM glucose.

**Disruption of AN6015**

The AN6015 gene (ndtA) was disrupted in an nkuAΔ strain (MH11036) so as to increase the frequency of gene targeting events. The entire predicted coding region of AN6015 (nucleotides 21661–23381, contig 103; Aspergillus Comparative Database) was replaced with the Aspergillus fumigatus pyroA gene using a similar strategy to the one described in Nayak et al. Gene disruption was confirmed by PCR and Southern blot analysis. Double mutants with lesions in AN6015 (ndtA) and hskC, hskD or xprG were generated in crosses and the presence of ndtA::A. fumigatus pyroA was confirmed by PCR using primers MK261 (5′-AACGGTTACCCTCCAATTGC-3′) complementary to sequences upstream of the A. nidulans ndtA coding region and MK323 (5′-GATGTTCTCGAAGTCGAC-3′) complementary to the A. fumigatus pyroA gene.

**Results**

**Transcriptional profiling**

A. nidulans microarrays provided by the Pathogen Functional Genomics Resource Center (PFGRC) were used to compare transcript levels in an xprG+ strain and an xprGΔ null strain after transfer to medium containing glucose as a carbon source or medium lacking a carbon source (carbon starvation) for 16 h. These four experiments (Figure 1) were designed to detect differences in transcript levels between the two strains (Experiments 2 and 4) and changes in transcript levels in each strain due to the different nutrient conditions (Experiments 1 and 3). The NCBI Gene Expression Omnibus (GEO) accession number for the microarray data reported in this paper is GSE36235 and is available at http://www.ncbi.nlm.nih.gov/geo/. A total of 516 probes that hybridized to differentially expressed transcripts were detected in Experiment 1, which examined the effect of carbon starvation in an xprG+ strain. One hundred and ninety seven were up-regulated and 319 were down-regulated during carbon starvation (Figure 2). The top five biological processes identified in the Gene Ontology analysis of Experiment 1 were sterigmatocystin biosynthesis, ergosterol biosynthesis, conidial spore wall assembly, the purine salvage pathway and...
autolysis. In the xprGΔ1 mutant, the number of transcripts that showed a significant change in response to carbon starvation was lower (Figure 2). All of the 73 up-regulated and 222 down-regulated transcripts in Experiment 3 showed similar responses (in direction) to carbon starvation in Experiment 1.

In Experiment 4, which examined the effect of the xprGΔ1 mutation on A. nidulans’ response to carbon starvation, 133 probes hybridized to transcripts that were either up- or down-regulated (Figure 2). Ninety-four probes hybridized to transcripts that were down-regulated in the xprGΔ1 mutant and 39 were up-regulated. Fifteen of the down-regulated transcripts, including four of the top five, belonged to the sterigmatocystin gene cluster (Table 2). The pathway for the synthesis of sterigmatocystin, a carcinogen closely related to aflatoxin, is encoded by a cluster of 25 co-regulated genes. Transcripts from an additional four genes from the cluster (aflR, stcA, stcO, and stcS) had lower levels in the xprGΔ1 mutant with p-values of less than 0.05 prior to applying the Benjamini-Hochberg correction. It is interesting that disruption of the aflR gene, which encodes another regulator of the aflatoxin, is encoded by a cluster of 25 co-regulated genes. Transcripts from an additional four genes from the cluster (aflR, stcA, stcO, and stcS) had lower levels in the xprGΔ1 mutant with p-values of less than 0.05 prior to applying the Benjamini-Hochberg correction. The tdiB gene, which is down-regulated in the xprGΔ1 mutant, belongs to another secondary metabolism gene cluster, tdiA-E, that controls the biosynthesis of the anti-tumor compound terrequinone A. A second gene in the cluster, tdiA, was down-regulated in the xprGΔ1 mutant with a p-value of 0.002 prior to adjustment and 0.073 after application of the Benjamini-Hochberg correction. It is interesting that disruption of the laeA gene, which encodes another regulator of the tdi gene cluster, produced similar effects on the members of the cluster; the reduction in tdiB transcript levels was greater than that of tdiA and the levels of the tdiC, D, and E transcripts were affected to an even lesser extent in the laeAΔ mutant.

Other genes with documented functions that showed differential expression in response to carbon starvation in the xprGΔ1 mutant include two genes encoding extracellular proteases (prtA and pepJ) which are known to be expressed during starvation. The expression of prtA in response to carbon or nitrogen starvation has been shown to be XprG-dependent. HxkC is involved in the regulation of extracellular protease production. Disruption of the hxkC gene, which is down-regulated in the xprGΔ1 mutant, increases extracellular protease production.

The microarray data indicated that a key regulator of conidiophore development brlA was down-regulated in the xprGΔ1 mutant, while the vveA gene, which activates sexual development, was up-regulated. Genes encoding a putative sex pheromone (ppgA) and pheromone receptor (preA) were also expressed at higher levels in the xprGΔ1 mutant. Carbon starvation is known to induce transcription of the brlA gene.

Autolysis is a process of hyphal fragmentation and digestion that occurs in stationary cultures of A. nidulans after carbon source depletion. Though autolysis and apoptotic cell death occur concurrently during carbon starvation, genetic evidence indicates that the two processes are regulated independently. The chitinase encoded by the chib gene plays an important role in autolysis while nagA is involved in apoptotic cell death. Both chib and nagA, which were up-regulated in response to carbon starvation in the xprGΔ1 strain in Experiment 1, are down-regulated in the xprGΔ1 mutant.

In contrast to Experiment 4, only two probes on the array showed significantly different intensities when hybridized with cDNA prepared from xprGΔ1 and xprGΔ1 strains grown in medium containing glucose in Experiment 2. This confirms that the role of XprG is mainly
confined to the starvation response. Only one of the two probes identified in Experiment 2 is annotated as a gene, *hpdA*, which encodes a putative 4-hydroxyphenylpyruvate dioxygenase with a predicted role in pyomelanin production. In *Aspergillus fumigatus*, disruption of the *hpdA* homolog (*hpdD*) abolished pyomelanin pigment production and no pigment was detected in mycelia or culture medium of the mutant when it was grown in liquid medium.

**qRT-PCR validation**

Three genes that were down-regulated (*brlA*, *chiB*, *tdiB*) and two that were up-regulated (*ppgA*, *veA*) in the *xprGΔ1* mutant (Experiment 4) were analyzed in qRT-PCR experiments using new preparations of RNA (Table 3), and by agarose gel electrophoresis of qRT-PCR products (Supplementary Figure 1). The housekeeping gene encoding actin (*actA*) was used as a control. The level of the actin transcript was lower in carbon-free medium than in glucose in both strains. In previous studies we have observed, using Northern blot analysis, that the level of the *actA* transcript is reduced (relative to tRNAs) during carbon starvation. The transcript levels in the three down-regulated genes were all higher in the *xprG*+ strain than in *xprGΔ1* mutant during carbon starvation and were higher during carbon starvation than in nutrient-sufficient conditions in a *xprG*+ strain as predicted by the microarray results. The qRT-PCR data for the up-regulated *ppgA* gene showed much higher expression in the *xprGΔ1* mutant than the wild-type strain during carbon starvation and higher levels in carbon-free medium than glucose for the *xprGΔ1* strain as predicted by the microarray results. The qRT-PCR data suggest that *ppgA* transcript levels are higher during carbon starvation in the *xprG*+ strain. For the *veA* gene, no differences between the wild-type and mutant strains were detected.

**Secondary metabolism in xprG mutants**

The results of the microarray experiments suggested that expression of genes in the sterigmatocystin gene cluster was reduced in the *xprGΔ1* mutant. To confirm that sterigmatocystin levels were altered, sterigmatocystin was extracted from the growth medium of strains carrying two different *xprG* mutations (*xprG1* and *xprG2*) and a strain carrying the *xprG1* gain-of-function mutation. The *xprG2* loss-of-function mutation is due to the insertion of two base pairs which causes a frameshift mutation in the ninth codon of the *xprG* gene.

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Penicillin is also a product of secondary metabolism in *A. nidulans*. Although no significant changes in the expression of penicillin biosynthetic genes were detected in the microarray experiments, this may have been due to the fact that the growth medium was not optimal for penicillin production. Bioassays were used to detect penicillin levels in broth cultures optimised for penicillin production.

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### Table 2. Genes that show altered expression in the *xprGΔ1* mutant during carbon starvation.

| Biological process                  | Genes                                      | Effect of *xprGΔ1* |
|-------------------------------------|--------------------------------------------|--------------------|
| Secondary metabolism                | Sterigmatocystin gene cluster: *stcB* (-3.7), *stcE* (-6.2), *stcl* (-5.4), *stcL* (-5.7), *stcN* (-4.5), *stcG* (-3.3), *stcT* (-4.4), *stcl* (-9.0), *stcv* (-4.8), *stcw* (-4.4), AN7808 (-4.5), AN7817 (-4.8), AN7819 (-3.8), AN11017 (-5.4), AN11021 (-4.1), tdIB (-4.8) | down               |
| Conidiophore development            | *brlA* (-4.9), *ivoC* (-4.4), hydrophobins: *rodA* (-3.2), AN0940 (-4.3), AN1873 (-5.1), AN6401 (-3.3) | down               |
| Sexual reproduction                 | *ppgA* (5.1), *preA* (5.2), *veA* (2.8)    | up                 |
| Extracellular protease production   | *hxcC* (-6.1), *pepJ* (-5.3), *ptA* (-3.2) | down               |
| Autolysis/apoptosis                 | *chiB* (-3.3), *nagA* (-4.6)               | down               |
| Sugar transport (high affinity)     | *mstA* (-4.0)                              | down               |
| Other                               | *actA* (2.9), *gabA* (3.2), *gta* (3.7), CYP680A1 (3.4) | up down            |

*a*The genes are described in the *Aspergillus Genome Database*. Only named genes (and genes with a similar function to the named genes) are listed. The fold change (log, scale) is given in parentheses, with a negative value indicating that the gene is down-regulated in the *xprGΔ1* mutant during carbon starvation. The full data set for differentially expressed genes is available through NCBI Gene Expression Omnibus (GEO) accession number GSE36235, (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36235).

*b*The effect of the *xprGΔ1* mutation on transcript levels during carbon starvation was determined in microarray experiments.
The results showed that penicillin levels, as measured by bacterial growth inhibition, were greatly reduced in an xprG2 loss-of-function mutant and increased in an xprG1 gain-of-function mutant (Figure 3B). When glucose was included in the growth medium, no penicillin was detected in the culture medium of any strains (Figure 3B).

**Effect of xprG mutations on conidiophore development**

BrlA is a DNA-binding protein that is required for conidiophore development\(^4\)\(^-\)\(^6\). The microarray and qRT-PCR data showed that expression of brlA is induced during carbon-starvation but is at lower levels in the xprGΔ1 mutant. The RNA used in the microarray and qRT-PCR experiments was extracted from mycelia grown in submerged cultures. While conidiation does not normally occur under these conditions, transfer to medium lacking a carbon source does induce conidiation in submerged cultures\(^1\). All xprG mutants produce conidia though they are abnormally pale in color (Figure 4A). The conidiophore structure of xprG mutants was examined and appeared to be normal (Figure 4A, Table 4). The conidiophore stalk length was highly variable in all strains but the difference between the xprG\(^+\) and xprG2 is marginally significant (p = 0.05). Asexual spore production was also highly variable in the gain- and loss-of-function mutants (Table 4). Both xprG1 and xprG2 mutants were slightly slower to initiate conidiophore development.

Expression of the ivoC gene was lower in the xprGΔ1 mutant. IvoC encodes a putative cytochrome P450 that is required for conidiophore pigmentation (A.J. Clutterbuck, personal communication). The ivoB gene also showed lower expression in the xprGΔ1 mutant with an unadjusted p-value of 0.002. Mutants lacking a functional copy of ivoA, B or C have ivory-coloured conidiophores\(^1\). Microscopic examination showed that the conidiophore stalks of xprG2 mutants display normal pigmentation (Figure 4A).

Initiation of conidiophore development occurs irrespective of nutrient limitation in *A. nidulans* cultures exposed to air\(^1\) and can be induced in submerged cultures by carbon starvation\(^4\). We found that conidiophore development occurred in carbon-starved submerged cultures of both the xprGΔ1 loss- and xprG1 gain-of-function mutants, though the number of metulae appeared to be reduced (Figure 4B). Thus, XprG is not essential for triggering conidiophore development in response to carbon starvation.

We investigated the genetic interactions between the xprG mutations and mutations in genes encoding key regulators of conidiophore development. VeA is a component of the light sensor which regulates the switch from sexual to asexual development. Laboratory strains of *A. nidulans* produce abundant asexual spores (conidia) in the absence of light because of a point mutation in the veA gene\(^4\). To investigate the interaction between the xprG and veA genes, strains carrying the xprG1 and xprG2 mutations were crossed to a ve\(^+\) strain, which requires light to trigger asexual spore formation. When xprG2 ve\(^+\) segregants were grown in complete darkness, the colonies produced even fewer conidia than xprG\(^+\) veA\(^+\) strains, whereas the xprG1 gain-of-function mutation partially suppressed VeA-mediated repression of conidiophore development (Figure 5). Programmed initiation of conidiation in surface cultures depends on FluG, but *fluG* mutants can be induced to undergo conidiophore development by nutrient stress\(^2\). We found that the xprG1 mutation partially suppresses the conidiophore development defect in the *fluG70I* mutants (Figure 5). In contrast, the xprG1 mutation did not suppress the brlA1 defect in conidiation.

**Table 3. Results of qRT-PCR validation experiments**\(^6\).

| Gene | Relevant genotype/carbon source | xprG\(^+\)/glucose | xprG\(^+\)/carbon-free | xprGΔ1/glucose | xprGΔ1/carbon-free |
|------|--------------------------------|-------------------|------------------------|----------------|-------------------|
| actA | Ct                             | 20.45 ± 0.18      | 22.15 ± 0.21           | 20.40 ± 0.20   | 22.66 ± 0.33      |
|      | REL                            | 1                 | 0.35                   | 1.02           | 0.34              |
| brlA | Ct                             | 32.51 ± 0.06      | 27.28 ± 0.04           | 34.45 ± 0.86   | 31.14 ± 0.27      |
|      | REL                            | 1                 | 1.69                   | 0.82           | 1.17              |
| chiB | Ct                             | 28.08 ± 0.27      | 20.37 ± 0.37           | 27.89 ± 0.26   | 23.79 ± 0.19      |
|      | REL                            | 1                 | 12.60                  | 0.72           | 3.13              |
| tdIB | Ct                             | 30.96 ± 0.07      | 28.23 ± 0.04           | 31.98 ± 0.20   | 31.34 ± 0.78      |
|      | REL                            | 1                 | 1.82                   | 0.75           | 0.86              |
| ppgA | Ct                             | 32.71 ± 1.70      | 29.26 ± 0.24           | 29.83 ± 0.38   | 24.51 ± 0.06      |
|      | REL                            | 1                 | 4.35                   | 2.68           | 24.3              |
| veA  | Ct                             | 24.41 ± 0.08      | 27.13 ± 0.10           | 24.32 ± 0.18   | 25.72 ± 0.22      |
|      | REL                            | 1                 | 0.72                   | 1.00           | 0.83              |

\(^6\)The average cycle threshold (Ct) values for threshold of 0.03 normalized fluorescence units and standard errors are shown. A lower Ct value indicates higher transcript levels. Relative expression levels (REL), based on the Takeoff point and reaction efficiency, were calculated using the Corbett Rotor-Gene Comparative Quantitation program, using the xprG\(^+\)/glucose reactions for each gene as the calibrator. The relative expression levels do not take into consideration the differences in the actA transcript levels.
tions on glucose transport was examined (Figure 6).

In the xprGΔ1 mutant, glucose uptake was significantly reduced when low levels of glucose were present but was unaltered when the concentration of glucose was high, indicating that only high-affinity glucose uptake was decreased. Both high- and low-affinity uptake of glucose was reduced in the xprG1 gain-of-function mutant.

Glucose uptake

The Aspergillus niger mstA gene encodes a high-affinity sugar transporter that is highly expressed during carbon starvation and repressed by glucose53. The A. nidulans homologue of mstA was among the top five genes that were up-regulated in response to carbon starvation in an xprGΔ strain in Experiment 1, and was down-regulated in the xprGΔ1 mutant. The effect of xprG loss- and gain-of-function muta-

tions on glucose transport was examined (Figure 6). In the xprGΔ1 mutant, glucose uptake was significantly reduced when low levels of glucose were present but was unaltered when the concentration of glucose was high, indicating that only high-affinity glucose uptake was decreased. Both high- and low-affinity uptake of glucose was reduced in the xprG1 gain-of-function mutant.

Autolysis

The chiB gene, which plays an important role in autolysis, was among the top five genes that were up-regulated in response to carbon starvation in the xprGΔ strain in Experiment 1, and was down-regulated in the xprGΔ1 mutant. Production of extracellular proteases also increases during autolysis54. The genes encoding two
extracellular proteases, PrtA and PepJ, were down-regulated in the xprG<sup>Δ1</sup> mutant. Cultures of the xprG<sup>1</sup> and xprG<sup>2</sup> mutants were observed over a period of eight days to determine whether XprG plays a role in autolysis, which occurs in stationary, submerged cultures of <i>A. nidulans</i> after carbon source depletion<sup>45</sup>. The disintegration of mycelial pellets, decline in mycelial mass, increase in culture medium turbidity due to hyphal fragmentation and accumulation of brown pigment which accompany autolysis occurred more rapidly in the xprG<sup>1</sup> gain-of-function mutant. In contrast, mycelial pellets were still present in the cultures of the xprG<sup>2</sup> and xprG<sup>Δ1</sup> mutants (the two xprG genotypes) after 8 days and there was no evidence of hyphal fragmentation or pigment accumulation (Figure 7). These results indicate that XprG is required for autolysis in response to carbon starvation. Thus, XprG, like Vib-1 of <i>N. crassa</i> has a role in regulating programmed cell death.

The microarray experiments showed that expression of the hpdA gene was reduced in the xprG<sup>Δ1</sup> mutant. The <i>A. fumigatus</i> hppD gene is the ortholog of the <i>A. nidulans</i> hpdA gene and has been shown to be essential for the production of pyomelanin<sup>49</sup>. A ΔhppD mutant has colourless mycelia and does not release pyomelanin in liquid medium. Thus, it is likely that the pale mycelia and absence of released pigment in the xprG mutants during autolysis is due to reduced hpdA expression.

**Figure 4.** Conidiophore morphology in xprG loss-of-function (middle) and xprG<sup>1</sup> gain-of-function (right) mutants. A. Conidiophores of strains MH2, MK198, and MK85 were photographed after 2 days growth at 37°C on solid complete medium on microscope slides followed by treatment with diluted Lactophenol Cotton Blue stain. For the lower set of pictures, conidia were scraped from MH2, MK422 and MK85 colonies on complete medium. Scale bars: 50 µm (upper row), 20 µm (lower row). B. Conidiophores of strains MH2, MK422 and MK85 after transfer to carbon-free liquid medium for 24 h. Scale bars: 10 µm. The full genotypes of the xprG<sup>+</sup> (MH2), xprG<sup>2</sup> (MK198), xprGΔ1 (MK422) and xprG<sup>1</sup> (MK85) strains are given in Table 1.
Table 4. Conidiophore development in xprG mutants.

| Phenotype                              | Relevant genotypea | xprG | xprG | xprG1 |
|----------------------------------------|--------------------|------|------|-------|
| Conidiophore morphology in surface cultures |                   | normal | normal | normal |
| Mean conidiophore stalk lengthb         |                   | 57.4 ± 19.5 µm | 62.5 ± 19.6 µmd | 55.7 ± 18.5 µm |
| Mean no. of conidia per mm²c            |                   | 1.23 ± 0.07 | 1.06 ± 0.33 | 0.76 ± 0.49 |
| Conidiophore development in submerged culturesd | yes | yes | yes |

aThe full genotypes are given in Table 1. Strains MH2 (xprG1) and MK85 (xprG1) were used for all analyses. Strain MK422 was used for all xprG analyses except for mean conidiophore stalk length, which used MK198 (xprG). Conidiophore morphology in surface cultures was examined in both MK198 and MK422.
bConidiophores were photographed at 400 x magnification after growth at 37°C on microscope slides. Measurements were carried out using the ImageJ program (http://rsbweb.nih.gov/ij). The mean length (± SD) for over 100 conidiophores are given. The difference between the xprG and xprG1 strains was marginally significant (unpaired t-test, p=0.05).cThe number of asexual spores (conidia) per mm² was determined by removing three plugs from colonies on complete medium containing 2.2% agar. The conidia from each plug were suspended in a solution of 0.01% TWEEN80 and counted in a haemocytometer. The number per mm² (± SD) is the mean from four experiments which used different batches of media. No significant differences were found using an unpaired t-test.dConidiophore development was monitored after transfer to carbon-free medium.

Role of other Ndt80-like proteins in filamentous fungi

Ndt80 is a transcriptional activator required for progression through meiosis in S. cerevisiae and A. nidulans, whereas A. nidulans mutants lacking a functional copy of the xprG gene are able to complete meiosis. S. cerevisiae is unusual among ascomycete fungi in that it possesses only one transcription factor in this class (Table 5). In A. nidulans, a second putative member of this class (AN6015) shows greater similarity to Ndt80 (17.1% identity overall and 23.5% in the DNA-binding domain) than does XprG (12.4% identity overall and 13.8% identity in the DNA-binding domain). To investigate the role of AN6015, the gene was disrupted. Strains carrying a disrupted copy of AN6015 could be crossed to wild-type strains but no cleistothecia (fruiting bodies) were observed when AN6015Δ mutants were crossed. These results suggest that AN6015 is required for sexual reproduction in A. nidulans and, as in S. cerevisiae, mutations in AN6015 are recessive. We suggest that AN6015 be named NdtA.

Unlike xprG loss-of-function mutants, ndtAΔ does not affect conidial pigmentation (Figure 8A), prevent extracellular protease production or suppress mutations in hxkC and hxkD (Figure 8B and 8C). If no ammonium is present, wild type strains produce a halo, due to extracellular protease activity, on medium containing milk as a nitrogen source. The ndtAΔ mutant also displays a halo but the xprG2 mutant, which is protease-deficient, does not when grown on medium containing milk as a nitrogen source (Figure 8B). Extracellular protease activity is low on medium containing milk as a carbon source, as carbon starvation is required to stimulate extracellular protease production when ammonium is present. The hxkCΔ and hxkDΔ mutants have elevated levels of extracellular protease and produce large halos on this medium. The xprG2 mutation suppresses this phenotype but the ndtAΔ mutation does not (Figure 8C). xprG2 ndtAΔ double mutants had the same pale conidia as xprG2 strains. Like the xprG2 single mutant, the xprG2 ndtAΔ double mutant produced no halo on medium containing milk as a carbon or nitrogen source and did not undergo autolysis in response to nutrient stress (Figure 7).

Discussion

The transcriptional profiling data reported here reveal that XprG plays a major role in the activation of gene expression in response to carbon starvation. More than 37% of the 197 probes that hybridized to transcripts that were significantly up-regulated during carbon starvation, were down-regulated in the xprGΔ1 mutant. This proportion is higher if less stringent criteria are used to identify differentially regulated transcripts; 60% of the transcripts up-regulated during carbon starvation show more than a two-fold decrease in transcript levels in the xprGΔ1 mutant and 91% show at least some decrease. In contrast, less than 5% of the 319 probes that hybridized to transcripts that were down-regulated during carbon starvation were up-regulated in the xprGΔ1 mutant and none were down-regulated. As XprG is a putative transcriptional activator, it is not surprising that it does not appear to be involved in repression of gene expression during carbon starvation. Secondary effects (e.g. downstream-regulation of repressors) may be responsible for the few transcripts that are down-regulated during carbon starvation and up-regulated in the xprGΔ1 mutant. XprG also does not appear to play a role in regulating gene expression during growth in medium containing glucose as a carbon source.

HxkC and HxkD are hexokinase-like proteins which are negative regulators of extracellular protease production and may modulate the activity of XprG. It has previously been reported that contrary to expectations, hxkD transcript levels increase during carbon...
starvation\textsuperscript{1}. The microarray data reported here showed that the \textit{hskC} gene, is also up-regulated during carbon starvation, and that increased expression of \textit{hskC} is dependent on XprG. It was not expected that \textit{hskC} and \textit{hskD} transcript levels would increase during carbon starvation, because HskC and HskD are negative regulators and production of extracellular proteases increases during carbon starvation. As noted previously, these results could indicate that HskC and D have other functions during carbon starvation\textsuperscript{1}.

We have shown here that XprG regulates the expression of \textit{brlA}, a key regulator of conidiophore development, in submerged cultures during carbon starvation. However, conidiophore development is essentially normal in \textit{xprG} mutants grown on solid media and can be induced by carbon starvation in submerged cultures. Thus, the reduction of \textit{brlA} expression observed in the \textit{xprG}\textit{Δ} mutant is not sufficient to block conidiophore development. Nevertheless, the genetic evidence suggests that XprG plays some role in triggering asexual development as the \textit{xprG}1 mutation stimulates conidiophore development in a \textit{veA}\textsuperscript{+} strain incubated in the dark and in a \textit{fluG701} mutant.

Secondary metabolism and asexual/sexual development are linked in filamentous fungi. XprG appears to be a member of a group of regulatory proteins that control both secondary metabolism and development (reviewed in Bayram \textit{et al.}\textsuperscript{55}). This group includes the light regulator VeA, which is required for sexual development\textsuperscript{61} and has been shown to regulate sterigmatocystin production\textsuperscript{62}. LaeA, the global regulator of secondary metabolism\textsuperscript{57} which is also required for asexual development\textsuperscript{63}, and components of a heterotrimeric G protein signaling pathway which is required for both asexual development and sterigmatocystin production\textsuperscript{64}. All of the proteins in this group act upstream of BrlA, the transcription factor that activates genes required for conidiophore development\textsuperscript{65}, but is not required for sterigmatocystin production\textsuperscript{64}. The \textit{A. nidulans} homologue of \textit{S. cerevisiae} Ime2 protein kinase is also a member of this group. An \textit{imeBA} null mutant does not produce sterigmatocystin and overproduces sexual fruiting bodies in light in a \textit{veA}\textsuperscript{+} strain\textsuperscript{65}. In \textit{S. cerevisiae} Ime2 activates transcription of Ndt80 and also controls Ndt80 activity through phosphorylation\textsuperscript{66}. XprG, as an Ndt80-like protein, could be a target of ImeB in \textit{A. nidulans}.

In addition to the link between asexual development and secondary metabolism in \textit{A. nidulans}, there is a link between a sexual development and autolysis\textsuperscript{66,67,68}. Thus, XprG may play a direct role in regulating autolysis through regulation of chitinase (ChiB), extracellular proteases (PrtA, PepJ) and other hydrolytic enzymes or XprG could act indirectly through BrlA, which is involved in the induction of autolysis\textsuperscript{69}.

The \textit{xprG}1 gain-of-function mutant had previously been shown to have the reverse phenotype to \textit{xprG} mutants with respect to extracellular protease and pigment production\textsuperscript{7}. Here we show that the \textit{xprG}1 mutation leads to accelerated autolysis and increased

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**Figure 5. Interactions between the \textit{xprG}, \textit{veA} and \textit{fluG} genes. A.** Conidiation is suppressed by VeA in the dark but XprG1 partially restores conidiation in a \textit{veA}\textsuperscript{+} strain. The plate was photographed after 3 days of growth on complete medium at 37°C. Light was excluded by wrapping the plate in aluminum foil. The full genotypes of the \textit{xprG} (MH2), \textit{xprG2} (MK198), \textit{xprG}1 (MK85), \textit{xprG} veA\textsuperscript{-} (WIM-126), \textit{xprG2} veA\textsuperscript{-} (MK565), and \textit{xprG1} veA\textsuperscript{-} (MK563) strains are given in Table 1. B. The \textit{fluG} gene is involved in producing an extracellular signal for the induction of conidiophore development\textsuperscript{51}. The \textit{fluG701} mutation is partially suppressed by the \textit{xprG}1 gain-of-function mutation. The full genotypes of the strains (top left MK593, top right MK592, bottom left MK595, bottom right MK594) are given in Table 1.**

**Figure 6. Glucose uptake, in 2.5 x 10\textsuperscript{7} germinating conidia, in the first 60 s after transfer to 25 \textmu M, 125 \textmu M, 500 \textmu M or 2 mM glucose.** The results are the average for four (\textit{xprG}Δ1, \textit{xprG}1) and five (\textit{xprG}+) experiments and standard errors are shown. The rate of glucose uptake was compared with the uptake of the \textit{xprG} strain at each concentration of glucose using an unpaired t-test. Values which differed significantly from the value for the \textit{xprG} strain are indicated with asterisks (*\textit{p < 0.5}, **\textit{p < 0.1}) The full genotypes of the \textit{xprG} (MH2), \textit{xprG}Δ (MK422) and \textit{xprG}1 (MK85) strains are given in Table 1.
penicillin production, whereas autolysis and penicillin production is reduced or absent in an xprG mutant. Likewise, conidiation is increased in an xprG veA+ strain but decreased in an xprG veA− strain. In contrast, glucose uptake and sterigmatocystin levels were reduced in both the xprG1 and xprG mutants. The reason for this difference in phenotypic effect is not known. The xprG1 allele contains a missense mutation (R186W) in the putative DNA-binding domain of XprG. It may be that this amino acid substitution increases the affinity of the XprG1 for some binding sites but decreases the affinity for others. Missense mutations with this type of gene specificity effect have been documented in the DNA-binding domain of AreA, the A. nidulans regulator of genes involved in nitrogen metabolism65.

We have shown that the two genes encoding Ndt80-like proteins in A. nidulans perform different functions. Among fungi, there is considerable variation in the number of genes in the NDT80 family (Table 5). Most basidiomycetes and the unicellular ascomycete Schizosaccharomyces pombe do not possess any genes encoding Ndt80/PhoG-like proteins. In contrast, the zygomycetes have large numbers of these genes. The number of NDT80-like genes varies within genera (e.g. Aspergillus) and even within the same species (e.g. Candida albicans). As most ascomycetes have a gene similar to NDT80 and one or more genes similar to xprG (data sourced from the Fungal Genome Initiative), it seems likely that the unicellular S. cerevisiae has lost the xprG-like gene.

The p53-like transcription factor superfamily (http://supfam.org/) is comprised of seven families containing the following DNA-binding domains: p53, Rel/Dorsal, T-box, STAT, Runt, Ndt80, and the LAG-1/CSL. Many of the proteins in this superfamily, including MRF (myelin gene regulatory factor), a mammalian member of the Ndt80 family, are involved in development. The Ndt80 and LAG-1 families include both animal and fungal proteins and the Ndt80 family is also found in the slime molds Dictyostelium discoideum and Dictyostelium purpureum. The Ndt80 family is present in all ascomycete fungi, with the exception of the Schizosaccharomyces

### Table 5. The Ndt80 class of p53-like transcriptional activators in fungi.

| Phylum             | No. of genes encoding Ndt80-like proteins | Species                             |
|--------------------|------------------------------------------|-------------------------------------|
| Basidiomycota      | 0                                       | Cryptococcus neoformans             |
|                    | 0                                       | Coprinus cinereus                   |
|                    | 0                                       | Phanerochaete chrysosporium         |
|                    | 0                                       | Postia placenta                     |
|                    | 0                                       | Puccinia graminis                   |
|                    | 1                                       | Ustilago maydis                     |
| Ascomycota         | 0                                       | Schizosaccharomyces pombe           |
|                    | 1                                       | Saccharomyces cerevisiae            |
|                    | 2                                       | Aspergillus nidulans                |
|                    | 2                                       | Aspergillus flavus                  |
|                    | 2-3                                     | Candida albicans                    |
|                    | 3                                       | Aspergillus fumigatus               |
|                    | 3                                       | Magnaporthe oryzae                  |
|                    | 3                                       | Neurospora crassa                   |
|                    | 3                                       | Fusarium graminearum                |
|                    | 4                                       | Fusarium oxysporum                  |
| Chytridiomycota    | 2                                       | Batrachochytrium dendrobatidis       |
|                    | 2                                       | Spizellomyces punctatus             |
| Zygomycota         | 5                                       | Phycomyces blakeleeanus             |
|                    | 6                                       | Mucor circinelloides                |
|                    | 7                                       | Rhizopus oryzae                     |

aGenome sequences were obtained from the Fungal Genome Initiative of the Broad Institute with the exception of the P. chrysosporium, P. placenta and P. blakeleeanus sequences which were from the DOE Joint Genome Institute.
It has recently been shown that Ndt80 is involved in resetting lifespan during meiosis and transient expression of *NDT80* extends the lifespan of aging yeast cells. Pathways responsible for the response to nutrient status appear to play an important role in controlling lifespan. We speculate that the ability of Ndt80 to sense nutrient status could be crucial in determining lifespan.

We have previously proposed that the common feature of fungal p53-like proteins is a role in nutrient sensing, and this may be the original role for this group of transcriptional activators. It has recently been shown that Ndt80 is involved in resetting lifespan during meiosis and transient expression of *NDT80* extends the lifespan of aging yeast cells. Pathways responsible for the response to nutrient status appear to play an important role in controlling lifespan. We speculate that the ability of Ndt80 to sense nutrient status could be crucial in determining lifespan.

**Figure 7.** Effect of the *xprG2/xprGΔ1* loss-of-function and *xprG1* gain-of-function mutations on autolysis. Loss of mycelial mass (A) and changes in the appearance of cultures (B) were monitored for 8 days in submerged cultures inoculated with the same number of conidia. The results in A are the average for the three experiments and standard errors are shown. The mycelial mass at each time point was compared with the mass of the *xprG*+ strain using an unpaired t-test. Values which differed significantly from the value for the *xprG*+ strain are indicated with asterisks (*p < 0.5, **p < 0.1, ***p < 0.001). The full genotypes of the *xprG*+ (MH2), *xprG1* (MK85), *xprG2* (MK198), *xprGΔ1* (MK422) and *xprG2 ndtAΔ* (MK505) strains are given in Table 1.
**Figure 8. Phenotype of the AN6015Δ gene disruption mutant.** Colony morphology and extracellular protease production of wild-type and mutant strains on (**A**) minimal medium (**B**) medium containing milk as a nitrogen source and (**C**) medium containing milk as a carbon source. The clear halo surrounding colonies on medium containing milk is due to extracellular protease activity. The full genotypes of strains MH97 (WT), MK198 (xprG2), MK481 (6015Δ), MK320 (hxkDΔ3), MK186 (hxkD1 xprG2), MK532 (hxkDΔ3 6015Δ), MK388 (hxkCΔ1), MK408 (hxkCΔ1 xprG2), and MK531 (hxkCΔ6015Δ) are given in Table 1.

**Author contributions**
MK conceived the study, MK, KB, and HN designed the experiments, MK, KB, GY, and SC carried out the experiments, CG analysed the microarray data, MK, HN and CG contributed to the preparation of the manuscript. All authors, except GY, were involved in the revision of the draft manuscript and have agreed to the final content. In spite of repeated attempts, MK has not been able to contact GY in China, but does not wish to omit him from the manuscript as he carried out important experimental work when he was visiting MK’s laboratory.

**Competing interests**
No competing interest have been disclosed.

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Supplementary figures

**Supplementary Figure 1.** Agarose gel electrophoresis of qRT-PCR products. The template was total RNA extracted from an xprG\(^+\) (+) and xprG\(\Delta\)1 (−) strains transferred to glucose (+) or carbon-free medium (−) for 16 h. A 100 bp ladder (Axygen) was used as a standard in the first and last lanes of the 2% agarose gel. The full genotypes of the xprG\(^+\) strain (MH2) and the xprG\(\Delta\)1 strain (MK422) are given in Table 1.

**Supplementary Figure 2.** Sterigmatocystin extracted from cultures grown on solid medium. Samples of two xprG\(^+\) strains (lane 1 MH2, Lane 2 MH97), two xprG\(\Delta\)1 strains (lane 3 MK85, lane 4 MK86) and four xprG\(^-\) strains (lane 5 MK198, lane 6 MK413, lane 7 MK414, lane 8 MK422) was analyzed using thin layer chromatography with a benzene: glacial acetic acid (95:5 vol/vol) as described in the experimental procedures. A sterigmatocystin (ST) standard (Sigma) was applied as standard.
## Supplementary table

### Supplementary Table 1. Oligonucleotides used in qRT-PCR experiments.

| Gene | Oligonucleotide sequence* | Positionb |
|------|--------------------------|-----------|
| actA | 5’- AGAGGAAGTTGCTGCTCTCG -3’ (F) | 6         |
|      | 5’- GGATACCACGCTTGGACTGT -3’ (R) | 193       |
| benA | 5’- CGTGAGATCGTACCCTTCA -3’ (F) | 4         |
|      | 5’- GAAGGCCGAGGTACCATTG -3’ (R) | 127       |
| briA | 5’- TCATCAAGCAGTGCAGTGCC -3’ (F) | 941       |
|      | 5’- CGTATATGCGGGCGTTGAGGT -3’ (R) | 1116      |
| chiB | 5’- ACGATAGCAGCTCAGAAC -3’ (F) | 425       |
|      | 5’- TCTCCCTAGCCGGAGCTTA -3’ (R) | 568       |
| ppgA | 5’- TGCCGCTGAATTACACAT -3’ (F) | 66        |
|      | 5’- CGGAACCTGCACCACATATT -3’ (R) | 212       |
| tdIB | 5’- GATGGACCTGATTGCTCGT -3’ (F) | 606       |
|      | 5’- TGTGCAGGTAGCATTTGACC -3’ (R) | 727       |
| veA  | 5’- GAGCTGTTGATGCAAGGTCC -3’ (F) | 23        |
|      | 5’- GACGCTCGGGTTTCAAGAGTG -3’ (R) | 196       |

*The sequences labeled (R) are complementary to the sense strand.

bThe position of the 5’ end of each primer in the coding region of the gene (excluding introns) is given. The sequences were obtained from the *Aspergillus Genome Database.*
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Carbon starvation is likely to be a common stress that fungi encounter in the environment. This group has previously identified the xprG gene, which contains a p53 like Ndt80 DNA binding domain, as being involved in the response to starvation. Here they have studied by microarrays the effects of xprG on the response to 16 hours of carbon starvation. They have verified some of the responses by qRT-PCR as well as physiological studies.

Effects on glucose uptake, conidial and hyphal pigmentation, secondary metabolite production and autolysis were verified and are consistent with previous studies. The effects of an xprG gain of function mutation support the results. This work therefore provides strong support for XprG playing an important role in the response to starvation – a novel and significant result which adds to the large body of data relating to genes involved in development and secondary metabolite production in A. nidulans.

A further significant result is the finding that a second Ndt80 domain containing gene, AN6015-designated ndtA, when deleted results in loss of sexual development. This may be related to the known role of Ndt80 in meiosis in Saccharomyces cerevisiae. Interestingly an Ndt80 homolog has been found to be involved in biofilm formation in Candida albicans (Cell 148, 126–138).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 25 March 2013

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Amir Sharon
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The title and abstract are appropriate for the paper. The work presented in this paper is well
planned, experiments are well designed and executed, and the analyses are comprehensive and
provide clear answers to the main questions. Particularly, the interpretation of the micro array
data, analysis of differentially expressed genes, and reference to most significantly changed
gene/gene clusters between the wild type and mutant are excellent.

The conclusions are all well supported by the data and interpreted in a conserved manner. The
results are novel and interesting. All experiments are detailed and clear.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of
expertise to confirm that it is of an acceptable scientific standard.