Diverse Regulation of the CreA Carbon Catabolite Repressor in *Aspergillus nidulans*

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ABSTRACT Carbon catabolite repression (CCR) is a process that selects the energetically most favorable carbon source in an environment. CCR represses the use of less favorable carbon sources when a better source is available. Glucose is the preferential carbon source for most microorganisms because it is rapidly metabolized, generating quick energy for growth. In the filamentous fungus *Aspergillus nidulans*, CCR is mediated by the transcription factor CreA, a C_2H_2 finger domain DNA-binding protein. The aim of this work was to investigate the regulation of CreA and characterize its functionally distinct protein domains. CreA depends in part on *de novo* protein synthesis and is regulated in part by ubiquitination. CreC, the scaffold protein in the CreB-CreC deubiquitination (DUB) complex, is essential for CreA function and stability. Deletion of select protein domains in CreA resulted in persistent nuclear localization and target gene repression. A region in CreA conserved between *Aspergillus* spp. and *Trichoderma reesei* was identified as essential for growth on various carbon, nitrogen, and lipid sources. In addition, a role of CreA in amino acid transport and nitrogen assimilation was observed. Taken together, these results indicate previously unidentified functions of this important transcription factor. These novel functions serve as a basis for additional research in fungal carbon metabolism with the potential aim to improve fungal industrial applications.

KEYWORDS *Aspergillus nidulans*; carbon catabolite repression; cellulases; ubiquitination; protein domains

GLUCOSE is the preferred carbon source for most microorganisms. Selecting the most energetically favorable carbon source is a survival strategy for microorganisms because it supports the rapid growth and development required for colonizing diverse habitats (Ruijter and Visser 1997). This process of carbon source selection is known as *carbon catabolite repression* (CCR). In the filamentous fungus *Aspergillus nidulans*, CCR is mediated by the transcription factor CreA, a C_2H_2 DNA-binding protein (Dowzer and Kelly 1991). On glucose detection, genes encoding enzymes (*e.g.*, xylanases, cellulases, and arabinases) required for the breakdown of alternative carbon sources, such as lignocellulose, are repressed. This is a disadvantage for second-generation (2G) biofuel production that aims to convert nonglucose saccharides (*e.g.*, xylose, arabinose, and cello- and xylooligosaccharides) to biofuels from lignocellulosic plant mass. In *A. nidulans*, CreA directly represses xylanases encoded by *xlnA* and *xlnD* via binding to the consensus DNA sequence 5′-SYGGRG-3′ in the promoter regions of these genes (Tamayo *et al.* 2008). The expression of *xlnR*, encoding the main inducer of xylanase and, to some extent, cellulase-encoding genes, is also under CreA regulatory control (Tamayo *et al.* 2008). Thus, CreA also indirectly represses all the genes that are under the regulatory influence of XlnR. Furthermore, CreA also represses genes involved in arabinose utilization (Ruijter and Visser 1997). Roy *et al.* (2008) previously described four different regions in CreA (Figure 1). CreA contains two C_2H_2-type zinc fingers required for DNA binding followed by a region containing seven alanine residues. Similar to *Trichoderma reesei*, the N-terminal part of CreA contains an acidic amino acid–rich region that is located adjacent to a highly conserved region among *A. nidulans*,

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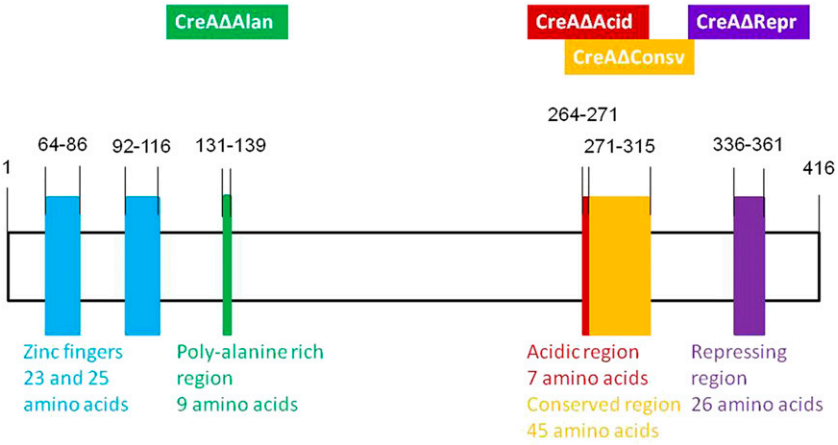

Figure 1 Schematic diagram of the CreA protein domains as proposed by Roy *et al.* (2008). The *creA* gene has 1251 base pairs and encodes a protein of 416 amino acids. CreA domains and corresponding sizes and gene locations are indicated. Above the diagram are the names used throughout this study of the strains with the different CreA regions deleted.

A. niger, and *T. reesei*. This conserved region is followed by a region shown to be essential for repression. Apart from the region important for mediating repression, the other three regions described have yet to be characterized. Previous studies (Ruijter and Visser 1997; Tamayo *et al.* 2008) investigated the repressive role of CreA at the transcriptional level in controlling the expression of genes encoding enzymes required for lignocellulose degradation. Yet few studies have examined how CreA is transcriptionally and post-translationally regulated.

Expression of *creA* was proposed to be autoregulated because CreA-binding sequences are present within its promoter region (Arst *et al.* 1990; Schroff *et al.* 1996; Strauss *et al.* 1999). Furthermore, regulation of CreA is thought to occur via the removal of ubiquitin molecules from the protein that leads to active CreA, a process that may be mediated by the CreB-CreC deubiquitination (DUB) complex (Lockington and Kelly 2002). CreB is a ubiquitin-specific processing (UBP) family protease that functions downstream of CreC; the latter is a WD-40 domain protein required for CreB stabilization (Lockington and Kelly 2002). Deubiquitinating enzymes are cysteine proteases that target the activation domains of specific transcription factors. Ubiquitination serves as a marker on proteins for targeting them to the proteasome, for macromolecular assembly, or for altering protein function (Lockington and Kelly 2002). DUB enzymes also interact with ubiquitin ligases, and together they likely control the amounts of transcription factors present during CCR (Kubicek *et al.* 2009). The CreB-CreC DUB complex has been proposed to be involved in CCR because mutations in CreB and CreC alleviate CCR (Hynes and Kelly 1977). Deletion of *creB* and *cre2* in *A. oryzae* and *T. reesei*, respectively, resulted in elevated levels of secreted hydrolytic enzymes (Denton and Kelly 2011; Hunter *et al.* 2013). Furthermore, recent studies have indicated that FbxA, a protein involved in ubiquitination of target proteins, is involved in *creA* messenger RNA (mRNA) accumulation (Colabardini *et al.* 2012).

Phosphorylation is another post-translational modification that may control the function and/or localization of CreA. The addition or removal of a phosphate group to target proteins by protein kinases and phosphatases regulates structure, localization, and function, playing a crucial role in many cellular processes such as cell fate, metabolism, secretion, and regulation (Ubersax and Ferrell 2007). In *Saccharomyces cerevisiae*, the nuclear localization of Mig1p, the functional homolog of CreA, is regulated by phosphorylation through the protein kinase Snf1p. On detection of phosphorylated glucose, Snf1p is inactivated, and Mig1p is dephosphorylated and localizes to the nucleus (Brown *et al.* 2014). The *T. reesei* CRE1 transcription factor is phosphorylated at Ser^{241} within its acidic domain by a casein kinase II; this post-translational modification is essential for DNA binding and to ensure full repression by CRE1 (Cziferszky *et al.* 2002). Although evidence is lacking for direct phosphorylation of *A. nidulans* CreA, research suggests that kinases are involved in controlling CreA cellular localization. For example, deletion of the two kinases SnfA (homolog of *S. cerevisiae* Snf1p) and SchA (homolog of *S. cerevisiae* Sch9p) prevents CreA from leaving the nucleus in glucose-rich conditions (Brown *et al.* 2013).

The aim of this work was to investigate the regulation of *A. nidulans* CreA and to characterize the distinct CreA domains. CreA depends in part on *de novo* protein synthesis and is regulated by ubiquitination. CreC, the scaffold protein in the CreB-CreC DUB complex, is observed to be essential for CreA function and stability. Deletion of specific regions in CreA results in an inability to leave the nucleus. A region in CreA that is conserved between *Aspergillus* spp. and *T. reesei* is identified as being essential for growth on carbon, nitrogen, and lipid sources. Consequently, a role of CreA in amino acid transport and nitrogen assimilation is also established.

Materials and Methods

Strains and media

A list of all strains used in this study is found in Supplemental Material, Table S1. Strains were grown at 37° (except where stated) in either liquid (without agar and shaking at 180 rpm) or solid (with 20 g/liter agar and no shaking) minimal medium [MM: 1% (w/v) carbon source, 50 ml of a 20× salt solution (120 g/liter $NaNO_3$, 10.4 g/liter KCl, 30 g/liter KH_2PO_4, and 10.4 g/liter $MgSO_4$), and 1 ml of

5× trace elements (22.0 g/liter $ZnSO_4$, 11 g/liter boric acid, 5 g/liter $MnCl_2$, 5 g/liter $FeSO_4$, 1.6 g/liter $CoCl_2$, 1.6 g/liter $CuSO_4$, 1.1 g/liter $(NH_4)_2MoO_4$, and 50 g/liter EDTA)] and adjusted to pH 6.5 with NaOH. Depending on the auxotrophy of the strain, uridine (1.2 g/liter), uracil (1.2 g/liter), or pyridoxine (0.005 mg/μl) was added.

All mycelia were filtered from the supernatant by Miracloth, rinsed with double deionized water (ddH_2O), and immediately snap frozen in liquid N_2. Alternatively, mycelia were filtered by Miracloth, washed two times with ddH_2O, and then transferred to MM supplemented with a different carbon source.

DNA manipulations

A list of primers used for gene fragment and transformation cassette amplifications, for quantitative RT-PCR (qRT-PCR), and for strain confirmation can be found in Table S2. All DNA fragments required for construction of specific strains were amplified by PCR from genomic DNA (gDNA) except where stated. Construction of the whole transformation cassette was carried out in *S. cerevisiae* Sc9721. To construct the CreA-truncated strains (CreAΔAlan, CreAΔAcid, CreAΔConsv, and CreAΔRepr), the following fragments were generated: CreA gene fragment 1 using primers pRS426-CreA-5UTR and CreA rv Alan, CreA rv Acid, CreA rv Consv, or CreA rv Repr to construct the ΔAlan, ΔAcid, ΔConserved, or ΔRepressing fragments and CreA gene fragment 2 using primers CreA fw Alan OH, CreA fw Acid OH, CreA fw Consv OH, or CreA fw Consv Repr and CreA spacer GFP rv to construct the second part of the ΔAlan, ΔAcid, ΔConserved, or ΔRepressing fragments. These two fragments then were followed by the *gfp* gene, amplified with primers Spacer GFP Fw and GFP Afu Rv from plasmid pMCB17apx (kindly provided by Vladimir P. Efimov), and by the *pyrG* gene, amplified with primers GFP PyrG Fw and PyrG Rv from plasmid pCDA21 (Chaveroche *et al.*, 2000). The *pyrG* fragment then was followed by the 3′ UTR of CreA amplified with primers 3UTR CreA Fw PyrG and 3UTR-CreA-pRS426. To construct the luciferase-containing strains (CreA::Luciferase, ClrA::Luciferase, and ClrB::Luciferase), the CreA, ClrA, and ClrB 5′ UTR and gene regions were amplified using primers pRS426-5UTR-CreA, CreA Rv Luc, 5UTR ClrA fw, ClrA Rv Luc, 5UTR ClrB fw, and ClrB Rv Luc. The *luciferase* gene was amplified from plasmid pUC19 containing the gene (kindly provided by Matthias Brock, Germany), whereas *pyrG* was amplified as described earlier. The 3′-UTR regions of the three genes were amplified with primers 3UTR PyrG CreA Fw, 3UTR-CreA-pRS426, 3UTR ClrA PyrG Fw, 3UTR ClrA rv, 3UTR ClrB PyrG Fw, and 3UTR ClrB rv. All 5′ and 3′ UTR DNA fragments contained plasmid pRS426 overhangs.

S. cerevisiae transformations and gDNA extraction

S. cerevisiae strain Sc9721 was used for cassette construction and was transformed with plasmid pRS426 (linearized with the restriction enzymes *Bam*HI and *Eco*RI) and the individual gene fragments using the lithium acetate method according to Schiestl and Gietz (1989). Positive yeast transformation colonies were selected from plates and grown in 5 ml of SC-URA$^-$ liquid medium for 2 days at 30° before DNA was extracted as described previously (Goldman *et al.*, 2003).

A. nidulans transformations and gDNA extraction

Cassettes for *A. nidulans* tranformations were amplified from yeast gDNA that was first checked by PCR to confirm the correct construction using the respective 5′ and 3′ UTR primers with the pRS426 overhang (see earlier) (Table S1). *A. nidulans* strain TN02a3 was used as the DNA recipient strain, and transformations were carried out according to Sambrook and Russell (2001).

Several candidates were selected, and colonies were purified over three rounds (select one colony and grow it on a new MM glucose plate, repeat three times). After purification, mycelia from strain candidates were grown in MM, gDNA was extracted, and PCR reactions were run to confirm the construction using the 5′ and 3′ UTR primers with the pRS426 overhang (as described earlier). gDNA was extracted according to Sambrook and Russell (2001). All strain constructions were confirmed by sequencing, and Southern blots were carried out to confirm single homologous integration (data not shown).

Strain construction by crossing

Strains CreA::GFPxCreB15, CreA::GFPxCreC27, and CreA::GFPxΔkap C, D, G, H, I, J, L, M, and N were constructed by sexually crossing the parental types (Table S1). Strains that were to be crossed were first grown next to each other on plates containing complete medium [0.5% (w/v) yeast extract, 2% glucose, 1 ml of 5× trace elements (see earlier), and 1.7% (w/v) agar]. Pieces of agar containing both strains then were cut out and transferred to plates containing MM without auxotrophic supplements. The plates were sealed hermetically and incubated at 37° for 10 days before cleistothecia were selected and plated. Cleistothecia that contained the recombinant ascospores able to grow on MM without the auxotrophic supplements were plated again to obtain single colonies. Candidate colonies were selected and grown in liquid MM, and gDNA was extracted. Phenotypical tests were carried out on the candidate colonies and/or PCR was run on the gDNAs to confirm the strains.

To confirm the CreB and CreC mutations, candidates were grown on MM plates supplemented with glucose and 20 mM allyl alcohol because mutations in *creB* and *creC* lead to increased sensitivity to allyl alcohol. To confirm the deletion of *kap I*, *J*, *N*, and *D*, candidates were grown on MM plates supplemented with 1% (w/v) xylose and 0.5, 1, and 2 mM 2-deoxyglucose (2DG). Deletion of these genes results in increased sensitivity to 2DG. The deletion of *kap C*, *G*, *H*, *L*, and *M* was confirmed by PCR using primers KapC Fw, KapC Rv, KapG Fw, KapG Rv, KapH Fw, KapH Rv, KapL Fw, KapL Rv, KapM Fw, and KapM Rv (Table S1). The *creA*::*gfp* gene construction was confirmed by PCR using primers pRS426-CreA-5UTR and GFP Afu Rv.

Table 1 Percentage of CreA::GFP that localized to the nucleus, as determined by microscopy, in the presence of glucose and Xylan and in the simultaneous presence of glucose and Xylan with and without cycloheximide (CH)

Glucose 16 hr (%)	Xylan 6 hr (%)	Xylan + glucose 30 min (%)	Xylan + CH + glucose 30 min (%)
93.9	8*	95.5	92.5

Between 100 and 300 nuclei were counted for biological duplicates for each condition (*$P < 0.001$ in a one-tailed equal-variance Student's *t*-test between the different conditions).

Strain complementation

To complement the CreAΔConsv strain, plasmid Af pyroA together with the *creA*::*gfp* cassette, which was PCR amplified from the *A. nidulans* CreA::GFP strain, were cotransformed into CreAΔConsv. Positive transformants did not present any prototrophy. Complemented strains were checked by PCR (as described earlier).

RNA extraction and cDNA synthesis

RNA from mycelia was extracted using TriZol (Invitrogen) and the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA using the Superscript III Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions.

qRT-PCR

qRT-PCR reactions were carried out using the 7500 Fast Real-Time PCR Thermocycler and the 7500 Fast System v1.4.0 (AB Applied Biosystems). Annealing temperature was set at 61°. All reactions were carried out in technical triplicates. Each 20-μl reaction contained 50 ng cDNA or different concentrations of standard-curve gDNA, 10 μl SYBR Green PCR Master Mix (AB Applied Biosystems), and 15 pmol/μl of the forward and reverse primers. Relative quantifications of the respective genes in the unknown samples were calculated as a reference to a standard curve. Gene expression of *creA* was quantified using primers CreA qRT Fw and CreA qRT Rv, whereas *xlnA*, *eglA*, and *xlnR* gene expressions were determined by using the corresponding qRT primers in Table S2.

Microscopic analysis

Coverslips were placed inside small Petri dishes containing 5 ml MM supplemented with the respective carbon source. Spores were added, and plates were incubated at 22° overnight or at 37° for 8 hr. All cellulose- and Xylan-grown hyphae were first fixed [3% (v/v) formaldehyde and 1.5% (v/v) DMSO in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4)] at room temperature for 4 min before nuclei were stained with 1 μg/ml Hoechst 33342 (Life Technologies) at room temperature for 5 min. Cycloheximide was added at a final concentration of 100 μg/ml for 1 hr at the desired temperature.

Mycelia were viewed under a Carl Zeiss (Jena, Germany) AxioObserver.Z1 fluorescent microscope equipped with a 100-W HBO mercury lamp using a 100× magnification oil immersion objective (EC Plan-Neofluar, NA 1.3). Phase-contrast bright-field and fluorescent images were taken with an AxioCam camera (Carl Zeiss) and processed using the AxioVision software (v3.1). Hoechst-stained hyphae were viewed with the same light spectrum used for DAPI staining. Between 150 and 300 nuclei were viewed for each condition. The number of nuclei that contained CreA::GFP was counted before the percentage of nuclei containing CreA::GFP was calculated.

Luciferase experiments

MM (10 ml) supplemented with different carbon sources was inoculated with 10^7 spores and 2 mM D-luciferin potassium salt (Gold Bio, St. Louis, MO). From this mix, twelve 350-μl replicates were pipetted into an all-white 96-well plate (Greiner Bio-One, Americana, SP, Brazil). Plates were incubated at 30° for the indicated amounts of time. Luminescence readings of the plates were taken at the specified time points in a SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA), and results were exported into Excel and analyzed.

Immunoprecipitation (IP)

Mycelia were ground to a fine powder under liquid N_2, and 500 mg was resuspended in 1 ml of 50 mM Tris-HCl (pH 7.6), 225 mM KCl, and 1% (v/v) IGEPAL (Sigma) supplemented with 1 mM sodium vanadate, 10 μl/ml phosphatase-inhibitor cocktail (Sigma), and EDTA-free protease-inhibitor cocktail tablets (1 tablet/10 ml; Roche). Samples were kept on ice for 30 min and mixed every 10 min before being centrifuged at maximum speed for 20 min at 4°. Supernatant was removed, and a Bradford assay (BioRad) was carried out to measure protein content. The same amount of protein for each sample was added to 20 μl of the GFP-Trap_A resin (ChromoTek, Planegg-Martinsried, Germany). The resin was washed three times with resuspension buffer prior to incubation. Cell extracts and resin then were incubated with shaking at 4° for 4 hr. After incubation, the resin was spun down for 30 ses at 5000 × *g* and washed three times in resuspension buffer. To release the proteins from the resin, samples (and all other nonimmunoprecipitated proteins samples) were incubated with NuPAGE Sample Buffer and reducing agent and boiled at 98° for 5 min before being run on premade gels according to the manufacturer's instructions (NuPAGE Bis-Tris Mini Gels, Novex Life Technologies).

Western blotting

Nonimmunoprecipitated samples were run on a 4% stacking and 12% resolving self-made gel according to the manufacturer's instructions (BioRad: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf), and immunoprecipitated samples were run on premade gel (NuPAGE Bis-Tris Mini Gels, Novex Life Technologies). Proteins were transferred to a membrane using the iBlot 2 Dry Blotting System (Life Technologies) according to the manufacturer's instructions. Membranes were blocked for 1 hr in 5% (w/v) dry skimmed milk in 1× TBS-T [0.14 M NaCl, 0.02 M Tris, and 0.1% (v/v) Tween 20, pH 7.6] at room temperature. Antibodies were

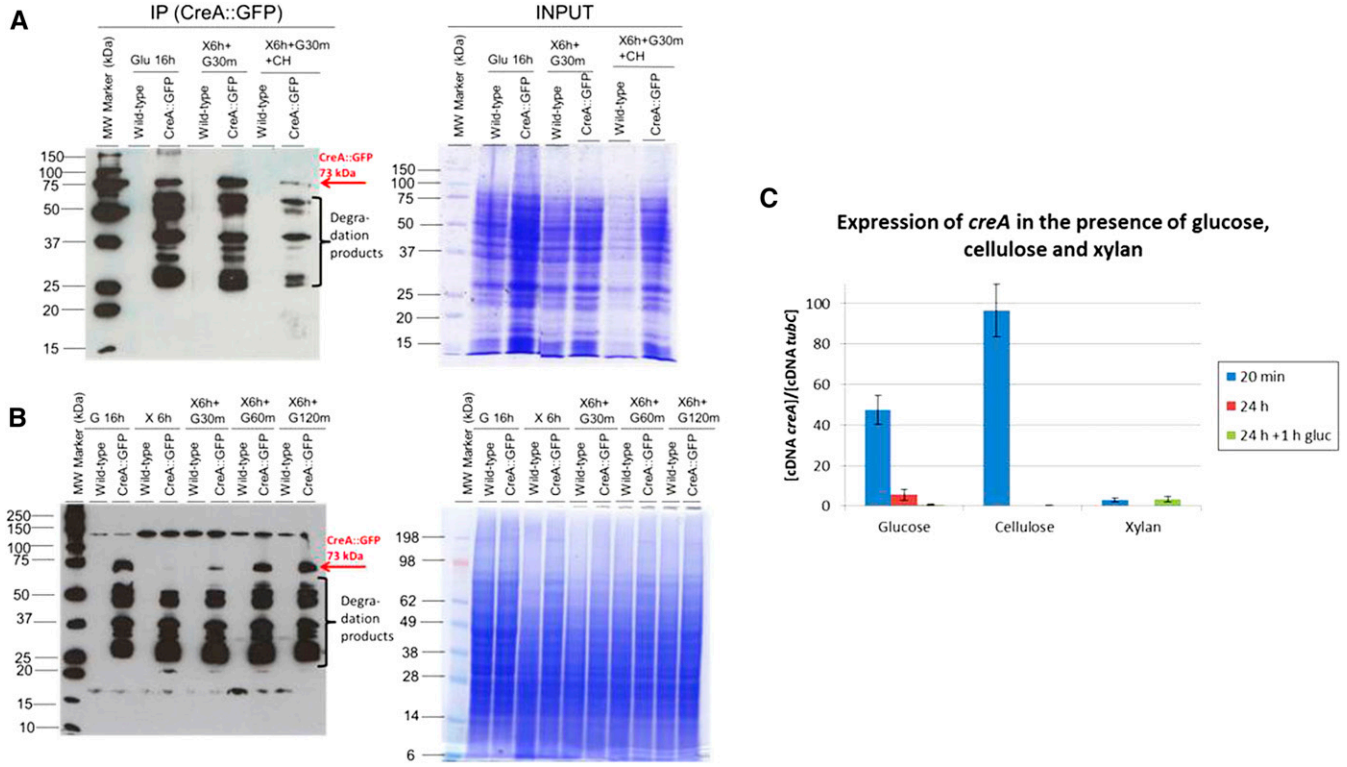

Figure 2 CreA does not require *de novo* protein synthesis and is regulated via partial degradation. (A) (Right) Western blot of immunoprecipitated CreA::GFP protein from whole-cell protein extracts of strains TN02a3 (wild-type) and CreA::GFP. Mycelia were grown from spores for 16 hr in glucose (Glu) and then transferred to Xylan for 6 hr before glucose was added for 30 min (X6h+G30m) in the absence or presence of cycloheximide (+CH). (Left) Coomassie-stained SDS-PAGE gel of whole-cell protein extracts before immunoprecipitation. CreA::GFP is indicated by a red arrow. (B) (Right) Western blot of immunoprecipitated CreA::GFP protein from whole-cell protein extracts. Mycelia were grown from spores for 16 hr in glucose (G) and then transferred to Xylan (X) for 6 hr before glucose was added for 30 min (X6h+G30m), 60 min (X6h+G60m), and 120 min (X6h+G120m). (Left) Coomassie-stained SDS-PAGE gel of whole-cell protein extracts before immunoprecipitation. CreA::GFP is indicated by a red arrow. (C) Expression of *creA* after 20 min in the presence of glucose, cellulose, and Xylan at 24 hr or after the addition of glucose for 1 hr, as determined by qRT-PCR. SD is shown for three technical replicates, and values were normalized by tubulin C (tubC) expression.

diluted in, and all membranes were washed at room temperature (3 × 5 min) with 1× TBS-T. After blocking, membranes were washed and incubated with a 1:1000 dilution of antibody against GFP (Abcam) or against ubiquitinylated proteins (anti-ubiquitinylated protein clone FK2; Upstate) overnight at 4°. Membranes were washed and incubated with a 1:10,000 dilution of anti-rabbit or anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, Beverly, MA) at room temperature for 1 hr. Membrane was washed and revealed using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific).

Chromatin IP (ChIP)

All strains were grown for 24 hr in MM supplemented with 1% (w/v) fructose before being transferred to MM supplemented with either 1% (w/v) glucose or 0.5% (w/v) sugarcane bagasse and shaken at 250 rpm for 6 hr at 37°. Samples were cross-linked with formaldehyde, sonicated, and immunoprecipitated as described previously (Chung *et al.* 2014). Briefly, mycelia were cross-linked for 15 min with 1% (v/v) formaldehyde at room temperature before the formaldehyde was quenched with 2 M glycine for 10 min at room temperature. Mycelia were harvested, snap frozen in liquid N_2, and resuspended in ChIP lysis buffer (Chung *et al.* 2014). Samples (2 ml) were sonicated for three cycles of 10 min with 30 sec on and 30 ec off at high power level with the Biorupter UCD-200 (Diagenode, Denville, NJ). After sonication, cell debris was spun down, and supernatants were stored at −80°. To check the sonication, 60 μl of the supernatant was reverse cross-linked. To prepare for IP, Dynabeads Protein A (Life Technologies) were incubated overnight at 4° with 1 μg/100 μl anti-GFP polyclonal antibody (ab290, Abcam) or IgG antibody with rotation. The following day, Dynabeads were washed with ChIP lysis buffer and incubated overnight at 4° with 100 μl (glucose samples) or 300 μl (bagasse samples) of sonicated supernatants with rotation. The Dynabeads were washed, and the protein-DNA complexes were eluted two times at 65° for 10 min with elution buffer. Samples were reverse cross-linked overnight at 65°. Sample DNA was purified using the GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. qRT-PCR was run on the ChIP DNA using primers ChIP 3 Fw and ChIP 3 Rv, which target the *xlnA* promoter region. ChIP–qRT-PCR reactions were carried out in a BioRad Thermocycler (BioRad MyiQ Single Color Real-Time PCR

Table 2 Expression of ClrA::Luciferase, ClrB::Luciferase, and CreA::Luciferase in the presence of glucose (G), cellulose (C), and Xylan (X)

Strain	G 0 hr	G 24 hr	G + G 1 hr	C 0 hr	C 24 hr	C + G 1 hr	X 0 hr	X 24 hr	X + G 1 hr
Wild type	247 ± 56	126 ± 50	207 ± 58	213 ± 42	201 ± 57	265 ± 51	507 ± 55	405 ± 60	405 ± 104
ClrA::Luciferase	337 ± 75	220 ± 71	261 ± 70	335 ± 85	562 ± 108	389 ± 90	290 ± 75	220 ± 66	231 ± 56
ClrB::Luciferase	462 ± 97	361 ± 88	448 ± 75	470 ± 81	1,715 ± 287	657 ± 104	338 ± 80	284 ± 72	310 ± 77
CreA::Luciferase	6608 ± 312	7944 ± 791	8097 ± 931	14,453 ± 475	11,722 ± 522	8319 ± 688	8347 ± 329	2890 ± 253	2007 ± 193

Luminescence was measured in spores (20 min) after 24 hr and before glucose was added to these carbon sources for 1 hr (+ G 1 hr). Luminescence values are given as relative luminescence units (RLU).

Detection System) at an annealing temperature of 60°. All reactions were carried out in technical duplicates. Each 20 μl contained 10 μl SYBR (BioRad iQ SYBR Green Supermix), 5 pmol/μl of the forward and reverse primers XlnA ChIP Fw and XlnA ChIP Rv, and 1 μl (∼25 ng) of gDNA recovered after IP.

Samples that were cross-linked but not immunoprecipitated (input) were used as positive controls, whereas samples incubated with IgG antibody were used as negative controls. Calculations were carried out using the percent input method (https://www.thermofisher.com/br/en/home/life-science/epigenetics-noncoding-rna-research/chromatin-remodeling/chromatin-immunoprecipitation-chip/chip-analysis.html), and all samples were normalized by the expression of the β-tubulin-encoding gene.

Cellulase and xylanase enzyme assays

Endoxylanase (endo-1,4-β-xylanase) and cellulase activities were measured using Azo-Xylan and Azo-cellulose (Birchwood; Megazyme International, Bray, Ireland) as a substrate. The enzyme assay was carried out according to the manufacturer's instructions. Briefly, the enzyme-containing supernatants were first diluted (as required) in 100 mM sodium acetate buffer (pH 4.5). Then 500 μl of the diluted enzyme preparation was mixed with 500 μl of substrate solution [1% (w/v) Azo-Xylan or 1% (w/v) Azo-cellulose], and samples were incubated at 40° for 10 min before the reactions were stopped via the addition of 2.5 ml of ethanol (95% v/v). Samples were centrifuged for 10 min at 1000 × *g*. The absorbance of the sample supernatants was measured at 590 nm. Enzymatic activity was determined using Mega-Calc software and the standard curve (Megazyme International).

Amino acid quantification

The concentration of amino acids in the supernatants of the CreA::GFP and CreAΔConsv::GFP strains, when grown for 16 hr in MM supplemented with glucose and then transferred to MM supplemented with 50 mM leucine or valine, was measured using the Branched Chain Amino Acid Kit (Sigma) according to the manufacturer's instructions. Experiments were carried out in triplicate.

Data availability

The authors state that all data necessary for confirming the conclusions presented in this article are represented fully within the article.

Results

CreA depends in part on de novo protein synthesis

To gain insight into the regulation of CreA, we investigated whether CreA requires *de novo* protein synthesis or whether cellular protein pools of this transcription factor are always available. Recently, it was observed that CRE1 in *T. reesei* does not require *de novo* biosynthesis and is imported into the nucleus from a preformed cytoplasmic pool (Lichius *et al.* 2014). Microscopy was carried out on *A. nidulans* CreA::GFP germlings, where the *creA* (AN6195) wild-type allele was replaced with the *creA*::*gfp* allele (strain TN02a3) (Table S1) (Brown *et al.* 2013), grown for 16 hr in glucose (CreA target genes repressed; CreA localizes to the nucleus), and then transferred to Xylan for 6 hr (CreA target genes derepressed; CreA leaves the nucleus) before glucose was added to the Xylan cultures for 30 min (Table 1). The protein synthesis inhibitor cycloheximide (which blocks the elongation step during protein translation) was added to the cultures (control condition without cycloheximide) during the last hour of the Xylan incubation to a final concentration of 100 mM. As a control, the efficiency of 100 mM cycloheximide was assessed and found to suppress hyphal growth after 7 hr at 37° in glucose (Figure S1). The amounts of CreA::GFP localized in the nuclei of fungal germlings in the presence and absence of cycloheximide were very similar (∼93%) (Table 1). To support the microscopy results, IP for GFP of whole-cell protein extracts, grown under the same conditions as described earlier, was accomplished, and Western blots with antibody against GFP were carried out. CreA::GFP, which has a predicted size of ∼73.5 kDa (compared to 44.7 kDa without GFP), was detected in the IP samples containing CreA::GFP but not in the wild-type strain (Figure 2A). CreA::GFP was detected in IP samples from mycelia treated with cycloheximide 30 min after the addition of glucose to the Xylan cultures (Figure 2A), supporting the results obtained with microscopy. In addition, CreA::GFP degradation products were recognized by the GFP antibody, and similar degradation products also were observed for CRE1 and XYR1 in *T. reesei* (Lichius *et al.* 2014). *creA* mRNA levels were similar for the wild-type and GFP-tagged strains under the previously described conditions (Figure S2A), indicating that transcription of *creA* was not affected by the *gfp* tagging or by cycloheximide. These results show that CreA is not totally dependent on *de novo* protein synthesis in *A. nidulans*, as also described for CRE1 in *T. reesei*.

Although CreA protein was detectable in the presence of cycloheximide, total protein abundance was lower, leading to the hypothesis that CreA may be partially degraded. To confirm this, Western blots of CreA were performed when strain CreA::GFP was grown for 16 hr under glucose-rich conditions and then transferred to Xylan-rich media for 6 hr before glucose was added for 30, 60, and 120 min. Results indicate that there is partial degradation of CreA because its abundance increases proportionally to the length of glucose incubation (Figure 2B). To validate the protein data, a CreA::Luc strain (*creA* fused to the *luciferase* gene) was constructed under the CreA native promoter. On expression, luciferase cleaves the substrate D-luciferin, and luminescence is emitted that can be measured in actively growing cells, allowing *in vivo* monitoring of CreA expression. This gene reporter system has been used previously to confirm gene expression and *in vivo* activity of proteins in *A. fumigatus* (Paul *et al.* 2012; Galiger *et al.* 2013) and *Neurospora crassa* (Larrondo *et al.* 2012). The wild-type strain, which does not contain the *luc* (*luciferase*) gene, was used as a negative control, whereas strains ClrA::Luc and ClrB::Luc were used as positive controls and served as comparisons for CreA expression. *A. nidulans* ClrA (AN5808) and ClrB (AN3369) are transcription factors important for cellulase induction (Coradetti *et al.* 2012). All strains were grown in 1% glucose, 1% Avicel cellulose, or 1% Xylan for 24 hr before glucose was added to a final concentration of 1% (w/v) for 1 hr. Results show that CreA is present in the conidia and actively growing cells in the presence of glucose and in the presence of derepressing carbon sources (Table 2). When compared to the luminescence levels of ClrB in all tested conditions (including 24-hr cellulose, where ClrB is induced), expression of CreA is almost 10 times higher (Table 2), indicating that CreA is more abundant than ClrB within the cell and that CreA expression remains high in derepressing carbon sources. In addition, the intensity of the luminescence is not the same for all conditions, indicating fluctuations in CreA activity/abundance (Table 2). A similar pattern of expression, as seen for the luminescence levels, also was observed at the transcriptional level when the wild-type and CreA::Luciferase strains were grown under the same conditions as described in Table 2 (Figure 2C). Differences between both sets of results show that protein activity is not necessarily reflected by the transcriptional activity of the corresponding gene. In summary, these results indicate that CreA is only partially dependent on *de novo* protein synthesis. As in *T. reesei*, small cytoplasmic pools of CreA are readily available, and more protein is synthesized once glucose is detected. This complexity in CreA protein turnover and synthesis also suggests that CreA is subject to post-translational modifications.

The scaffold protein CreC is important for CreA function

Previous work has shown that (de)ubiquitination, carried out by the CreB-CreC DUB complex, plays a role in CCR, and it was suggested that CreA is deubiquitinylated by the CreB-CreC complex (Hynes and Kelly 1977; Lockington and Kelly 2002).

Table 3 Percentage of CreA::GFP localized to the nucleus, as determined by microscopy, in the wild-type, *creB15*, and *creC27* background strains in the presence of glucose and Xylan after transfer from glucose to Xylan or after the addition of glucose to Xylan-grown cultures

Strain (%)	Glucose 16 hr (%)	Xylan 16 hr (%)	Glucose 16 hr + Xylan 6 hr (%)	Xylan 16 hr + glucose 30 min (%)
CreA::GFP	93.6	30.0	8.0	98.3
CreA::GFP *creB15*	86.4	28.3	16.5	100.0
CreA::GFP *creC27*	29.6**	8.1*	10.2	15.0**

Between 100 and 200 nuclei were counted for biological duplicates in each condition (*$P < 0.01$, **$P < 0.001$ in a one-tailed equal-variance Student's *t*-test between the wild-type and mutated strains for each condition).

CreB (AN3587) is the deubiquitinating enzyme, whereas CreC (AN4166) works as a scaffold protein (Lockington and Kelly 2002). To investigate this hypothesis, the CreA::GFP strain was crossed with the *creB15* and *creC27* mutant strains to generate strains that contain *creA*::*gfp* (expressed from the native promoter) in the *creB15* and *creC27* background strains. CreA cellular localization was assessed by microscopy in strains grown in glucose or Xylan for 16 hr, transferred to Xylan for 6 hr, or after the addition of glucose to the Xylan-grown hyphae for 30 min. In the *creC27* mutant background, a significant reduction in CreA nuclear localization was observed in the presence of glucose, whereas CreA cellular localization was not affected in the *creB15* mutant (Table 3 and Figure S3).

Western blots of immunoprecipitated CreA::GFP in the wild-type and *creB15* and *creC27* mutant strains were carried out. The wild-type strain, which does not contain CreA::GFP, was used as a negative control. In agreement with the microscopy results, CreA was detected after 16 hr of growth in glucose, in Xylan, and after the addition of glucose to Xylan in the wild-type and *creB15* strains (Figure 3). In contrast, CreA was not at all or very weakly detected in the *creC27* strain under all conditions (Figure 3), indicating that the mutation of the gene that encodes this scaffold protein possibly affected the stability of CreA. To check whether the mutation in *creC* affected transcription of *creA*, qRT-PCR on this gene was carried out when the wild-type, CreA::GFP, CreA::GFP × CreB15, and CreA::GFP × CreC27 strains were grown for 17 hr in glucose. The levels of *creA* expression in the CreB and CreC background strains were similar to those obtained for the wild-type strain (Figure S2B).

CreA is regulated by ubiquitination

These results suggest that ubiquitination plays a role in regulating CreA function. CreA could be directly or indirectly (via another protein that is subject to ubiquitination) ubiquitinylated. To investigate this, the wild-type and CreA::GFP strains were grown under the same conditions as in Figure 2B, and then Western blots were performed against ubiquitinylated proteins of IP samples. Membranes were first incubated with anti-GFP antibody to confirm that CreA::GFP was successfully

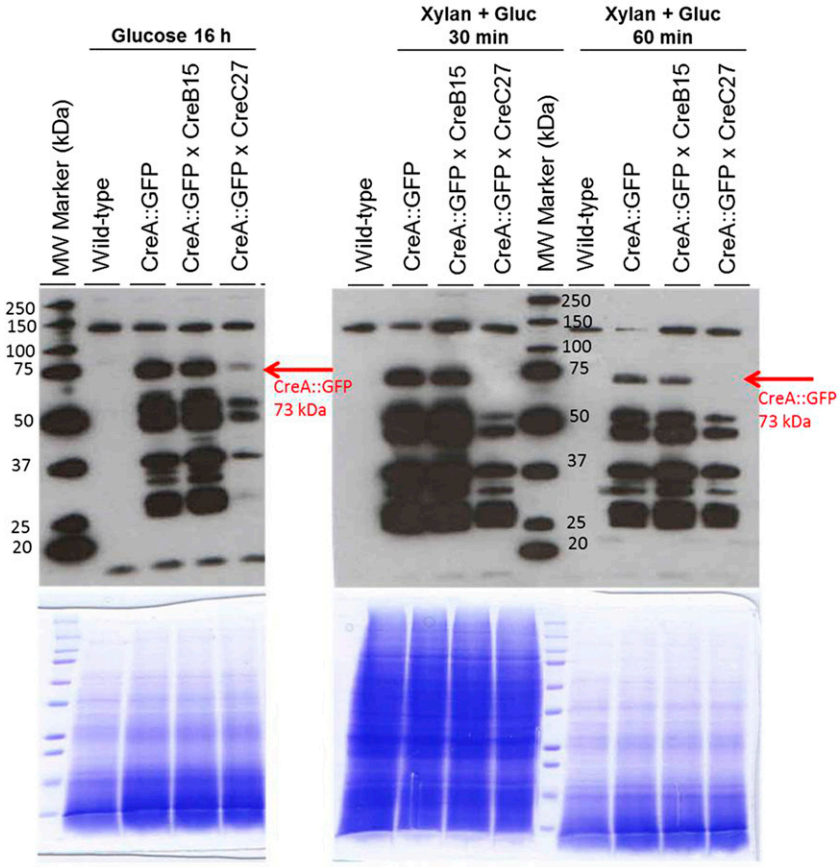

Figure 3 The DUB complex scaffold protein CreC is important for CreA function. (Top) Western blot of immunoprecipitated CreA::GFP protein from whole-cell protein extracts of different strains. Mycelia were grown from spores for 16 hr in glucose and then transferred to Xylan for 6 hr before glucose was added (Xylan + Gluc) for 30 and 60 min. (Bottom) Coomassie-stained SDS-PAGE gel of whole-cell protein extracts before immunoprecipitation. CreA::GFP is indicated by a red arrow.

immunoprecipitated before incubation with an anti-ubiquitin antibody (Figure 4). Results show that CreA and a protein that was immunoprecipitated together with CreA is ubiquitinylated because the smear of ubiquitination was much stronger in the CreA::GFP strain than in the negative-control strain (Figure 4). Furthermore, the intensity of the ubiquitination smear was greater after 6 hr of incubation in Xylan and for the 6-hr Xylan + 30-min glucose samples than when compared to longer incubations (1 and 2 hr) with glucose, suggesting that ubiquitination increases when CreA is derepressed and localized to the cytoplasm. Furthermore, it appears that the ubiquitination smear is between 60 and 100 kDa, suggesting that CreA itself may be subjected to ubiquitination, as well as another protein that is co-immunoprecipitated with CreA. Analysis of the CreA protein sequence in two ubiquitin prediction site programs (CKSAAP_UBSITE and BDM_PUB) (Chen *et al.* 2011; Ao *et al.*, unpublished results) detected one low-confidence site at K47 and one high-confidence site at K275. In addition, CKSAAP_UBSITE identified another low-confidence ubiquitination site at K126. Taken together, these results indicate that CreA may be subject to ubiquitination and that it is interacting with other proteins that are regulated by post-translational modifications.

Truncation of CreA results in it being unable to leave the nucleus in the presence of complex carbon sources

Roy *et al.* (2008) previously described an alanine-rich region, an acidic region, a conserved region, and a region important for repression in CreA (Figure 1). To investigate the function of each region, four CreA-truncated constructions (CreA-ΔAlan, CreAΔAcid, CreAΔConsv, and CreAΔRepr) (see Figure 1) that use *creA* endogenous promoters replacing the wild-type alleles were generated with these regions deleted and fused to *gfp*. All strain truncations/constructions were confirmed by PCR and DNA sequencing (data not shown). Western blots were carried out to confirm that the truncated CreA proteins were present and stable within the respective strains (Figure S4). Defects in CCR were assessed by growing the CreA-truncated strains in the presence of varying concentrations of the glucose analog 2DG or allyl alcohol (AA). Treatment with 2DG inhibits the ability of the cells to complete glycolysis, whereas AA is converted to the cytotoxic compound acrolein by alcohol dehydrogenase. All strains showed, with varying degrees, increased sensitivity to 2DG and increased resistance to AA when compared to the wild-type strain (Figure 5A) when grown in a repressing (glucose) and derepressing (xylose) carbon source. This indicates that CreA mutations result in an inability of CreA to derepress in the presence of complex carbon sources.

To assess whether this inability to derepress depends on CreA cellular localization, microscopy of the wild-type CreA::GFP and the CreA-truncations::GFP was carried out. All strains were grown for 16 hr in glucose and then transferred to cellulose for 6 hr, or they were grown under reverse conditions (16 hr in cellulose before glucose was added for

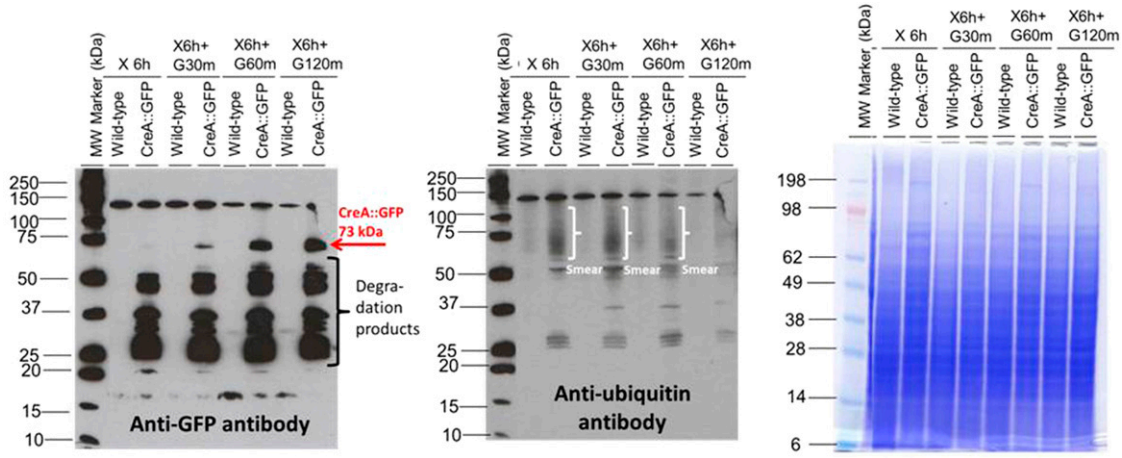

Figure 4 CreA is regulated by ubiquitination. Western blot of immunoprecipitated CreA::GFP protein from whole-cell protein extracts. Mycelia were grown from spores for 16 hr in glucose and then transferred to Xylan for 6 hr before glucose was added for 30 min (X6h+G30m), 60 min (X6h+G60m), and 120 min (X6h+G120m). Membranes were incubated with anti-GFP antibody (right) or anti-ubiquitin antibody (center). (Left) Coomassie-stained SDS-PAGE gel of whole-cell protein extracts before immunoprecipitation. CreA::GFP is indicated by a red arrow and the ubiquitination smears by white braces.

30 min). All CreA-truncated strains, except for CreAΔAcidi::GFP, were unable, to varying degrees, to leave the nucleus under cellulase-inducing conditions (Table 4 and Figure S5). Furthermore, deletion of the conserved region resulted in *A. nidulans* conidia being unable to germinate in the presence of cellulose (Table 4 and Figure 6A). Surprisingly, the CreAΔAcid::GFP strain also was unable to germinate (~93%) in cellulose. Strains also were grown for 16 hr in the presence of Xylan, and similar to cellulose, CreA::GFP was unable to leave the nucleus. In contrast, the CreAΔConsv and CreAΔAcid strains were able to germinate and grow in the presence of Xylan. In summary, truncation of CreA results in this protein being partially "locked" in the nucleus, thus conferring sensitivity to 2DG and resistance to AA. These results also suggest that the mechanism of induction/repression mediated in the presence of cellulose and Xylan appears to be different for each carbon source. Moreover, the expression of the xylanase-encoding gene *xlnA* (AN3613), the cellulase-encoding gene *eglA* (AN1285), and *xlnR* (AN7610), encoding the main inducer of xylanolytic and cellulolytic genes, was reduced in all CreA-truncated strains, except for the CreAΔAlan strain when incubated in the presence of sugarcane bagasse for 6 hr (Figure 5B).

The effect of truncating CreA::GFP on lignocellulosic enzyme secretion then was investigated. Cellulase and xylanase activities were measured in the supernatant of cultures grown in fructose for 24 hr (to get fungal biomass) and transferred to cellulose for 5 days before being transferred again to glucose-rich medium for 24 hr. Cellulase and xylanase activities, which were normalized by intracellular protein concentration, were reduced in all CreA-truncated strains except for the CreAΔAlan::GFP strain (Table 5). The defect in cellulose and xylanase activities was especially severe in the strains with the conserved and repressing regions deleted. This reduction in the secretion of cellulases and xylanases in the CreAΔConsv strain is in agreement with the germination defect observed in the presence of cellulose as well as with the transcriptional data. It has been described previously that in the presence of complex carbon sources such as cellulose and Xylan, CreA leaves the nucleus in order to allow the induction of genes encoding enzymes required for the degradation of these carbon sources (Brown *et al.* 2013). The inability of CreA to leave the nucleus when truncated may result in the continuing repression of cellulose- and xylanase-encoding genes, resulting in very low levels of secreted enzymes.

The CreA conserved region is important for mediating growth on different carbon, nitrogen, and lipid sources

Given the inability of the CreAΔConsv strain to germinate in the presence of cellulose, growth of all strains was examined on a variety of carbon and nitrogen sources and the triglyceride tributyrin (Figure 6B). Deletion of the alanine and acidic regions did not have a major effect on growth in the presence of these carbon, nitrogen, and lipid sources. In contrast, deletion of the conserved region affected growth on complex carbon sources such as Xylan, cellulose, ethanol, tributyrin, and hydrolyzed casein (amino acids) and individual amino acids (Figure 6B). Furthermore, when grown on milk powder, the halo of secreted proteases by the CreAΔConsv strain (and the CreAΔRepr and CreAΔAcid strains) was much smaller than that of the wild-type strain (Figure 6, C and D). Deletion of the repressing region did not result in as severe a reduction in growth as the CreAΔConsv strain but still presented reduced growth in the presence of hydrolyzed casein and other amino acids. These results indicate that the C-terminus of CreA, and especially the conserved region, is important for mediating growth on a wide array of carbon, nitrogen, and lipid sources. Complementing the CreAΔConsv strain restored growth on cellulose, ethanol, and tributyrin (Figure S6), confirming that the reduction in growth is associated with the conserved region.

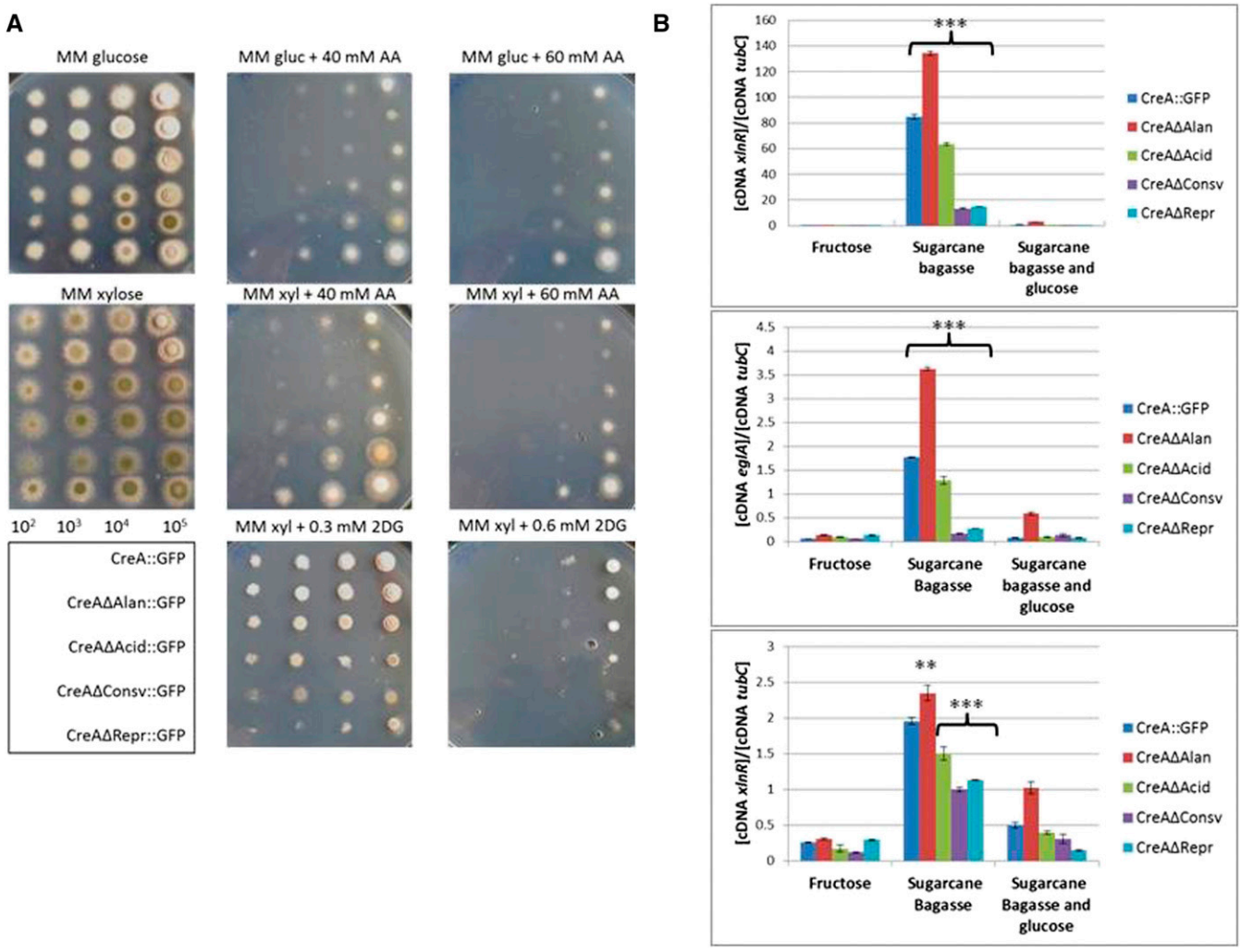

Figure 5 Truncation of CreA results in it being unable to leave the nucleus and in reduced cellulase and hemicellulase gene expression. (A) Growth of CreA-truncated strains on MM containing 1% (w/v) glucose (gluc) or xylose (xyl) supplemented with different concentrations of 2DG and AA. (B) Expression of *xlnA*, *eglA*, and *xlnR* in the wild-type and CreA-truncated strains as determined by qRT-PCR. Strains were grown for 24 hr in fructose and then transferred to sugarcane bagasse for 6 hr before glucose was added to a final concentration of 2% (w/v) for 1 hr. Gene expression was normalized by tubulin C (tubC) expression. SD was calculated for three technical replicates (**$P < 0.005$, ***$P < 0.001$ in an equal-variance paired Student's *t*-test).

To confirm the growth defect associated with deletion of the conserved region, the wild-type CreA::GFP and the CreAΔConsv::GFP strains were grown overnight in MM supplemented with 50 mM leucine or valine. Deletion of the conserved region resulted in the inability of most of the conidia to germinate (81% in leucine and 92% in valine in the CreAΔConsv strain compared with 44% in leucine and 52% in valine in the wild-type strain) (Figure 6A), hence explaining the observed growth defect in the presence of various amino acids.

ChIP qRT-PCR

Deletion of various regions in CreA resulted in the inability of this transcription factor to leave the nucleus. This may cause a permanent repression of genes encoding enzymes required for lignocellulose deconstruction. To determine whether truncation of CreA inhibited it from binding to its DNA target sequences or whether it was still capable of binding to the promoter regions of its target genes, ChIP was carried out on the *xlnA* (AN3613) gene for the wild-type and CreA-truncated strains when grown for 24 hr in fructose-rich medium and then transferred for 6 hr to MM supplemented with either glucose or sugarcane bagasse. Three CreA-binding sites were previously identified in the promoter region of *xlnA*, encoding a xylanase-degrading enzyme (Orejas *et al.* 1999) (Figure 7A). Results show that all strains, except for the CreAΔRepr strain, were able to bind to the *xlnA* promoter region at the third site in the presence of glucose (Figure 7B). In bagasse, however, CreA did not bind to the *xlnA* promoter region in all strains (Figure 7B). These preliminary DNA-binding results indicate that DNA binding in the CreA-truncated strains may not be responsible for the observed decrease in enzyme secretion but rather that protein-protein interactions and post-translational modifications may govern CreA cellular localization.

Table 4 Percentage of CreA::GFP localized to the nucleus, as determined by microscopy, in the wild-type and CreA-truncated strains in the presence of different carbon sources and after transfer from glucose to cellulose or after the addition of glucose to cellulose-grown cultures

Strain	Glucose 16 hr	Avicel 16 hr	Xylan 16 hr	Glucose 16 hr + transfer to Avicel 6 hr	Cellulose 16 hr and glucose 30 min
CreA::GFP	92.6%	16.3%	30.0%	28.1%	100%
CreA ΔAlan::GFP	100%	48.4%*	40.7%	63.5%*	100%
CreA ΔAcid::GFP	100%	No germination	28.2%	4.0%	No germination
CreA ΔConsv::GFP	97%	No germination	90.4%**	80.6%**	No germination
CreA ΔRepr::GFP	98.6%	83.9%**	76.8%**	81.4%**	100%

Between 200 and 300 nuclei were counted for biological duplicates in each condition (*$P < 0.01$, **$P < 0.001$ in a one-tailed equal-variance Student's *t*-test between the wild-type and truncated strains for each condition).

CreA is involved in amino acid transport and metabolism

In addition to severe growth defects in the presence of lignocellulosic carbon sources, the CreAΔConsv strain also showed reduced growth in the presence of various amino acids (Figure 6, A and B). In both cellulose and amino acids, most of the CreAΔConsv conidia were unable to germinate. To determine whether CreA cellular localization is involved in the observed growth defect in the presence of different amino acids (in the presence of complex carbon sources, deletion of the conserved strain resulted in CreA being constantly in the nucleus), microscopy of the wild-type CreA::GFP strain when grown overnight under glucose-rich conditions and then transferred to either leucine or valine for 30, 60, 180, or 360 min was carried out. During the first hour after transfer, around 60–75% of CreA is inside the nucleus, whereas longer incubations (3–6 hr) resulted in 80–90% of CreA localizing to the nucleus (Table 6). Cellular localization of CreA alone therefore cannot explain the observed growth phenotypes of the CreAΔConsv::GFP strain because CreA localized to the nucleus during growth in the presence of leucine and valine.

CreA may play a role in the germination process because deletion of the conserved region resulted in the inability of the fungus to germinate in the presence leucine and valine. To test this hypothesis, the CreA::GFP and the CreAΔConsv::GFP strains were first inoculated directly on plates containing glucose or amino acids as sole carbon sources or on plates containing both glucose and amino acids. Alternatively, both strains were first allowed to germinate in glucose-rich liquid medium for 4 hr at 37° before being transferred to plates containing the same combinations of carbon sources as described earlier. There was no difference in growth of both strains with and without transfer (Figure 8A), indicating that CreA is not required for germination in the presence of amino acids as the sole carbon source.

As a third possibility, the observed growth defect could be due to CreAΔConsv being unable to take up and metabolize the respective amino acids. The wild-type and CreAΔConsv strains therefore were grown for 16 hr in glucose-rich medium before being transferred to MM containing either 50 mM leucine or 50 mM valine. The concentration of amino acids in the supernatant was measured after 15, 30, 60, and 120 min for both strains. The CreAΔConsv strain appeared to take up less leucine and valine into the cell than the wild-type strain (Figure 8B). This deficiency in transport may explain the previously observed growth defect and suggests a role for CreA in amino acid transport.

Alternatively, CreA also could be critical for amino acid metabolism. To test this hypothesis, the wild-type and CreA-truncated strains were grown on agar plates containing glucose and milk powder, with the latter being the only available nitrogen source, supplemented with either sodium nitrate or casamino acids. After 48 hr, the colony and protease halo diameters were measured, and the halo-colony ratio was calculated (Figure 8C). Proteases are secreted by the fungus to degrade proteins, thus providing the fungus with a nitrogen source. When nitrate or casamino acids (mixture of essential and nonessential amino acids) are added, the fungus primarily uses these nitrogen sources because they are more energetically favorable than synthesizing and secreting proteases. Nitrate is reduced by nitrate reductase to nitrite, the first step in nitrate assimilation that ultimately results in the incorporation of nitrogen into cellular substances (Hall and Tomsett 2000). Similarly, the amino acids contained within the casamino acids can be easily taken up and metabolized. Once both nitrogen sources are consumed, the fungus switches back to secreting proteases. As seen in Figure 8C, the halo-colony ratio is reduced in the control condition (glucose and milk), showing a reduction in protease secretion, indicating that CreA is involved in nitrogen catabolite repression. Furthermore, after incubation with nitrate or casamino acids, protease secretion is severely reduced in the CreA-truncated strains, especially in the CreAΔConsv strain, supporting the proposed hypothesis that CreA is involved in the process of nitrogen assimilation/metabolism.

In summary, these results suggest that CreA has a role not only in carbon metabolism but also in amino acid metabolism, including transport and assimilation.

CreA nuclear translocation is not solely dependent on any of the nine nonessential karyopherins

These results show that in addition to post-translational modifications, cellular localization of CreA regulates the

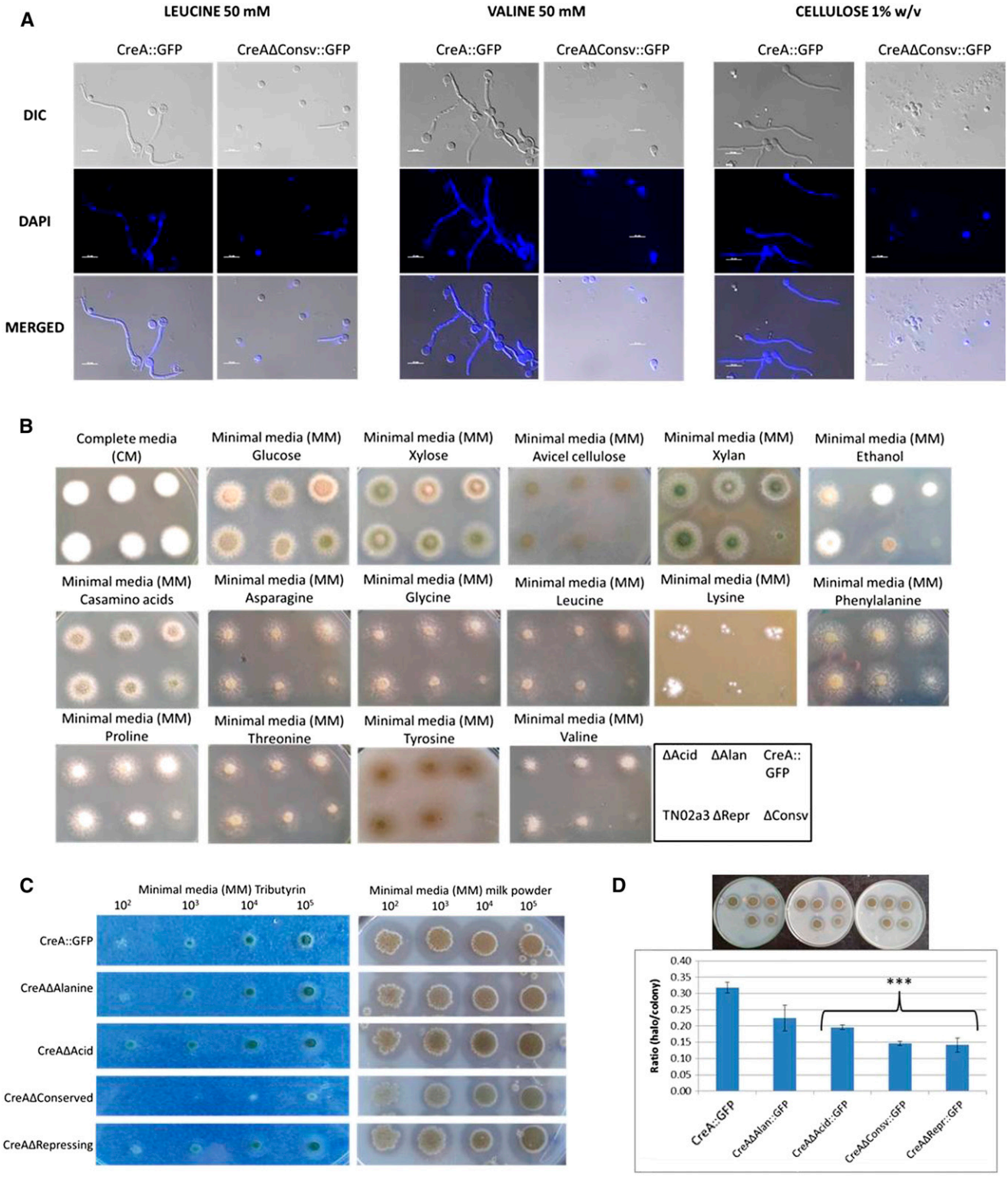

Figure 6 The CreA-conserved region is important for mediating growth in the presence of different carbon, nitrogen, and lipid sources. (A) Deletion of the CreA-conserved region results in the spores being unable to germinate. Photographs were taken by microscopy in the absence (DIC, differential interference contrast) and presence (DAPI) of fluorescence of the wild-type CreA::GFP and CreAΔConsv::GFP strains when grown overnight in MM supplemented with 50 mM leucine (left) or 50 mM valine (middle) or 1% (w/v) cellulose (right). Nuclei were stained with Hoechst and viewed under the DAPI filter. (B) Strains were grown on agar plates containing 1% (w/v) various carbon sources, 1% (w/v) casamino acids, 1% (v/v) ethanol, and 50 mM of individual amino acids or (C) on plates containing 1% (v/v) tributyrin and 1% (w/v) milk powder supplemented with 0.05% (v/v) Triton X-100. (D) Halo-colony ratio of the wild-type and CreA-truncated strains when grown on plates containing 1% (w/v) milk powder supplemented with 0.05% (v/v) Triton X-100. The SD was measured between biological triplicates (***$P < 0.001$ in a one-tailed equal-variance Student's *t*-test).

Table 5 Cellulase and xylanase activities of the CreA::GFP wild-type and truncated strains when grown for 24 hr in fructose-rich medium and transferred for 5 days to cellulose-rich medium before being transferred again to MM supplemented with glucose for 24 hr

Cellulase activity (units/μg intracellular protein)			
Strain	Fructose 24 hr	Avicel 120 hr	Glucose 24 hr
CreA::GFP	0.009 ± 0.002	2.245 ± 0.166	0.012 ± 0.005
CreAΔAlan::GFP	0.001 ± 0.000	11.594 ± 0.711	0.000 ± 0.000
CreAΔAcid::GFP	0.006 ± 0.002	1.609 ± 0.072	0.000 ± 0.000
CreAΔConsv::GFP	0.004 ± 0.001	0.059 ± 0.074	0.000 ± 0.000
CreAΔRepr::GFP	0.009 ± 0.002	0.487 ± 0.281	0.014 ± 0.006
Xylanase activity (units/μg intracellular protein)			
Strain	Fructose 24 hr	Avicel 120 hr	Glucose 24 hr
CreA::GFP	0.002 ± 0. 001	1.407 ± 0.041	0.008 ± 0.001
CreAΔAlan::GFP	0.002 ± 0.001	12.468 ± 0.785	0.003 ± 0.001
CreAΔAcid::GFP	0.004 ± 0.001	3.740 ± 0.140	0.012 ± 0.001
CreAΔConsv::GFP	0.003 ± 0.001	0.300 ± 0.163	0.002 ± 0.000
CreAΔRepr::GFP	0.003 ± 0.000	0.241 ± 0.046	0.005 ± 0.001

expression of genes encoding lignocellulose-degrading enzymes. Recently, Ghassemi *et al.* (2015) identified the β-importin KAP8 to be important for XYR1 nuclear translocation in *T. reesei*. Nuclear transport is carried out by specific receptors or transporters, of which most belong to the karyopherin (Kap) β superfamily (Markina-Iñarrairaegui *et al.* 2011). A total of 14 karyopherins (KapA–N) were identified in *A. nidulans*, and five of them are essential (KapA, -B, -E, -F, and -K) (Markina-Iñarrairaegui *et al.* 2011). Homologs of KapC, -D, -G, -H, -I, and -J (AN0926 , AN6006 , AN2163 , AN4053 , AN5717 and AN2120) are considered importins; a homolog of KapM (AN8787)is an exportin; and homologs of KapL and -N (AN3012 and AN7731)can transport molecules bidirectionally (Markina-Iñarrairaegui *et al.* 2011).

The nine nonessential karyopherin deletion strains were crossed with the CreA::GFP strain, and microscopic studies were carried out. Strains were grown for 8 hr at 37° in MM supplemented with Xylan before glucose was added. In the presence of Xylan, most of the CreA is localized outside the nucleus (90–100%), whereas on addition of glucose, CreA localized back into the nucleus (96–100%) (Table S3). CreA::GFP cellular localization was similar under both conditions between the wild-type and *kap* deletion strains (Table S3). This indicates that none of these nine nuclear transporters is specific for CreA nuclear bidirectional transport.

Discussion

One of the drawbacks of the conversion of lignocellulosic plant biomass to biofuels is the inhibition of genes encoding lignocellulose-degrading enzymes when easily metabolized sugars such as glucose, released during enzymatic degradation of lignocellulose, are detected by the cell (Hsieh *et al.* 2014). Glucose is the preferred carbon source for most microorganisms because it provides a means of obtaining the quick energy required for growth, niche colonization, and survival (Ruijter and Visser 1997). In *A. nidulans*, CCR, mediated by the transcription factor CreA, is a mechanism that represses the use of alternative carbon sources in the presence of glucose (Tamayo *et al.* 2008). Studies of CreA have so far been limited to investigating its role in CCR at the transcriptional level, although some studies suggest a more cell-wide regulatory role for this transcription factor (Portnoy *et al.* 2011). The aim of this work was to study the regulation of CreA at the protein level and to characterize its distinct protein domains in relation to xylanase and cellulase production.

We first investigated whether CreA function depends on *de novo* protein synthesis. Recently, *T. reesei* CRE1 was shown to not require *de novo* protein synthesis and to be imported into the nucleus from a preformed cytoplasmic pool (Lichius *et al.* 2014). Similar to the study carried out in *T. reesei*, *A. nidulans* CreA depends in part on *de novo* protein synthesis. Microscopic studies and Western blots detected the CreA::GFP protein in cell extracts treated with the protein synthesis inhibitor cycloheximide. In addition, Western blots showed that CreA increased proportionally to the incubation time in glucose-rich medium. These results suggest that a small preformed pool of CreA remains in the cytoplasm and that total levels depend in part on *de novo* protein synthesis. Accordingly, the expression of a CreA::Luc strain in the presence of xylanase- and cellulase-repressing and -inducing carbon sources showed major fluctuations in CreA protein levels, but the expression of CreA remained relatively high under all tested conditions, including nonglucose complex carbon sources. The readily available CreA protein pools therefore would allow the fungus to quickly adjust gene expression and metabolism once glucose is detected. Once glucose is detected, protein synthesis occurs and increases cellular CreA pools to optimize energy generation. Furthermore, Western blot results suggest a steady protein degradation rate because CreA::GFP degradation products were observed under all tested conditions. Degradation products also were observed for CRE1 and XYR1 in *T. reesei* (Lichius *et al.* 2014), indicating a dynamic turnover of these important cellular transcription factors.

In addition, these results suggest that regulatory mechanisms other than degradation govern CreA function when it is present in the cytoplasm. Proteins are subjected to post-translational modifications such as ubiquitination that regulate protein function, activity, and cellular localization (Karve and Cheema 2011). Ubiquitination is a post-translational modification in which one, two, or more ubiquitin molecules are added to a target protein (Komander 2009). The DUB complex, formed by CreB-CreC, has been shown to be involved in CCR and CreA regulation (Hynes and Kelly 1977; Lockington and Kelly 2002), and it is thought that deubiquitination prevents CreA from being degraded. This study shows for the first time direct evidence of the involvement of the CreB-CreC DUB complex in CreA cellular localization and stability. The smear caused by the different states of protein ubiquitination in the CreA region appeared to be

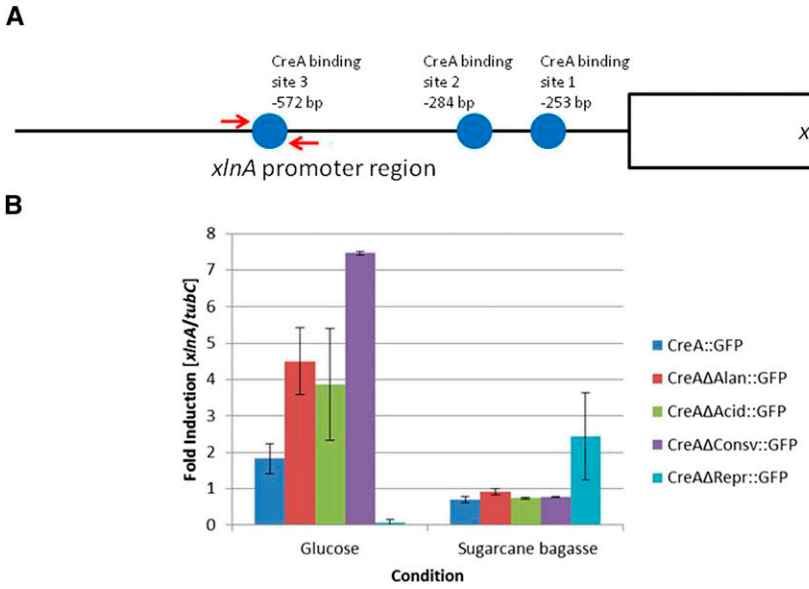

Figure 7 Binding of the wild-type and CreA-truncated strains to the *xlnA* promoter region. (A) Schematic diagram of the CreA-binding sites in the *xlnA* (encoding xylanase A) promoter region. Red arrows indicate the primer pair used in the ChIP–qRT-PCR. (B) Quantity of *xlnA* detected by ChIP–qRT-PCR on CreA binding site 3 in the wild-type and CreA-truncated strains when grown for 24 hr in fructose and then transferred to either glucose or sugarcane bagasse for 6 hr. All *xlnA* expression values were normalized by the quantity of tubC (β-tubulin) in each sample. SDs are shown for technical duplicates.

stronger in the presence of Xylan (CreA-repressing condition) than when the fungus was grown in glucose-rich conditions (CreA-inducing condition), suggesting increased CreA degradation in the presence of Xylan. The ubiquitination smear did not correspond to the CreA::GFP degradation products because overlaying both Western blots (anti-GFP and antiubiquitin blots) did not match. Rather, the ubiquitination smear appears to be localized in the CreA region and below it, indicating ubiquitination, and therefore also deubiquitination, of CreA itself and another protein that may interact with CreA. The identity of this protein is unclear and will be the subject of further investigation. Ubiquitination of CreA appears to take place on three sites within the protein (K47, K126, and K275), which could be a signal for proteasome targeting and subsequent protein degradation. In agreement, ubiquitination of K275, a site that was predicted to be ubiquitinylated with high confidence in CreA, was associated with protein degradation of the human Hsp90 chaperone protein (Kundrat and Regan 2009) and of human liver cytochrome P450 2E1 (CYP2E1) peptides (Wang *et al.* 2011).

This work also uncovered a crucial role of CreC in the regulation and cellular localization of CreA: mutation in CreC, but not CreB, results in CreA not being detected by Western blotting and in the absence of CreA from the nucleus in the presence of glucose. CreC is the scaffold protein required for stabilization of the deubiquitinating enzyme CreB (Lockington and Kelly 2002). A BLAST search of CreC shows high similarity to the CreC proteins from other *Aspergillus* spp. and to a mitochondrial Rho GTPase in *Neosartorya udagawae* and *A. parasiticus* (Miro-2; *E*-value 0.0, identity 76 and 78%, respectively) that is important for mitochondrial homeostasis (Interpro IPR021181 and IPR029506). CreC contains a WD-40 domain that is associated with protein-protein interactions; WD-40 proteins are involved in a wide range of cellular functions (Xu and Min 2011). A BLAST search of CreB and CreC against the *A. nidulans* genome revealed that CreB has some similarity to a region found in three ubiquitin-specific proteases (An5186, An3711, and An2027; *E*-value between 2×10^{-19} and 3×10^{-13}, similarity around 40%) and in a protein with ubiquitinyl hydrolase activity (An6164; *E*-value 1×10^{-13}, similarity 41.8%), proteins that are all involved in diverse deubiquitination processes. CreC has similarity (*E*-value between 5×10^{-13} and 5×10^{-3}, similarity around 45%) to a region in proteins with unknown function or to proteins involved in various cellular functions such as polarized growth, histone H3-K4 methylation, spliceosome components, SAGA complex components, metal ion transport, or mitotic spindle components. It is therefore possible that some redundancy exists between enzymes involved in different deubiquitination processes, whereas CreC is the only WD-40 scaffold protein involved in CreA-related carbon metabolism. Currently, additional work is being carried out to further characterize CreC and identify interaction partners of this protein.

Next, this work aimed at characterizing the different regions of CreA that were previously described by Roy *et al.* (2008). Deletion of these regions resulted in CreA not being able to leave the nucleus under carbon catabolite derepressing conditions. The percentage of CreA that remained in the nucleus depended on each deletion, but overall, deletion of the C-terminal regions (conserved and repressing regions) resulted in the majority of CreA (~90%) being in the nucleus in the presence of Xylan and cellulose or when transferred from glucose- to cellulose-rich conditions. In contrast to Roy *et al.* (2008), all experiments here were carried out under the CreA native promoter (in their paper, the *gpdA* promoter from the glyceraldehyde dehydrogenase was used), which may explain discrepancies between the two studies. This work was not able to identify a nonessential nuclear transporter (karyopherin) specific for CreA nucleocytoplasmic shuttling. This is probably the result of the redundancy that exists between the different nuclear transporters and/or that more than one nuclear transporter is responsible for CreA

Table 6 Percentage of CreA::GFP localized in the nucleus, as determined by microscopy

Condition	CreA in the nucleus (%)	Number of nuclei
Glucose 16 hr	91.3	391
Transfer to leucine 30 min	60.6*	188
Transfer to leucine 60 min	67.5*	268
Transfer to leucine 180 min	88.3	290
Transfer to leucine 360 min	85.5	303
Transfer to valine 30 min	75.7	305
Transfer to valine 60 min	68.1*	254
Transfer to valine 180 min	88.2	254
Transfer to valine 360 min	88.0	217

Strains were grown overnight in glucose-rich medium and then transferred to 50 mM leucine or 50 mM valine for 30–360 min (*$P < 0.01$ in a one-tailed equal-variance Student's *t*-test between the different amino acid conditions when compared to the glucose control condition).

nucleocytoplasmic transport. In this way, the cell ensures that CreA is always correctly localized. Similar observations have been made for the alkaline pH response transcription factor and for CrzA, a transcription factor involved in modulating the cellular response to calcium levels and alkaline pH stress (Fernández-Martínez *et al.* 2003; Markina-Iñarrairaegui *et al.* 2011; Hernández-Ortiz and Espeso 2013). No nuclear localization signal (NLS) was predicted to be contained within CreA in this work or in previous studies (Roy *et al.* 2008). The mechanism of CreA nuclear import therefore remains unknown. In contrast, a nuclear export signal (NES) was predicted (La Cour *et al.* 2004) to be contained between residues 325 and 333, which are located between the conserved and repressing regions. It is possible that deletion of this region (and other regions) causes protein conformational changes that render the NES inaccessible, therefore preventing CreA nuclear export.

Furthermore, the expression of a cellulase- and a xylanase-encoding gene (*eglA* and *xlnA*, respectively) as well as the transcriptional activator *xlnR* was severely reduced in the CreA-truncated strains, especially when the conserved and repressing regions were deleted. In agreement, *A. nidulans* still secreted enzymes, although at lower levels, when the acidic and alanine-rich regions were deleted. In contrast, deletion of the C-terminal regions (conserved and repressing) resulted in extremely low secreted cellulase and xylanase levels. This reduction in enzyme secretion could be the result of CreA cellular localization (and hence DNA binding) because this work showed that truncation of CreA resulted in persistent nuclear localization under derepressing conditions. Thus, to determine whether CreA was still able to bind to specific sites in the promoter regions of its target genes, ChIP–qRT-PCR was carried out on the *xlnA* promoter region. All CreA-truncated strains, with the exception of the CreAΔRepr strain, were able to bind to the *xlnA* promoter region in the presence of glucose. The repressing region, as already suggested by its name, is therefore important for CreA-mediated repression of target genes. This is in agreement with the study carried out by Roy *et al.* (2008). Surprisingly, in the presence of sugarcane bagasse, a derepressing carbon source, CreA did not bind to the *xlnA* promoter region in all the CreA mutant strains. These results indicate that CreA is able to release the DNA (thus responding to the derepressing signal) but cannot, as discussed earlier, leave the nucleus. In addition, these results also suggest that DNA binding alone does not cause repression (and subsequent reduction in xylanase and cellulase secretion) and that additional signals are required for CreA regulation and CreA-mediated repression. One such signal could be ubiquitination because the lysine that was predicted to be ubiquitinylated (K275) is located within the CreA-conserved region. Deletion of this region and the other protein regions therefore could cause a decrease in ubiquitination and protein degradation, hence keeping the CreA protein levels high even in the presence of derepressing carbon sources. The regulatory network in which CreA is embedded therefore is extremely complex and governed by various mechanisms such as cellular localization and nucleocytoplasmic shuttling, the presence of different food sources, and protein-protein interactions.

Interestingly, deletion of the conserved region inhibited germination and growth in the presence of cellulose but not in the presence of Xylan in liquid medium. In solid medium, growth of the same strain was severely reduced in the presence of these two carbon sources. These results indicate that the derepression mechanism differs between complex carbon sources. Although this has not been proposed previously for CreA, XlnR-mediated induction of xylanase- and cellulase-encoding genes is thought to be (at least partially) different from each other (Noguchi *et al.* 2011; Mach-Aigner *et al.* 2012; Kobayashi and Kato 2010). Deletion of the acidic region also resulted in the fungus being unable to grow in the presence of cellulose in liquid medium. It is possible that the acidic region, which is located directly adjacent to the conserved region, is required for function of the latter or that deletion of the acidic region caused conformational changes in the protein that rendered the conserved region inaccessible.

The same defect in growth of the CreAΔConsv strain was observed when grown in the presence of various amino acids. This study showed that a reduction in amino acid transport (at least for leucine and valine) may be the cause for this growth defect. In *T. reesei*, CRE1 was shown to control genes encoding amino acid transporters (Portnoy *et al.* 2011). Furthermore, CreA was shown to play a role in nitrogen assimilation and metabolism because the secretion of proteases, required for the degradation of nitrogen sources, was severely impaired in all the truncated strains. It is possible that the defect in derepression observed for the CreA-truncated strains in carbon metabolism (*e.g.*, Xylan and cellulose) also may affect nitrogen metabolism. The results found in this work therefore suggest that CreA is regulated by various signals related to growth and that the conserved region in CreA may be responsible for responding to this signal. This region is definitely of interest for further study because it is found in industrially relevant *Aspergillus* spp. and *T. reesei*. In the filamentous fungus *Beauvaria bassiana*, deletion of *creA* caused cell lysis and

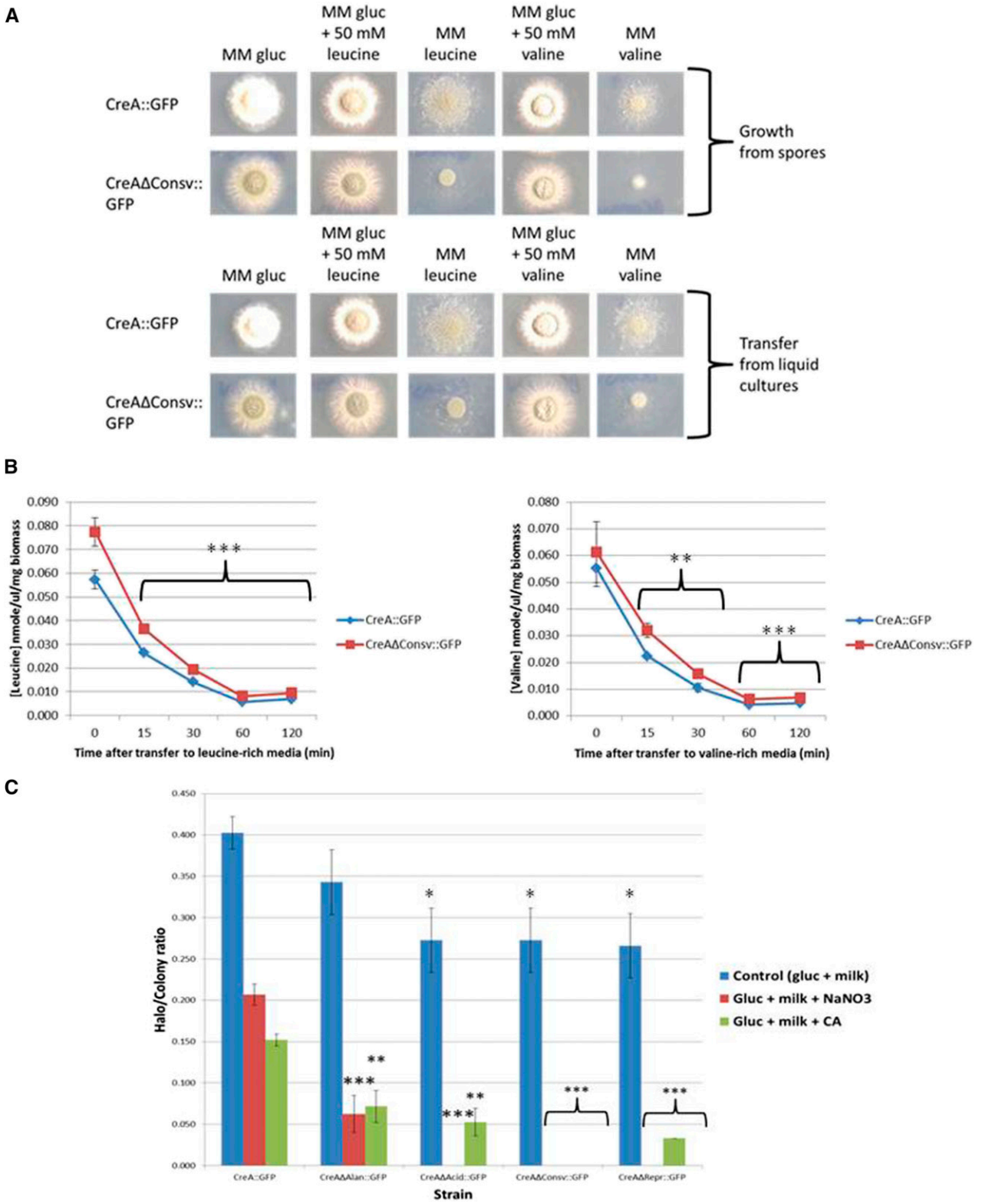

Figure 8 CreA is involved in amino acid transport and metabolism. (A) Growth of CreA::GFP and CreAΔConsv on solid medium supplemented with different carbon and nitrogen sources without and with transfer from liquid cultures (4 hr at 37°). (B) Amino acid uptake of the wild-type and CreAΔConsv strains during a 2-hr incubation in medium supplemented either with 50 mM leucine or valine. Concentrations of the amino acids were measured in the supernatants of biological triplicates and normalized by fungal dry weight (**$P < 0.01$, ***$P < 0.001$ in a paired equal-variance Student's *t*-test). (C) Halo-colony ratio of the growth of wild-type (CreA::GFP) and CreA-truncated strains on plates containing 1% glucose and 1% milk (control) or in the presence of 50 mM $NaNO_3$ or 1% casamino acids (CA). SD was calculated for biological triplicates (*$P < 0.01$, **$P < 0.001$, ***$P <$ 0.0001 in a paired equal-variance Student's *t*-test).

growth impairment when the fungus was grown in the presence of specific amino acids (Luo *et al.* 2014). Cell lysis was not observed for the *A. nidulans* CreA-truncated strains, but deletion of *creA* results in the fungus having severe growth defects on a wide range of carbon and nitrogen sources (data not shown), supporting the proposed hypothesis. CreA therefore seems to be important for growth on many carbon and nitrogen sources, where it regulates the expression of genes required for correctly taking up, degrading, and metabolizing these nutrient sources. The role of CreA is therefore not limited to only CCR but has a cell-wide role in ensuring growth and fungal survival in the presence of diverse carbon and nitrogen sources—functions that could be useful for future strain engineering to improve biotechnological processes such as biofuel production from lignocellulosic biomass.

In conclusion, this study provides an in-depth description of the regulation of CreA at the protein level and characterization of distinct previously identified CreA protein regions. CreA is under the control of a complex regulatory network consisting of partial protein degradation, post-translational modifications, and other signals that occur directly or indirectly (via protein-protein interactions) on CreA. Furthermore, CreA cellular localization is important for carbon metabolism and the expression of genes involved in complex carbon-source utilization. This work also uncovered a role of CreA in amino acid transport and metabolism, attributing a global function in metabolism for CreA not restricted to CCR in the presence of various food sources. These additional functions, which were uncovered in this study, are subject to further investigations into carbon and nitrogen metabolism of filamentous fungi with the aim to engineer fungal strains that will improve biotechnological applications such as second-generation biofuel production from plant biomass.

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Diverse Regulation of the CreA Carbon Catabolite Repressor in *Aspergillus nidulans*

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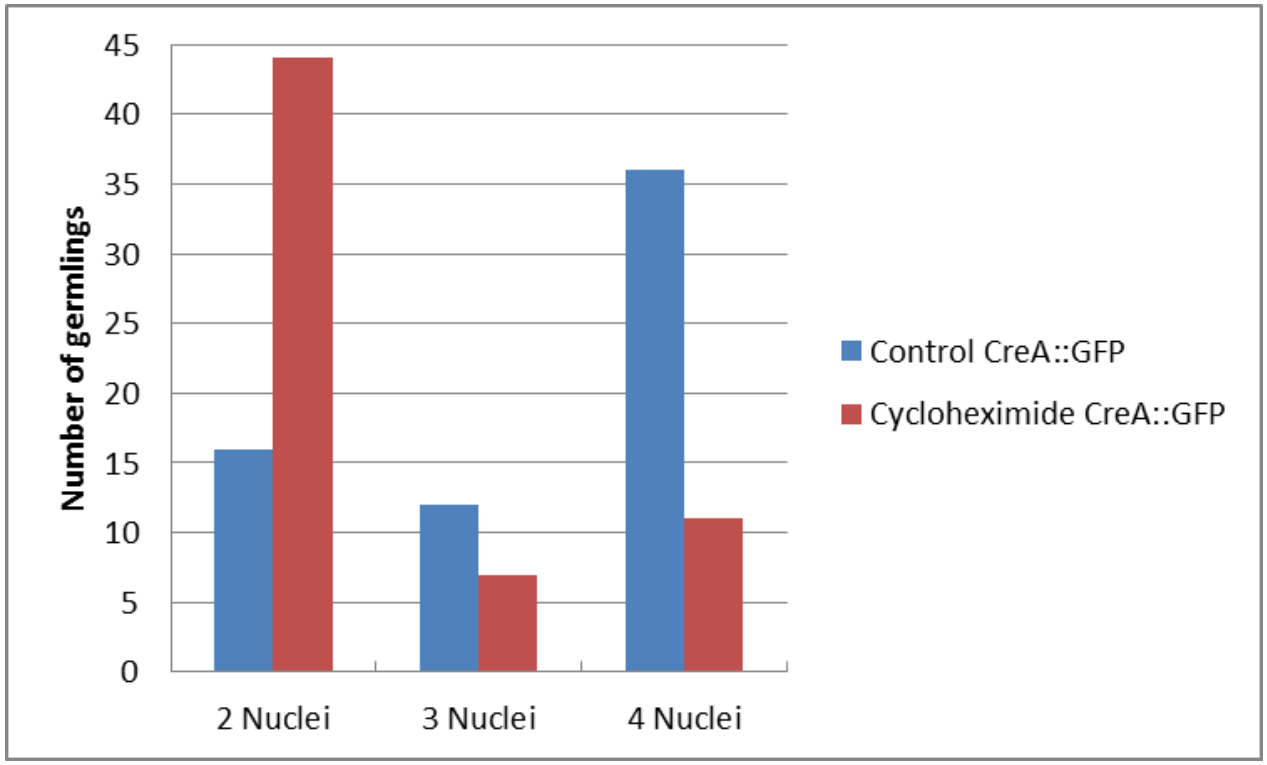
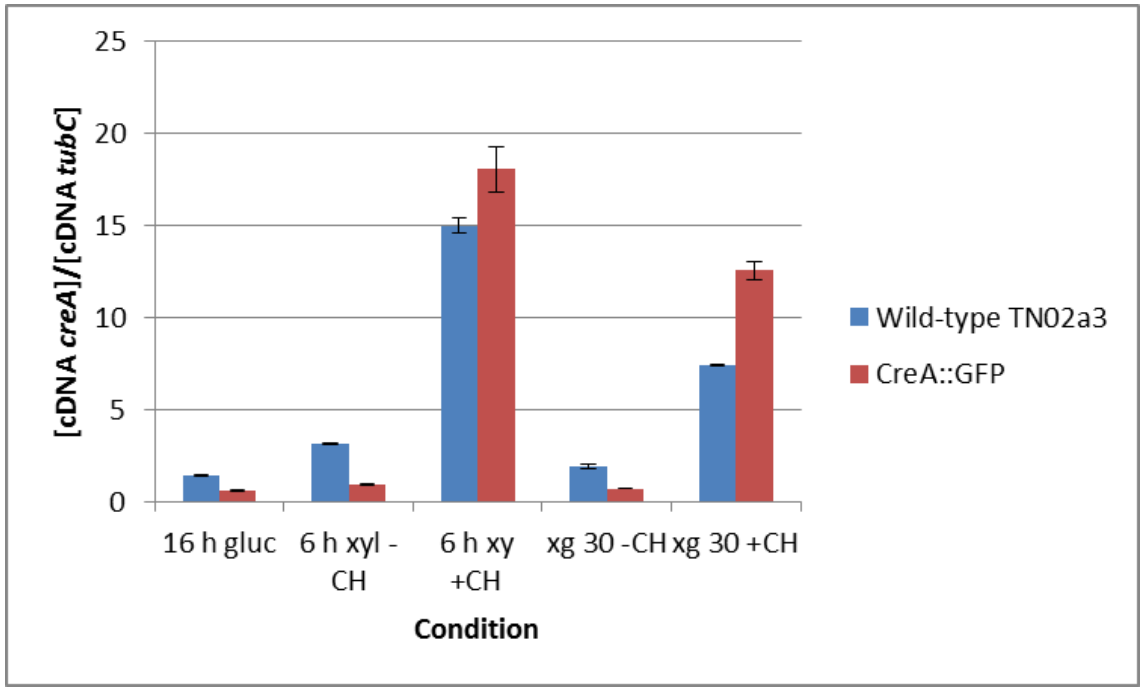


Figure S1: The effect of cycloheximide on hyphal growth. Germlings were grown for 6 h at 37°C before cycloheximide (100 mM) was added for 1 h (control = no cycloheximide). Hyphal nuclei were then stained and germlings with 2, 3 or 4 nuclei respectively were counted. A total of 65 hyphae for each condition were counted.

A.



B.

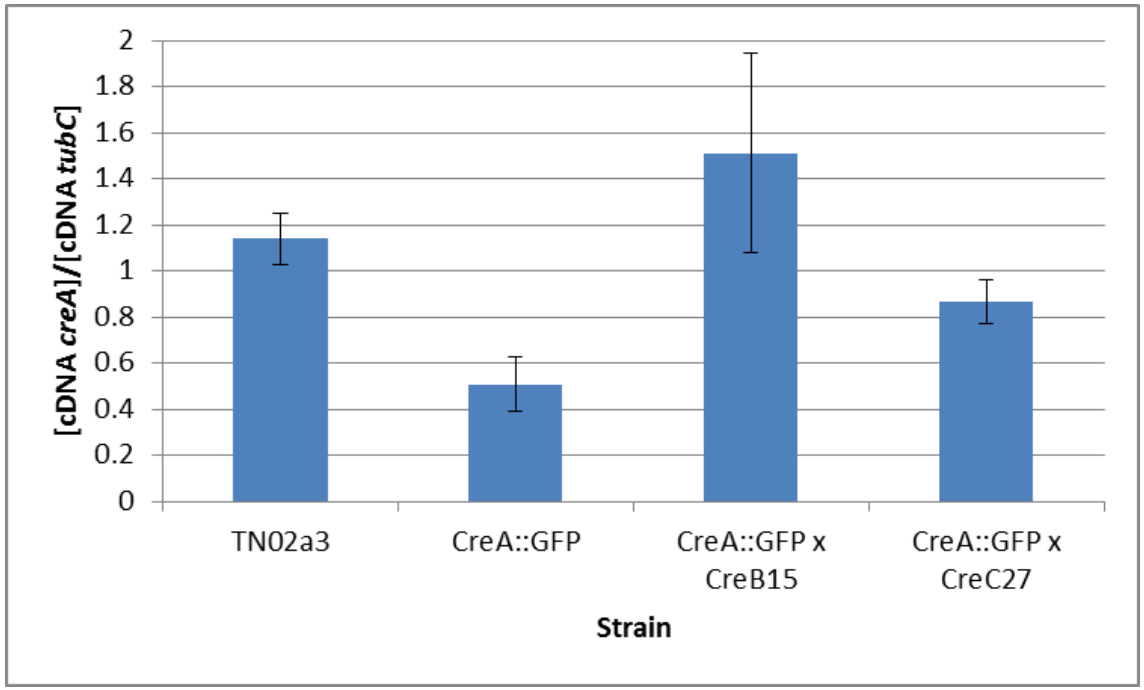


Figure S2: Expression of *creA* in mycelia grown for **A.** 16 h in glucose (16 h gluc), transferred to xylan for 6 h (6 h xyl) with or without cycloheximide (CH), which was added during the last hour of the xylan incubation, before glucose was added to a final concentration of 1% (w/v) for 30 min (xg 30); or **B.** in mycelia from different strains grown overnight in glucose-rich conditions. *creA* gene expression was normalised by *tubC* expression. Standard deviations were measured between biological duplicates and technical triplicates.

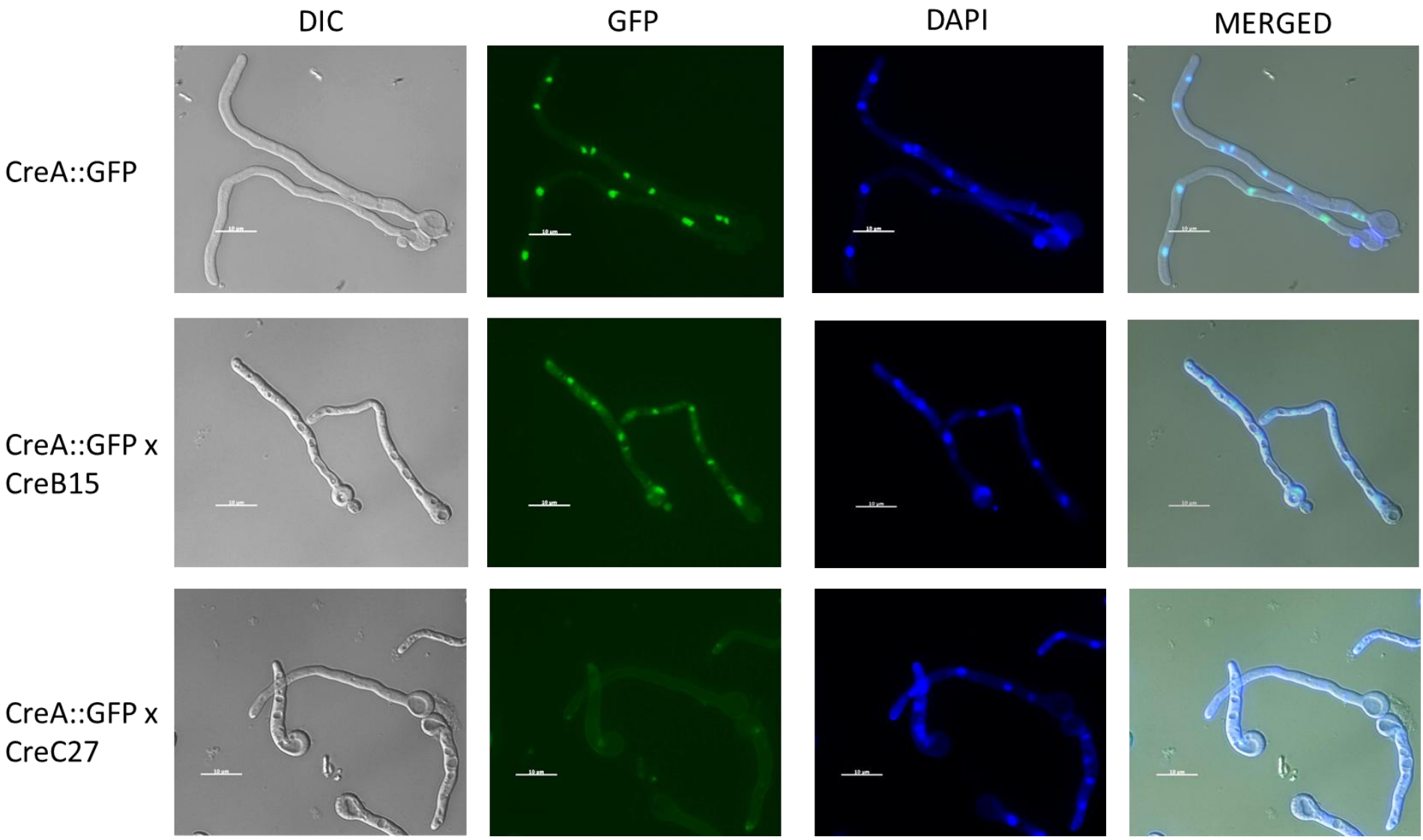


Figure S3: CreA::GFP cellular localisation in the wild-type (CreA::GFP) and the CreB (CreA::GFP x CreB15) and CreC (CreA::GFP x CreC27) mutant strains when mycelia were grown from spores overnight in glucose at 22°C. Pictures were taken in the absence of fluorescence (DIC = differential interference contrast) and in the presence of fluorescence (GFP, DAPI). Fungal nuclei were stained with Hoechst and viewed with the DAPI filter.

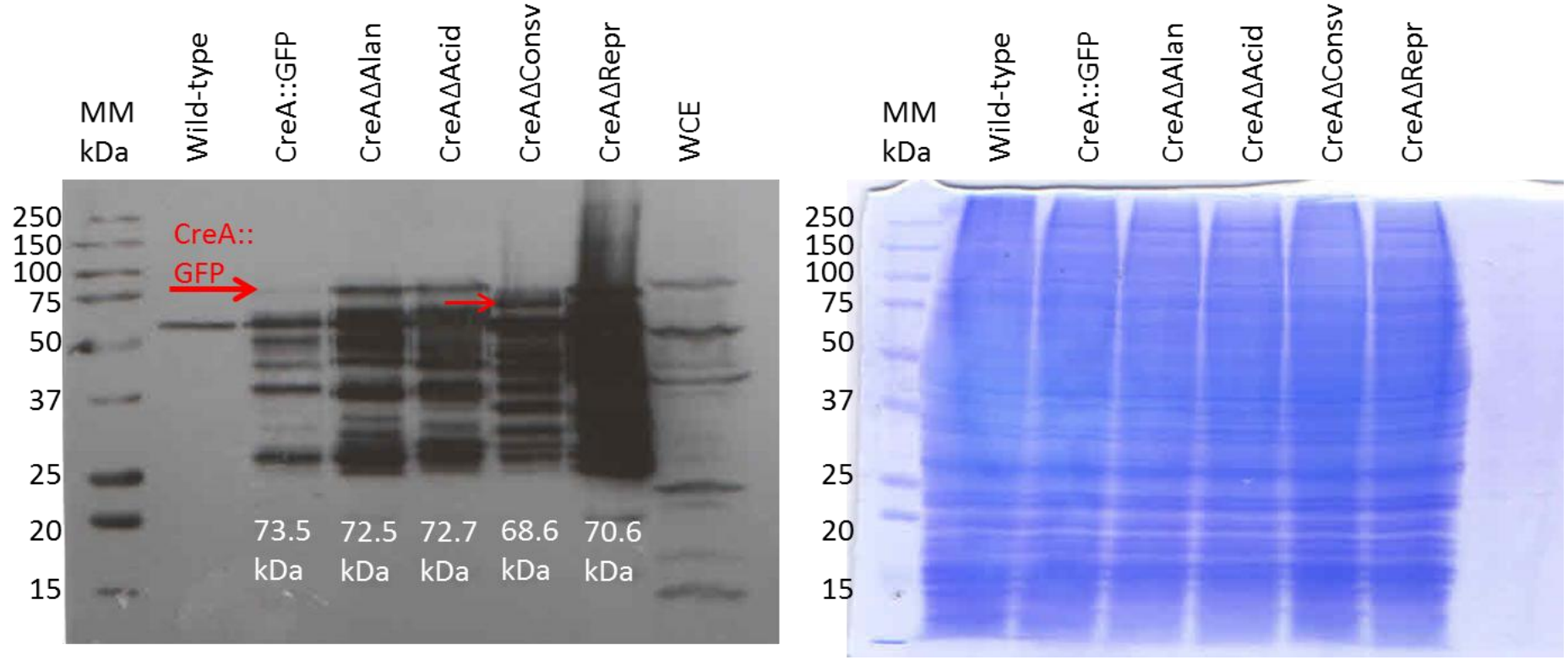


Figure S4: CreA::GFP is present in the CreA truncated background strains. Left: Western blot against GFP of immunoprecipitated CreA::GFP from various strains, including the wild-type strain which serves as the negative control and from whole cell extracts (WCE). All strains were grown for 16 h in glucose-rich conditions. CreA::GFP is indicated by a red arrow and the expected sizes of the truncations are indicated. CreA::GFP is also indicated by a red arrow in the CreAΔConsv strain as this truncations results in a considerably smaller protein. Right: Coomassie stained gel of whole cell protein extracts before immunoprecipitation.

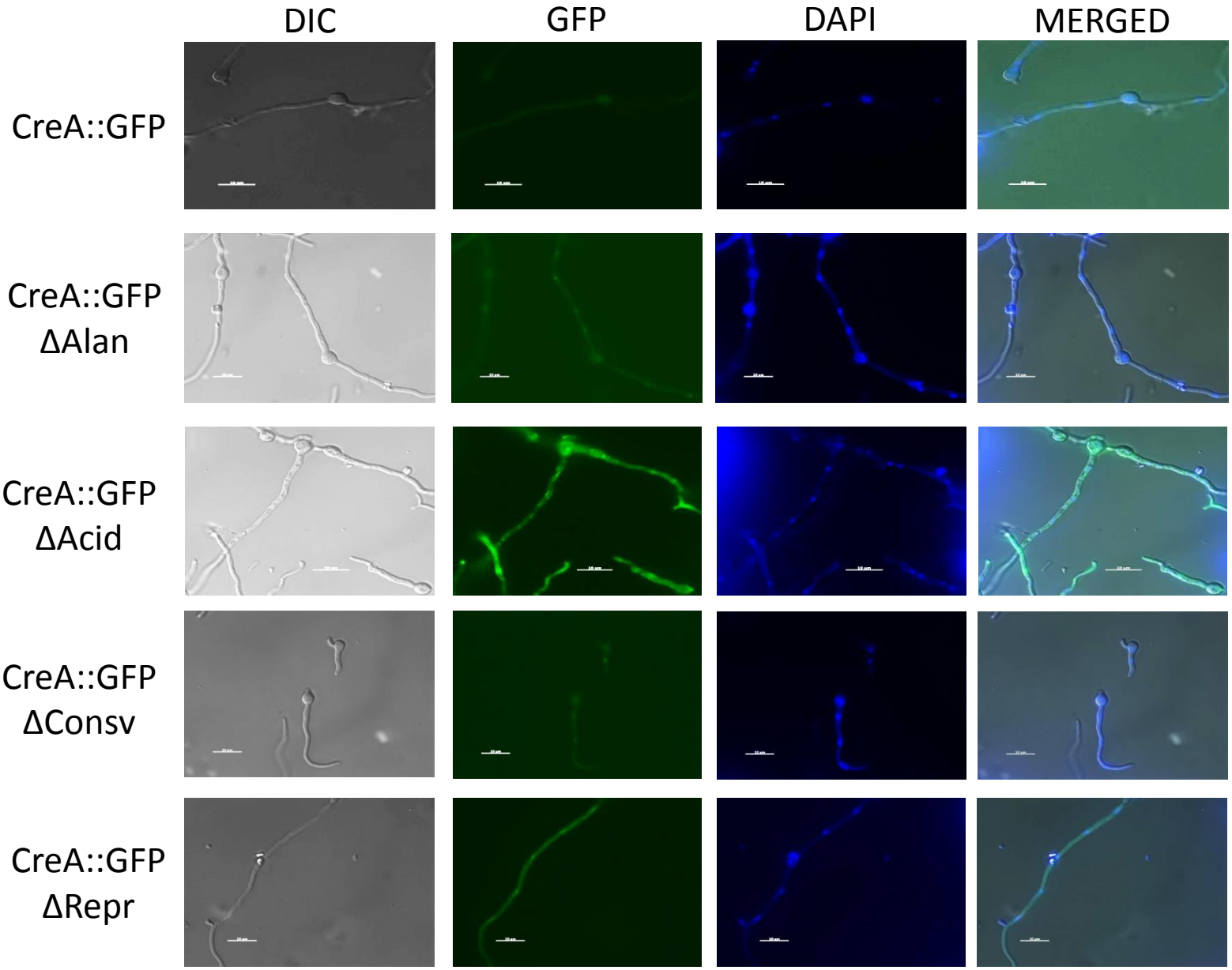


Figure S5: In the presence of cellulose, truncated CreA::GFP cannot leave the nucleus. Pictures were taken by microscopy in the absence (DIC = differential interference contrast) and presence (GFP, DAPI) of fluorescence of the wild type CreA::GFP and CreA truncated strains when grown overnight in minimal medium supplemented with 1% w/v glucose and then transferred to cellulose-rich media for 6 h. Nuclei were stained with Hoechst and viewed under the DAPI filter.

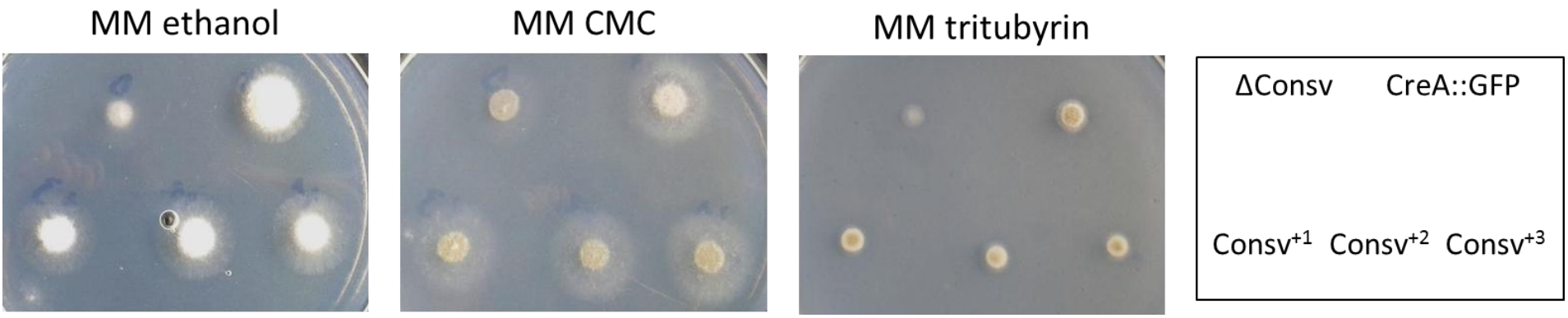


Figure S6: Growth of the CreA::GFP, CreAΔConsv and three CreAΔConserved complemented strains (Consv^{+1}, Consv^{+2}, Consv^{+3}) in the presence of 1% (v/v) ethanol, 1% (w/v) carboxymethylcellulose (CMC) and 1% (v/v) tributyrin.

Table S1: Strains and their respective genotypes used in this study.

Strain name	Genotype	Reference
TN02a3	*pyrG89; argB2 ΔnkuA::argB; pyroA4 veA1*	Hoffmann et al., 2001
CreA::GFP	*pyrG89; pyroA4; Δnku70::argB; creA::gfp::pyrG*	Brown et al., 2013
CreB15	*creB15; biA1; wA3*	Hynes and Kelly, 1977
CreC27	*creC27; niiA4; biA1; wA3*	Hynes and Kelly, 1977
ΔKapC	*pyrG89, ΔkapC::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapD	*pyrG89, ΔkapD::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapG	*pyrG89, ΔkapG::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapH	*pyrG89, ΔkapH::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapI	*pyrG89, ΔkapI::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapJ	*pyrG89, ΔkapJ::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapL	*pyrG89, ΔkapI::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapM	*pyrG89, ΔkapM::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
ΔKapN	*pyrG89, ΔkapN::pyrGAf; pyroA4, ΔnkuA::argB (argB2)*	Markina-Iñarrairaegui et al., 2011
CreA::Luciferase	*pyrG89; pyroA4; Δnku70::argB; creA::luciferase::pyrG*	This work
CreA::GFPxCreB15	*pyrG89; Δnku70::argB; creA::gfp::pyrG; biA1; creB15; wa3*	This work
CreA::GFPxCreC27	*pyrG89; Δnku70::argB; creA::gfp::pyrG; biA1; creC27; niiA4; wa3*	This work
CreAΔAlan	*pyrG89; pyroA4; Δnku70::argB; creA$^{131-139}$::pyrG*	This work
CreAΔAcid	*pyrG89; pyroA4; Δnku70::argB; creA$^{264-271}$::pyrG*	This work
CreAΔConsv	*pyrG89; pyroA4; Δnku70::argB; creA$^{271-315}$::pyrG*	This work
CreAΔRepr	*pyrG89; pyroA4; Δnku70::argB; creA$^{336-361}$::pyrG*	This work
ClrA::Luciferase	*pyrG89; pyroA4; Δnku70::argB; clrA::luciferase::pyrG*	This work
ClrB::Luciferase	*pyrG89; pyroA4; Δnku70::argB; clrB::luciferase::pyrG*	This work
CreA::GFPxΔkap C	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapC::pyrGAf*	This work
CreA::GFPxΔkap D	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapD::pyrGAf*	This work
CreA::GFPxΔkap G	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapG::pyrGAf*	This work
CreA::GFPxΔkap H	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapH::pyrGAf*	This work
CreA::GFPxΔkap I	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapI::pyrGAf*	This work
CreA::GFPxΔkap J	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapJ::pyrGAf*	This work
CreA::GFPxΔkap L	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapL::pyrGAf*	This work
CreA::GFPxΔkap M	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapM::pyrGAf*	This work
CreA::GFPxΔkap N	*creA::gfp::pyrG; pyrG89; pyroA4; Δnku70::argB; ΔkapI::pyrGAf*	This work

Table S2: Primer pairs used in this study.

Primer name Fw	Fw sequence 5' to 3'	Primer name Rv	Rv sequence 5' to 3'
pRS426-CreA-5UTR	GTAACGCCAGGGTTTTCCCAGTCACGACGTCTTTTCTTTTTGCCCTTTCG	CreA Rv Luc	ATGTTCTTGGCGTCCTCCATGAACCTCTCAGCCAAGTC
Luc Fw	ATGGAGGACGCCAAGAAC	Luc Rv PyrG	TGCCTCCTCTCAGACAGAATCTAGACGGCGATCTTGCC
3UTR CreA Fw PyrG	GCATTGTTTGAGGCGAATTCTCCGGCCAAAAAACTTCG	3UTR-CreA-pRS426	GTAACGCCAGGGTTTTCCCAGTCACGACGCCGCAATACAGAAATACACTGG
5UTR ClrA Fw	GTAACGCCAGGGTTTTCCCAGTCACGACGGGTTATAGAGTTGCACCAGG	ClrA Rv Luc	ATGTTCTTGGCGTCCTCCATTACTCTGCTTCCCGAGGATCTGAGA
3UTR ClrA PyrG Fw	GCATTGTTTGAGGCGAATTCTTTTGGACGTAGGAATCAACT	3UTR ClrA Rv	GTAACGCCAGGGTTTTCCCAGTCACGACGCATATGCCGATTCGCTCTT
5UTR ClrB Fw	GTAACGCCAGGGTTTTCCCAGTCACGACGGGCCTCAATCTGATGCAA	ClrB Rv Luc	ATGTTCTTGGCGTCCTCCATCGAATAATATGGAATAGATGAATTAGTGAAAAAAGGCGGTTC
3UTR ClrB PyrG Fw	CATTGTTTGAGGCGAATTCTCAGGCTTATGTGGATACCA	3UTR ClrB Rv	GTAACGCCAGGGTTTTCCCAGTCACGACGAGAGAAGAACTGTGCGGA
CreA fw Alan OH	GAAACAAGGCTCAACACCTGCAAGATGGTAGCGCGA	CreA rv Alan	CAGGTGTTGAGCCTTG
CreA fw Acid OH	TGAGCCGTTCCCATTCGCACTCACATCGCGTCAAGCGTTC	CreA rv Acid	GAACGCTTGACGCGATGTGAGTGCGAATGGGAACGGCTCA
CreA fw Consv OH	AGGATGAGGATTCTTATGCGAAGCCATTGTCGCCG	CreA rv Consv	CGCATAAGAATCCTCATCC
CreA fw Repr OH	TCACCTATCGCTTCACCACAGCATAAGCGATATCATGTCT	CreA rv Repr	TGTGGTGAAGCGATAGG
Spacer GFP Fw	GGAACACGGGGAATGAGTAAAGGAGAAGAACT	GFP Afu Rv	CTCAGACAGAATACGCCAAGCTTGCATGC
GFP PyrG Fw	GCATGCAAGCTTGGCGTATTCTGTCTGAGAGGAGGC	PyrG Rv	GAATTCGCCTCAAACAATGCTCTTCACC
PyrG CreA 3UTR Fw	ATTGTTTGAGGCGAATTCTCCGGCCAAAAAACTTCG	CreA spacer GFP Rv	GGTGACTTGGCTGAGAGGTTCGGAACACGGGGAATGAGTAAAGGAGAAGAACT
KapC Fw	AGAATGGTTGCTCTATCAGAC	KapC Rv	CCTGAACCTAGCGAGTATCA
KapG Fw	GCAGCAGTGAGGGGA	KapG Rv	TCGCCCAATAAATCACGC
KapH Fw	GCTGTCTCAATATGGTCTAGC	KapH Rv	TCATTGTCGGCTGTCATTT
KapL Fw	ATACGGTCGACTTGCTCT	KapL Rv	GGGTATATGCGTATCGGAGA
KapL Fw	CAACTCAGCCTCTCTCCATA	KapL Rv	TACTTATCTGCTTTCTCGTGC
XlnA ChIP Fw	CAGTAAGTGGTCTAGCGGT	XlnA ChIP Rv	TCAATCAGAGTTTAGGGCAATC
CreA qRT Fw	GAGGATGAGGATTCTTATGCG	CreA qRT Rv	TATCGCTTATGCTTGGGC
XlnA qRT Fw	ATCAACTACGGCGGAAG	XlnA qRT Rv	CAGTAATAGAAGCCGACCC
EglA qRT Fw	CTCACCGATCCCCAAGATA	ElgA qRT Rv	CCCAGTTTCCCGTTCTCT
XlnR qRT Fw	AATAGCCTCGCCCTAGC	XlnR qRT Rv	GAGGAATGACTCGGAAGAGA

Table S3: CreA nuclear localisation (%) in the presence of xylan and xylan and glucose in the wild-type and *kap* deletion strains.

Strain	8 h xylan	8 h xylan + 15 min glucose
CreA::GFP	8.3	96.4
CreA::GFP x Δ*kap C*	0.0	97.1
CreA::GFP x Δ*kap D*	7.3	99.4
CreA::GFP x Δ*kap G*	4.3	97.0
CreA::GFP x Δ*kap H*	11.8	97.9
CreA::GFP x Δ*kap I*	7.4	98.6
CreA::GFP x Δ*kap J*	0.0	96.4
CreA::GFP x Δ*kap L*	3.0	100.0
CreA::GFP x Δ*kap M*	9.5	100.0
CreA::GFP x Δ*kap N*	2.2	98.1