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Diverse regulation of the CreA carbon catabolite repressor in *Aspergillus nidulans*

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Aspergillus nidulans carbon catabolite repressor CreA

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ABSTRACT

Carbon catabolite repression (CCR) is a process that selects the energetically most favourable carbon source in an environment. CCR represses the utilization of less favourable carbon sources when a better source is available. Glucose is the preferential carbon source for most microorganisms as it is rapidly metabolised 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 characterise its functionally distinct protein domains. CreA is partially dependent 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 the *Aspergilli* and *T. 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, this study describes 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.

INTRODUCTION

Glucose is the preferred carbon source for most microorganisms. Selecting the most energetically favourable carbon source is a survival strategy for microorganisms as it supports rapid growth and development required for colonising 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). Upon glucose detection, genes encoding enzymes (e.g. xylanases, cellulases, arabinases) required for the breakdown of alternative carbon sources, such as lignocellulose, are repressed. This is a disadvantage for 2nd generation (2G) biofuel production that aims to convert non-glucose saccharides (e.g. xylose, arabinose, cello- and xylooligosaccharides) to biofuels from lignocellulosic plant mass. In *A. nidulans*, CreA directly represses xylanases encoded by *xlnA* and *xlnD* through 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 utilisation (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 *A. nidulans* CreA contains an

acidic amino acid rich region that is located adjacent to a highly conserved region among *A. nidulans*, *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 auto-regulated as CreA binding sequences are present within its promoter region (Arst *et al*. 1990; Schroff *et al*. 1996; Strauss *et al*. 1999). Furthermore, the regulation of CreA is thought to occur via the removal of ubiquitin molecules from the protein that lead to active CreA, a process that may be mediated by the CreB-CreC deubiquitination (DUB) complex (Lockington and Kelly 2002). CreB is an UBP (ubiquitin-specific-processing protease) family ubiquitin protease that functions downstream of CreC; the latter is a WD-40 domain protein required for CreB stabilization (Lockington and Kelly 2002). De-ubiquitinating 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 macro-molecular assembly, or for altering protein function (Lockington and Kelly 2002). DUB enzymes also interact with ubiquitin ligases and together this likely controls the amounts of transcription factors present during CCR (Kubicek *et al*. 2009). The CreB/CreC de-ubiquitination complex has been proposed to be involved in CCR since 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* 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 homologue of CreA, is regulated by phosphorylation through the protein kinase Snf1p. Upon detection of phosphorylated glucose, Snf1p is inactivated and Mig1p is de-phosphorylated 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 (Czifersky *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 (homologue of *S. cerevisiae* Snf1p) and SchA (homologue 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 is partially dependent 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 the *Aspergilli* 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 transport is also established.

MATERIALS AND METHODS

Strains and media

A list of all strains used in this study is found in Table S1. Strains were grown at 37°C (except where stated) in either liquid (without agar and shaking at 180 rpm) or solid (with 20 g/l agar and no shaking) minimal medium [MM: 1% (w/v) carbon source, 50 ml of a 20 × salt solution (120 g/l $NaNO_3$, 10.4 g/l KCl, 30 g/l KH_2PO_4, 10.4 g/l $MgSO_4$), 1 ml of 5 × trace elements (22.0 g/l $ZnSO_4$, 11 g/l boric acid, 5 g/l $MnCl_2$, 5 g/l $FeSO_4$, 1.6 g/l $CoCl_2$, 1.6 g/l $CuSO_4$, 1.1 g/l $(NH_4)_2MoO_4$, 50 g/l ethylenediaminetetraacetic acid (EDTA)] and adjusted to pH 6.5 with NaOH. Depending on the auxotrophy of the strain, uridine (1.2 g/l), uracil (1.2 g/l) or pyridoxine (0.005 mg/µl) were added.

All mycelia were filtered from the supernatant by miracloth, rinsed with 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 qRT-PCRs and for strain confirmation can be found in Table S2. All DNA fragments required for construction of specific strains were amplified by PCR from gDNA except where stated. Construction of the whole transformation cassette was carried out in *Saccharomyces 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; 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 were then 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 which was amplified with primers “GFP PyrG Fw” and “PyrG Rv” from plasmid pCDA21 (Chaveroche et al., 2000). The *pyrG* fragment was then 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 above. 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 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 Sc URA$^-$ liquid medium for 2 days at 30°C before DNA was extracted as previously described (Goldman *et al.*, 2003).

A. nidulans *transformations and gDNA extraction*

Cassettes for *A. nidulans* tranformations were amplified from yeast gDNA which was first checked by PCR to confirm the correct construction using the respective 5' and 3' UTR primers with the pRS426 overhang (Table S1; see above). *A. nidulans* strain TN02a3 was used as 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, repeated 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 above). gDNA was extracted according to Sambrook and Russell, 2001. All strain constructions were confirmed by sequencing and Southern blots were carried out in order 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, 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 above), 1.7% (w/v) agar]. Pieces of agar containing both strains were then cut out and transferred to plates containing MM without auxotrophic supplements. The plates were sealed hermetically and incubated at 37°C 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 in order to obtain single colonies. Candidate colonies were selected, grown in liquid minimal medium and gDNA was extracted. Phenotypical tests were carried out on the candidate colonies and/or PCRs were run on the gDNAs in order 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 as 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 mM, 1 mM and 2 mM 2DG (2-deoxyglucose). 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”, “Kap M Fw” and “Kap M Rv” (Table S1). The *creA*::*gfp* gene construction was confirmed by PCR, using primers “pRS426-CreA-5UTR” and “GFP Afu Rv”.

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 co-transformed into CreAΔConsv. Positive transformants did not present any prototrophy. Complemented strains were checked by PCR (as described above).

RNA extraction and cDNA synthesis

RNA from mycelia was extracted as previously described utilizing TriZol (Invitrogen) and RNeasy® Mini Kit (Qiagen) (REFERENCE). RNA (1 µg) was reverse transcribed to cDNA using the Superscript® III Reverse Transcriptase kit (Invitrogen) according to manufacturer’s instructions.

Real-time PCR (qRT-PCR)

qRT-PCR reactions were carried out using the 7500 Fast Real-Time PCR thermocycler and the 7500 Fast system v.1.4.0 (AB Applied Biosystems). Annealing temperature was set at 61°C. 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 gene in the unknown samples were calculated as a reference to a standard curve. Gene expression of *creA* was quantified by using primers “CreA qRT Fw” and “CreA qRT Rv” whereas *xlnA*, *eglA* and *xlnR* gene expression was determined by using the corresponding qRT primers in Table S2.

Microscopy 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°C overnight or at 37°C for 8 h. All cellulose and xylan-grown hyphae were first fixed [3% v/v formaldehyde and 1.5% v/v ml DMSO in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4)] at RT for 4 min before nuclei were stained with 1 µg/ml Hoechst 33342 (Life Technologies) at RT for 5 min. Cycloheximide was added at a final concentration of 100 µg/ml for one hour 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 the 100× magnification oil immersion objective (EC Plan-Neofluar, NA 1.3). Phase-contrast brightfield and fluorescent images were taken with an AxioCam camera (Carl Zeiss) and processed using the AxioVision software (version 3.1). Hoechst-stained hyphae were viewed with the same light spectrum than is used for DAPI staining. Between 150 and 300 nuclei were viewed for each condition. The number of nuclei, which contained CreA::GFP, was counted before the percentage of nuclei containing CreA::GFP was calculated.

Luciferase experiments

10 ml of MM supplemented with different carbon sources, was inoculated with 10^7 spores and 2 mM D-luciferin potassium salt (Gold Bio, St. Louis, MO, U.S.A.). 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°C for the indicated amounts of time. Luminescence readings of the plates were taken at the specified time points in a SpectraMax®i3 device (Molecular devices, Sunnyvale, CA, U.S.A.) and results were exported into excel and analysed.

Immunoprecipitation (IP)

Mycelia were ground to a fine powder under liquid N_2 and 500 mg were re-suspended 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, 10µl/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 for 20 min at 4°C, maximum speed. 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 re-suspension buffer prior to incubation. Cell extracts and resin were then incubated with shaking at 4°C for 4 h. After incubation, the resin was spun down for 30 s at 5000 x g and washed three times in re-suspension buffer. To release the proteins from the resin, samples (and all other non-immunoprecipitated proteins samples) were incubated with NuPAGE® sample buffer and reducing agent and boiled at 98°C for 5 min before being run on pre-made gels, according to manufacturer's instructions (NuPAGE® Bis-Tris Mini Gels, Novex Life Technologies).

Western blotting

Non-immunoprecipitated samples were run on a 4% stacking and 12% resolving self-made gel according to manufacturers' instructions (BioRad http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf) and immunoprecipitated samples were run on pre-made gel (NuPAGE® Bis-Tris Mini Gels, Novex Life Technologies). Proteins were transferred to a membrane using the ibolt® 2 dry blotting system (Life Technologies) according to manufacturer's instructions. Membranes were blocked for 1 h in 5% (w/v) dry-skimmed milk in 1 x TBS-T (0.14 M

NaCl, 0.02 M Tris, 0.1% v/v Tween 20, pH 7.6) at RT. Antibodies were diluted in and all membranes were washed at RT (3 x 5 min) with 1 x 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 proteins clone FK2, Upstate) overnight at 4°C. Membranes were washed and incubated with a 1:10000 dilution of anti-rabbit or anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) at RT for 1 h. Membrane was washed and revealed using the SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific).

Chromatin Immunoprecipitation (ChIP)

All strains were grown for 24 h 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 for 6 h at 37°C, 250 rpm.

Samples were crosslinked 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 RT before the formaldehyde was quenched with 2 M glycine for 10 min at RT. Mycelia were harvested, snap frozen in liquid N_2 and re-suspended in ChIP Lysis buffer (Chung *et al*. 2014). Samples (2 ml) were sonicated for 3 cycles of 10 min with 30 sec ON and 30 sec OFF at power level high with the Biorupter UCD-200 (Diagenode, USA). After sonication, cell debris were spun down and supernatants stored at -80°C. 60 µl of the supernatant was reverse cross-linked and used for checking the sonication. To prepare for immunoprecipitation,

Dynabeads Protein A (Life Technologies) were incubated overnight at 4°C 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°C with 100 µl (glucose samples) or 300 µl (bagasse samples) of sonicated supernatants with rotation. Dynabeads were washed and the protein/DNA complexes eluted two times at 65°C for 10 min with elution buffer. Samples were reverse-cross-linked overnight at 65°C. Sample DNA was purified using the GeneJET Gel Extraction kit (Thermo Scientific) according to manufacturer's instructions. qPCRs were run on the chromatin immunoprecipitated DNA using primers "ChIP 3 Fw" and "ChIP 3 Rv" which target the *xlnA* promoter region. ChIP-qPCR reactions were carried out in the BioRad Thermocycler (BioRad MyiQ Single Color Real-Time PCR Detection System) at an annealing temperature of 60°C. 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 immunoprecipitation.

Samples that were cross-linked but not immuno-precipitated (input) were used as positive control 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 normalised 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 from 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 substrate solution (1% w/v Azo-Xylan or 1% w/v Azo-cellulose) and samples were incubated at 40°C for ten minutes before the reactions were stopped via the addition of 2.5 ml ethanol (95% v/v). Samples were centrifuged for 10 min at 1,000 × *g*. The absorbance was measured at 590 nm of the sample supernatants. Enzymatic activity was determined using the Mega-Calc™ software and 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 h 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 manufacturer’s instructions. Experiments were carried out in triplicates.

RESULTS

CreA is partially dependent on de novo protein synthesis

In order 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* wild-type allele was replaced with the *creA*::*gfp* allele (strain TN02a3, Table S1 and Brown *et al*. 2013), grown for 16 h in glucose (CreA target genes repressed, CreA localizes to the nucleus) then transferred to xylan for 6 h (CreA target genes de-repressed, CreA leaves the nucleus), before glucose was added to the xylan-cultures for 30 min (Table 1). The protein synthesis inhibitor cycloheximide (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 h at 37°C in glucose (Figure S1). The amount of CreA::GFP localized in the nuclei of fungal germlings in the presence and absence of cycloheximide was very similar (about 93 %; Table 1). To support the microscopy results, immunoprecipitation for GFP of whole cell protein extracts, grown in the same conditions as described above, were collected and Western blots with antibody against GFP were carried out. CreA::GFP, that has a predicted size of ~73.5 kDa (compared to 44.7 kDa without GFP), was detected in the immunoprecipitated

samples containing CreA::GFP but not in the wild-type strain (Figure 2A). CreA::GFP was detected in immunoprecipitated 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 recognised by the GFP antibody and similar degradation products were also 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 in the above 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 h in glucose-rich conditions, then transferred to xylan-rich media for 6 h before glucose was added for 30 min, 60 min and 120 min. Results indicate that there is partial degradation of CreA, because its abundance increases proportionally with 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. Upon 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 previously been used to confirm gene expression and *in vivo* activity of proteins in *A. fumigatus* (Galiger *et al*. 2013; Paul *et al*. 2012) and *Neurospora crassa* (Larrondo *et al*. 2012).

The wild-type strain that 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 and ClrB 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 h before glucose was added to a final concentration of 1% (w/v) for 1 h. Results show that CreA is present in the conidia and actively growing cells in the presence of glucose and in the presence of de-repressing carbon sources (Table 2). When compared to the luminescence levels of ClrB in all tested conditions (including 24 h 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 de-repressing 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, was also observed at the transcriptional level when the wild-type and CreA::Luciferase strains were grown in the same conditions than 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. Like in *T. reesei*, small cytoplasmic pools of CreA are readily available and more protein is synthesised once glucose is detected. This complexity in CreA protein turnover and synthesis also suggest 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 de-ubiquitinylating complex, plays a role in CCR and it was suggested that CreA is de-ubiquitinylated by the CreB/CreC complex (Hynes and Kelly 1977; Lockington and Kelly 2002). CreB is the de-ubiquitinating enzyme whereas CreC 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 h, transferred to xylan for 6 h 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 localisation was not affected in the *creB15* mutant (Table 3 and Figure S3).

Western blots of immunoprecipitated CreA::GFP in the wild-type, *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 h 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 in 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 x CreB15 and CreA::GFP x CreC27

strains were grown for 17 h 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

The above results suggest that ubiquitination plays a role in regulating CreA function. CreA could be directly or in-directly (via another protein which is subject to ubiquitination) ubiquitinylated. In order to investigate this, the wild-type and CreA::GFP strains were grown in the same conditions as in Figure 2B, before Western blots were performed against ubiquitinylated proteins of immunoprecipitated samples. Membranes were first incubated with anti-GFP antibody in order to confirm that CreA::GFP was successfully 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 as 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 is greater after 6 h incubation in xylan and for the 6 h xylan + 30 min glucose samples than when compared to longer incubations (1 h and 2 h) with glucose, suggesting that ubiquitination increases when CreA is de-repressed and localized to the cytoplasm. Furthermore, it appears that the ubiquitination smear is between 60 kDa 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, Chen *et al*. 2011 and BDM_PUB, Ao *et al*. manuscript submitted), detected one low

confidence site at K47 and one high confidence site at K275. In addition, the 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 region important for repression in CreA (Figure 1). In order to investigate the function of each region, four CreA-truncated constructions (CreAΔAlan, CreAΔAcid, CreAΔConsv, and CreAΔRepr; see Figure 1) that use *creA* endogenous promoters replacing the wild-type alleles, were generated with the above described 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 analogue 2-deoxy-glucose (2DG) or allyl alcohol (AA). Treatment with 2DG inhibits the ability of the cells to complete glycolysis whereas allyl alcohol is converted to the cytotoxic compound acrolein by alcohol dehydrogenase. All strains showed, with varying degrees, increased sensitivity to 2DG and increased resistance to allyl alcohol when compared to the wild-type strain (Figure 5A) when grown in a repressing (glucose) and de-repressing (xylose) carbon

source. This indicates that CreA mutations result in an inability of CreA to de-repress in the presence of complex carbon sources.

In order to assess whether this inability to de-repress 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 h in glucose then transferred to cellulose for 6 h or they were grown in the reverse conditions (16 h in cellulose before glucose was added for 30 min). All CreA-truncated strains, except for CreAΔAcidic::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 was also unable to germinate (~93 %) in cellulose. Strains were also grown for 16 h in the presence of xylan and similarly 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 allyl alcohol. 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*, the cellulase-encoding gene *eglA* and the expression of *xlnR*, 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 h (Figure 5B).

The effect of truncating CreA::GFP on lignocellulosic enzyme secretion was then investigated. Cellulase and xylanase activities were measured in the supernatant of cultures grown in fructose for 24 h (to get fungal biomass), transferred to cellulose for 5 days, before being transferred again to glucose-rich medium for 24 h. Cellulase and xylanase activities, that were normalised 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 and also with the transcriptional data. It has been previously described 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 cellulase 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

Due to 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 as well as on hydrolysed casein (amino acids) and individual amino acids (Figure 6B). Furthermore, when grown on milk powder, the halo of secreted proteases by the CreAΔConsv (and the CreAΔRepr and CreAΔAcid strains) was much smaller than when compared to the wild-type (Figures 6C and 6D). Deletion of the repressing region did not have as severe of a reduction in growth as when compared to the CreAΔConsv strain but still presented reduced growth in the presence of hydrolysed 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.

To confirm the growth defect associated with the deletion of the conserved region, the wild-type CreA::GFP and the CreAΔConsv::GFP strains were grown overnight in minimal medium supplemented with 50 mM leucine or valine. Deletion of the conserved region resulted in the inability of the majority of conidia to germinate (81% in leucine, 92% in valine in the CreAΔConsv strain compared to 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.

Chromatin Immunoprecipitation (ChIP) qPCR

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. In order 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 (chromatin immunoprecipitation) was carried out on the *xlnA* gene for the wild-type and CreA truncated strains when grown for 24 h in fructose-rich media and then transferred for 6 h to either minimal medium supplemented with glucose or with 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 on the other hand, 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 above observed decrease in enzyme secretion but rather protein-protein interactions and post-translational modifications may govern CreA cellular localization.

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 (Figures 6A and 6B). In both cellulose and amino acids, the majority of the CreAΔConsv conidia were unable to germinate. In order 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 in glucose-rich conditions and then transferred to either leucine or valine for 30 min, 60 min, 180 min or 360 min, was carried out. During the first hour after the transfer, around 60% - 75% of CreA is inside the nucleus whereas longer incubations (3 h – 6 h) resulted in 80% - 90% of CreA localizing to the nucleus (Table 6). Cellular localization of CreA alone can therefore not explain the observed growth phenotypes of the CreAΔConsv::GFP strain, as CreA localized to the nucleus during growth in the presence of leucine and valine.

CreA may play a role in the germination process as 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 h at 37°C before being transferred to plates containing the same combinations of carbon sources as described above. There is 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 sole carbon source.

As a third possibility, the observed growth defect could be due to CreAΔConsv being unable to take up and metabolise the respective amino acids. The wild-type and CreAΔConsv strains were therefore grown for 16 h in glucose-rich media 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 min, 30 min, 60 min 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 above observed growth defect and suggests a role for CreA in amino acid transport.

Alternatively, CreA could also be critical for amino acid metabolism. To test this hypothesis, the wild-type and the 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 h, the colony and protease halo diameters were measured and the halo/colony ratio was calculated (Figure 8C). Proteases are secreted by the fungus in order to degrade proteins, thus providing the fungus with a nitrogen source. When nitrate or casamino acids (mixture of essential and non-essential amino acids) are added, the fungus primarily uses these nitrogen sources as they are more energetically favourable 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 easily be 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 the process of nitrogen assimilation/metabolism.

In summary these results suggest that CreA not only has a role 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 non-essential karyopherins

The above results show that, in addition to post-translational modifications, cellular localization of CreA regulates the 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 the majority belong to the karyopherin (Kap) β-superfamily (Markina-Iñarrairaegui *et al*. 2011). A total of 14 karyopherins (KapA to N) were identified in *A. nidulans* and 5 of them are essential (Kap A, B, E, F and K) (Markina-Iñarrairaegui *et al*. 2011). Homologues of karyopherins C, D, G, H, I and J are considered importins; of karyopherin M an exportin and karyopherin L and N homologues can transport molecules bi-directionally (Markina-Iñarrairaegui *et al*. 2011).

The 9 non-essential karyopherin deletion strains were crossed with the CreA::GFP strain and microscopy studies were carried out. Strains were grown for 8 h at 37°C in minimal media supplemented with xylan before glucose was added. In the presence of xylan, the majority of CreA is localised outside the nucleus (90% - 100%), whereas upon the addition of glucose, CreA localised back into the nucleus (96% to 100%) (Table S3). CreA::GFP cellular localisation was similar in both conditions

between the wild-type and *kap* deletion strains (Table S3). This indicates that none of these 9 nuclear transporters are specific for CreA nuclear bi-directional 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 metabolised 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 as it provides a means of obtaining quick energy required for growth, niche colonisation and survival (Ruijter and Visser 1997). In *A. nidulans*, carbon catabolite repression (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 with 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 pre-formed cytoplasmic pool (Lichius *et al*. 2014). Similarly to the study carried out in *T. reesei*, *A. nidulans* CreA is partially

dependent on *de novo* protein synthesis. Microscopy 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 media. These results suggest that a small pre-formed pool of CreA remains in the cytoplasm and that total levels partially depend 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 non-glucose, complex carbon sources. The readily available CreA protein pools would therefore 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 as CreA::GFP degradation products were observed under all tested conditions. Degradation products were also 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 localisation (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 de-ubiquitination (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 de-ubiquitination 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 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 as overlaying both Western blots (anti-GFP and anti-ubiquitin blots) did not match. Rather, the ubiquitination smear appears to be localized in the CreA region and below it, indicating ubiquitination, and therefore also de-ubiquitination, of CreA itself and another protein which may interact with CreA. The identity of this protein is unclear and will be subject to further investigation. Ubiquitination of CreA appears to take place on three sites within the protein (K47, K126 and K275) that 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 CYP2E1 (human cytochrome P450 2E1) 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 the stabilisation of the de-ubiquitinating 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-values 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 3 ubiquitin-specific proteases (An5186, An3711, An2027; E-values between $2e^{-19}$ and $3e^{-13}$ and similarity around 40%) and in a protein with ubiquitinyl hydrolase activity (An6164; E-value $1e^{-13}$, similarity 41.8%); proteins that are all involved in diverse de-ubiquitination processes. CreC has similarity (E-values between 5e-13 and 5e-3, similarity around 45%) to a region in proteins with unknown function or to proteins involved in various cellular functions such as polarised 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 de-ubiquitination processes, whereas CreC is the only WD-40 scaffold protein involved in CreA-related carbon metabolism. Currently, additional work is being carried out in order 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 de-repressing 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, 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 manuscript 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 non-essential nuclear transporter (karyopherin) specific for CreA nucleocytoplasmic shuttling. This is probably due to redundancy that exists between the different nuclear transporters and/or that more than one nuclear transporter is responsible for CreA nucleocytoplasmic transport. In this way, the cell assures 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-Martinez *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 325-333 that are located between the conserved and repressing regions. It is possible that deletion of these regions (and the other regions) causes protein conformational changes that render the NES inaccessible, therefore preventing CreA nuclear export.

Furthermore, the expression of a cellulase- (*eglA*) and a xylanase (*xlnA*)-encoding gene 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 due to CreA cellular localization (and hence DNA binding), as this work showed that truncation of CreA resulted in persistent nuclear localization under de-repressing conditions. Thus, in order to determine whether CreA was still able to bind to specific sites in the promoter regions of its target genes, ChIP-qPCR was carried out of 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 is therefore, as already suggested by its name, 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 de-repressing 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 de-repressing signal) but cannot, as discussed above, 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 as 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 could therefore cause a decrease in ubiquitination and protein degradation, hence keeping the CreA protein levels high even in the presence of de-repressing 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 de-repression mechanism differs between complex carbon sources. Although this has not previously been proposed 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 *et al*. 2010). Deletion of the acidic region also resulted in the fungus being unable to grow in the presence of cellulose in liquid media. It is possible that the acidic region, that is located directly adjacent to the conserved region, is required for the 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 as 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 de-repression observed for the CreA truncated strains in carbon metabolism (e.g. xylan, cellulose) may also 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 as it is found in industrially relevant *Aspergillus spp*. and *T. reesei*. In the filamentous fungus *Beauvaria bassiana*, deletion of *creA* caused cell lysis and growth impairment when 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 metabolising 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 which could be useful for future strain engineering in order 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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Figure legends

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.

Figure 2. CreA does not require *de novo* protein synthesis and is regulated through 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 h in glucose (Glu) then transferred to xylan for 6 h 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 h in glucose (G) then transferred to xylan (X) for 6 h 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* in the presence of glucose, cellulose and xylan after 20 min, 24h or after the addition of glucose for 1 h as determined by qRT-PCR. Standard deviation is shown for three technical replicates and values were normalised by tubulin C expression (tubC).

Figure 3. The de-ubiquitination complex scaffold protein CreC is important for CreA function. **Above:** Western blot of immunoprecipitated CreA::GFP protein from whole

cell protein extracts of different strains. Mycelia were grown from spores for 16 h in glucose then transferred to xylan for 6 h before glucose was added (Xylan + Gluc) for 30 and 60 min. **Below:** Coomassie stained SDS-PAGE gel of whole cell protein extracts before immunoprecipitation. CreA::GFP is indicated by a red arrow.

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 h in glucose then transferred to xylan for 6 h 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 (**middle**). **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.

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 minimal media containing 1% (w/v) glucose (gluc) or xylose (xyl) supplemented with different concentrations of 2-deoxy-glucose (2DG) and allyl alcohol (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 h in fructose, and then transferred to sugarcane bagasse for 6 h before glucose was added to a final concentration of 2% w/v for 1 h. Gene expression was normalised by tubulin C (*tubC*) expression. Standard deviations were calculated for 3 technical replicates (**=P-value < 0.005, ***= P-value < 0.001 in an equal-variance, paired student t-test).

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. Pictures 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 minimal medium 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 of different 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 standard deviation was measured between biological triplicates. *** = P-value < 0.001 in a one-tailed, equal variance student t-test.

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-qPCR. **B.** Quantity of *xlnA* detected by ChIP-qPCR on the CreA binding site 3 in the wild-type and CreA truncated strains when grown for 24 h in fructose and then transferred to either glucose or sugarcane bagasse for 6 h. All *xlnA* expression values were normalised by the quantity of *tubC* (β-tubulin) in each sample. Standard deviations are shown for technical duplicates.

Figure 8. CreA is involved in amino acid transport and metabolism. **A**. Growth of CreA::GFP and CreAΔConsv on solid media supplemented with different carbon and nitrogen sources without and with transfer from liquid cultures (4 h at 37°C). **B.** Amino acid uptake of the wild-type and CreAΔConsv during a 2 h incubation in media supplemented either with 50 mM leucine or valine. Concentrations of the amino acids were measured in the supernatants of biological triplicates and normalised by fungal dry weight. **P<0.01, ***P<0.001 in a paired, equal variance student t-test. **C.** Halo/colony ratio of the growth of the wild-type (CreA::GFP) and CreA truncated strains on pates containing 1% glucose and 1% milk (control) or in the presence of 50 mM $NaNO_3$ or 1% casamino acids (CA). Standard deviations were calculated for biological triplicates (*P<0.01, **P<0.001, ***P<0.0001 in a paired, equal variance student t-test).

Tables

Table 1. Percentage of CreA::GFP which localized to the nucleus, as determined by microscopy, in the presence of glucose, xylan and in the simultaneous presence of glucose and xylan with and without cycloheximide (CH). Between 100 and 300 nuclei were counted for biological duplicates for each condition (*P-value < 0.001 in a one-tailed, equal variance Student T-test between the different conditions).

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

Table 2. Expression of ClrA::Luciferase, ClrB::Luciferase and CreA::Luciferase in the presence of glucose (G), cellulose (C) and xylan (X). Luminescence was measured in spores (20 min), after 24 h and before glucose was added to these carbon sources for 1 h (+ G 1h). Luminescence values are given as Relative Luminescence Units (RLU).

Strain	G 0 h	G 24 h	G+G 1 h	C 0 h	C 24 h	C + G 1 h	X 0 h	X 24 h	X + G 1h
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	1715 ± 287	657 ± 104	338 ± 80	284 ± 72	310 ± 77
CreA::Luciferase	6608 ± 312	7944 ± 791	8097 ± 931	14453 ± 475	11722 ± 522	8319 ± 688	8347 ± 329	2890 ± 253	2007 ± 193

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, xylan, after transfer from glucose to xylan or after the addition of glucose to xylan-grown cultures. Between 100 and 200 nuclei were counted for biological duplicates in each condition (*P-value < 0.01, **P-value < 0.001 in a one-tailed, equal variance Student T-test between the wild-type and mutated strains for each condition).

Strain **(%)**	**Glucose 16 h** **(%)**	**Xylan 16 h** **(%)**	**Glucose 16 h + Xylan 6 h** **(%)**	**Xylan 16 h+ 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**

Table 4. Percentage of CreA::GFP localised to the nucleus, as determined by microscopy, in the CreA wild-type and 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. Between 200 and 300 nuclei were counted for biological duplicates in each condition (*P-value < 0.01, *P-value < 0.001 in a one-tailed, equal variance Student T-test between the wild-type and truncated strains for each condition).

Strain	Glucose 16 h	Avicel 16 h	Xylan 16 h	Glucose 16 h transfer Avicel 6 h	Cellulose 16 h 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%

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

Cellulase Activity (U/µg intracellular protein)			
Strain	**Fructose 24 h**	**Avicel 120 h**	**Glucose 24 h**
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 (U/µg intracellular protein)			
Strain	**Fructose 24 h**	**Avicel 120 h**	**Glucose 24 h**
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

Table 6. Percentage of CreA::GFP localised in the nucleus as determined by microscopy. Strains were grown overnight in glucose-rich medium and then transferred to 50 mM leucine or 50 mM valine for 30 min to 360 min (*P-value < 0.01 in a one-tailed, equal variance Student T-test between the different amino acid conditions when compared to the glucose control condition).

Condition	CreA in the nucleus (%)	Number of nuclei
16 hours Glucose	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

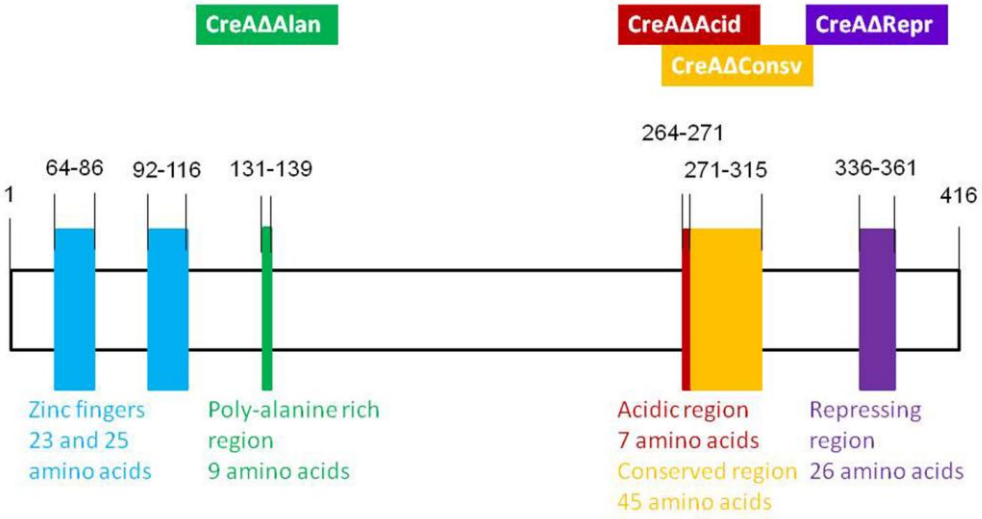

CreAΔAlan
CreAΔAcid
CreAΔRepr
CreAΔConsv
1
64-86
92-116
131-139
264-271
271-315
336-361
416
Zinc fingers
23 and 25
amino acids
Poly-alanine rich
region
9 amino acids
Acidic region
7 amino acids
Conserved region
45 amino acids
Repressing
region
26 amino acids

Figure 1

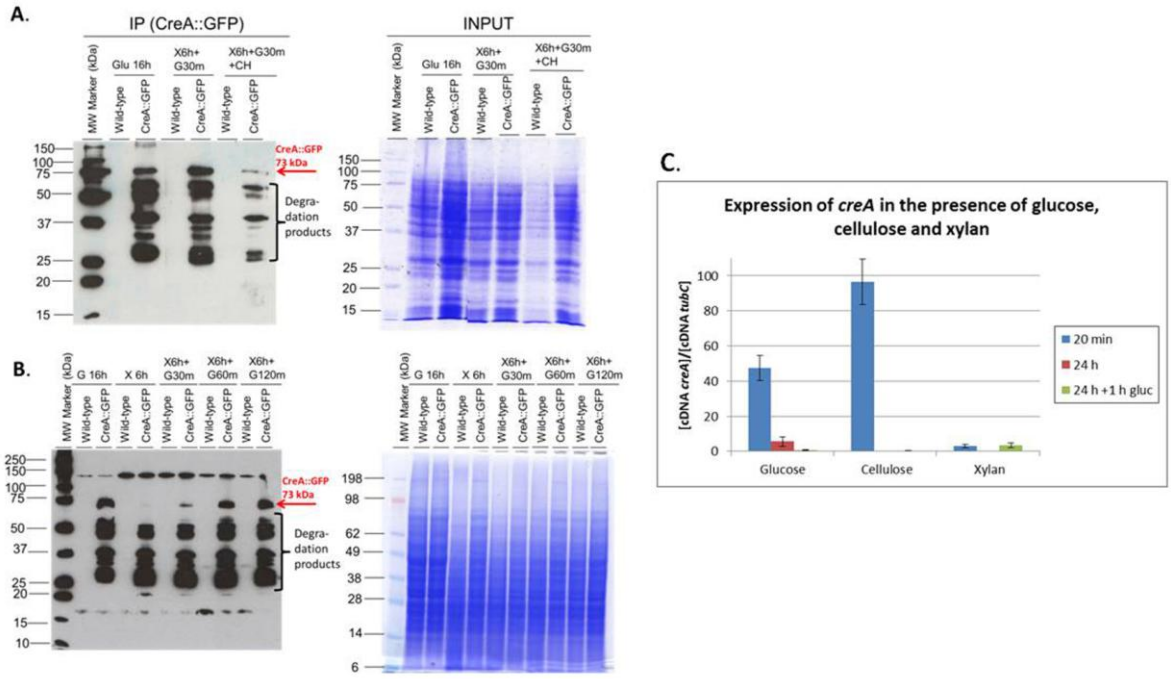

A.
IP (CreA::GFP)
INPUT
MW Marker (kDa)
Glu 16h
X6h+ G30m
X6h+G30m +CH
Wild-type
CreA::GFP
CreA::GFP 73 kDa
Degra-dation products
B.
G 16h
X 6h
X6h+ G30m
X6h+ G60m
X6h+ G120m
C.
Expression of creA in the presence of glucose, cellulose and xylan
[cDNA creA]/[cDNA tubC]
20 min
24 h
24 h +1 h gluc
Glucose
Cellulose
Xylan

Figure 2

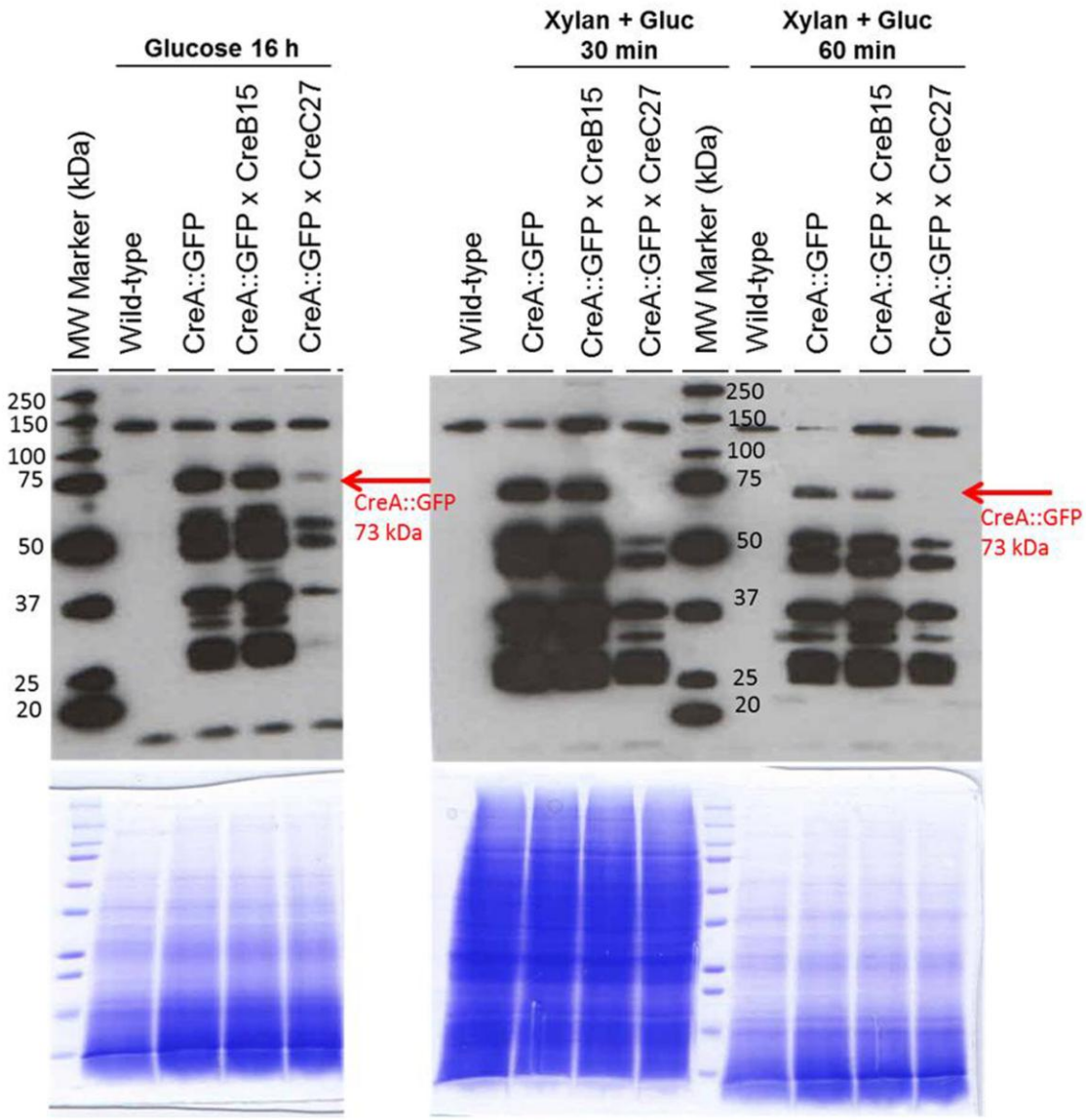

Glucose 16 h
Xylan + Gluc 30 min
Xylan + Gluc 60 min
MW Marker (kDa)
Wild-type
CreA::GFP
CreA::GFP x CreB15
CreA::GFP x CreC27
250
150
100
75
50
37
25
20
CreA::GFP
73 kDa

Figure 3

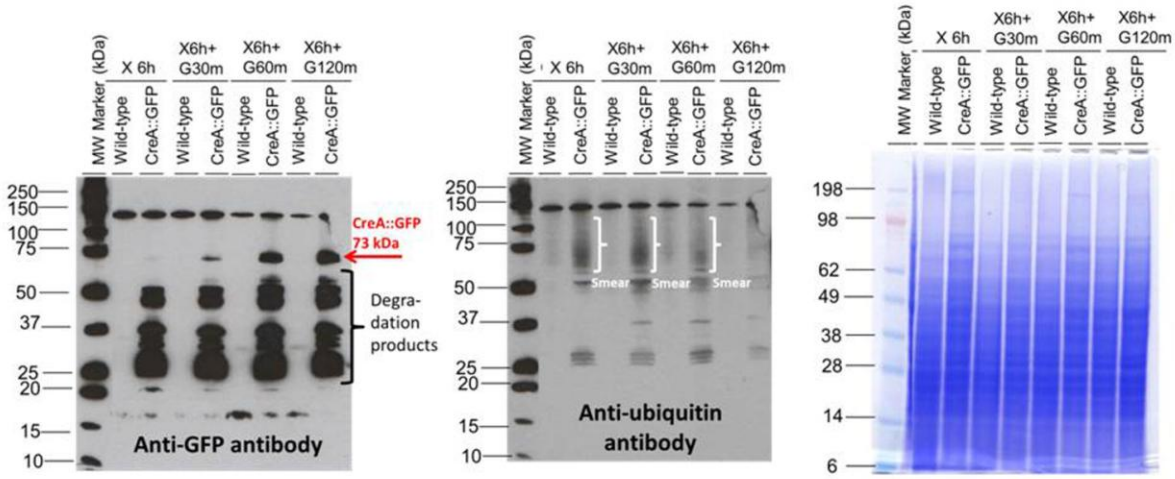

MW Marker (kDa)
X 6h
X6h+ G30m
X6h+ G60m
X6h+ G120m
Wild-type
CreA::GFP
250
150
100
75
50
37
25
20
15
10
CreA::GFP 73 kDa
Degra-dation products
Anti-GFP antibody
Smear
Anti-ubiquitin antibody
198
98
62
49
38
28
14
6

Figure 4

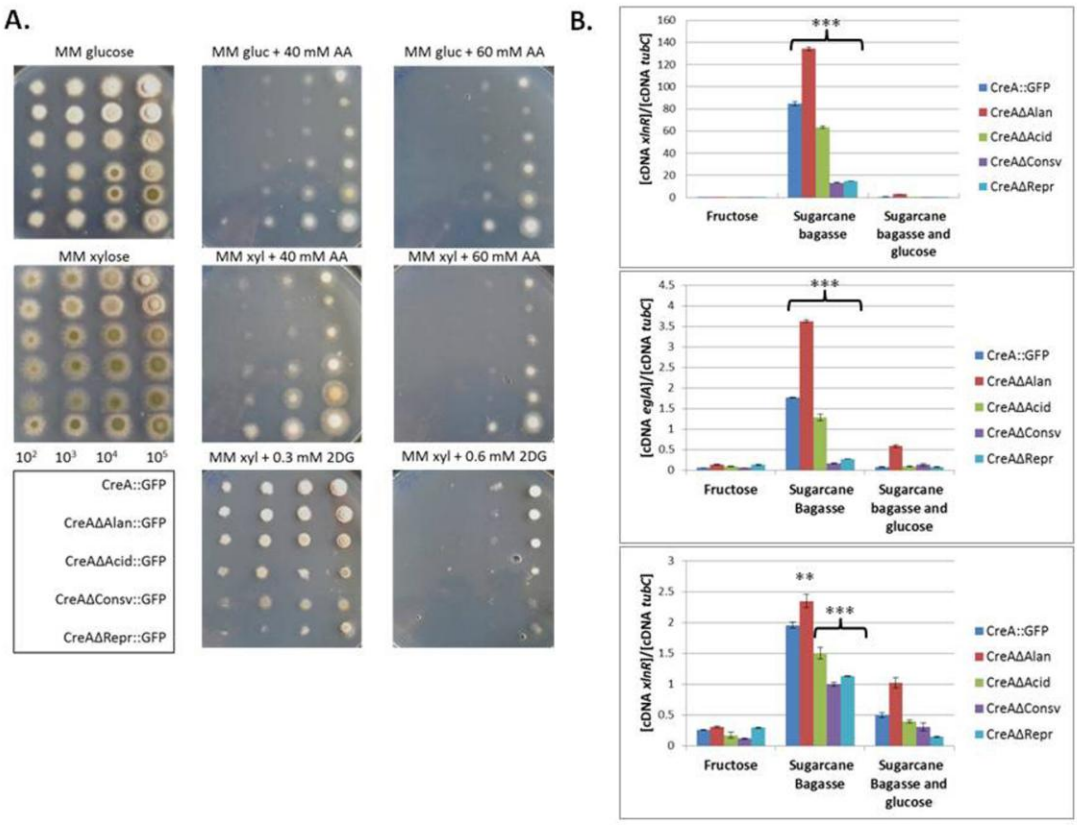

A.
MM glucose
MM gluc + 40 mM AA
MM gluc + 60 mM AA
MM xylose
MM xyl + 40 mM AA
MM xyl + 60 mM AA
10² 10³ 10⁴ 10⁵
MM xyl + 0.3 mM 2DG
MM xyl + 0.6 mM 2DG
CreA::GFP
CreAΔAlan::GFP
CreAΔAcid::GFP
CreAΔConsv::GFP
CreAΔRepr::GFP
B.
[cDNA xlnR]/[cDNA tubC]
[cDNA eglA]/[cDNA tubC]
[cDNA xlnR]/[cDNA tubC]
Fructose
Sugarcane bagasse
Sugarcane bagasse and glucose
Sugarcane Bagasse
CreA::GFP
CreAΔAlan
CreAΔAcid
CreAΔConsv
CreAΔRepr
***
**

Figure 5

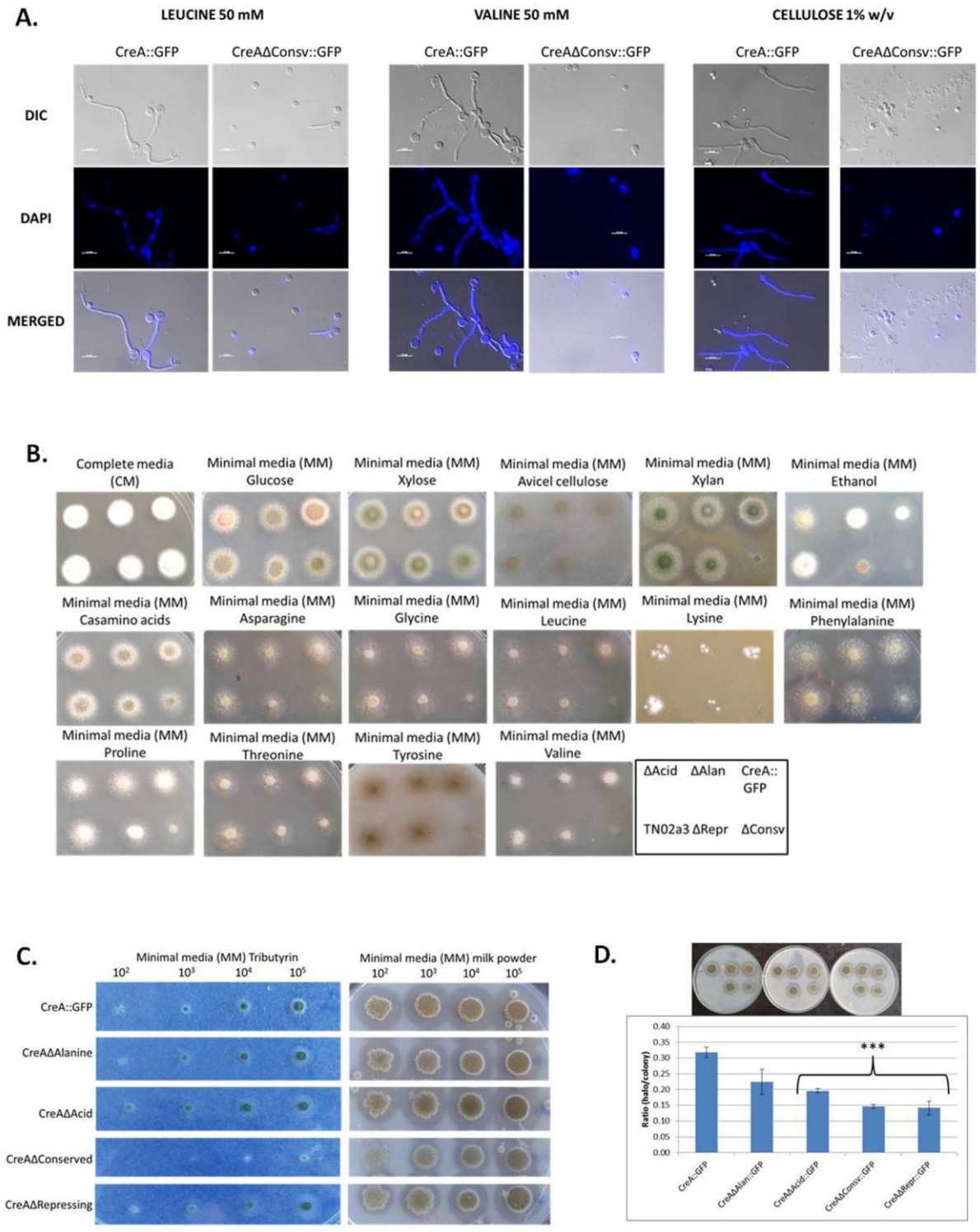
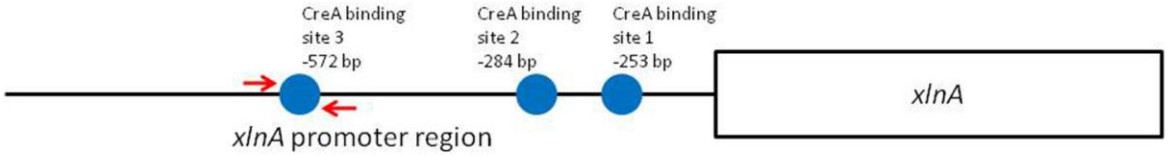

A.
LEUCINE 50 mM
VALINE 50 mM
CELLULOSE 1% w/v
CreA::GFP
CreAΔConsv::GFP
DIC
DAPI
MERGED
B.
Complete media (CM)
Minimal media (MM) Glucose
Minimal media (MM) Xylose
Minimal media (MM) Avicel cellulose
Minimal media (MM) Xylan
Minimal media (MM) Ethanol
Minimal media (MM) Casamino acids
Minimal media (MM) Asparagine
Minimal media (MM) Glycine
Minimal media (MM) Leucine
Minimal media (MM) Lysine
Minimal media (MM) Phenylalanine
Minimal media (MM) Proline
Minimal media (MM) Threonine
Minimal media (MM) Tyrosine
Minimal media (MM) Valine
ΔAcid ΔAlan CreA:: GFP
TN02a3 ΔRepr ΔConsv
C.
Minimal media (MM) Tributyrin
Minimal media (MM) milk powder
10² 10³ 10⁴ 10⁵
CreA::GFP
CreAΔAlanine
CreAΔAcid
CreAΔConserved
CreAΔRepressing
D.
Ratio (halo/colony)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
***
CreA::GFP
CreAΔAlan::GFP
CreAΔAcid::GFP
CreAΔConsv::GFP
CreAΔRepr::GFP

Figure 6

A.


CreA binding
site 3
-572 bp
CreA binding
site 2
-284 bp
CreA binding
site 1
-253 bp
xlnA
xlnA promoter region

B.

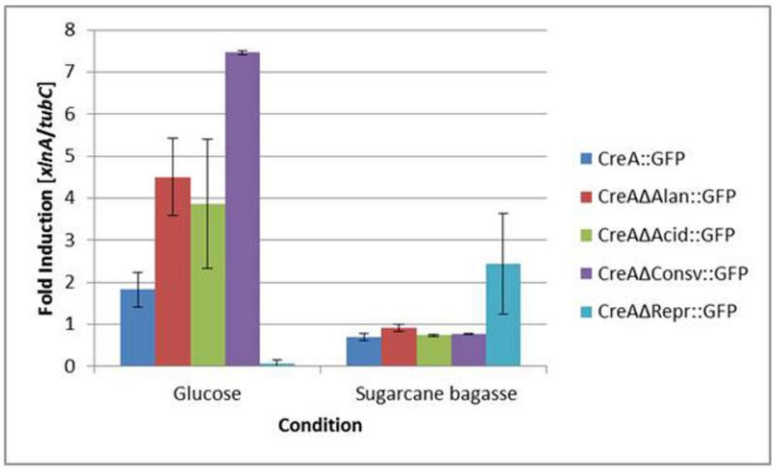

Fold Induction [xlnA/tubC]
8
7
6
5
4
3
2
1
0
Glucose
Sugarcane bagasse
Condition
CreA::GFP
CreAΔAlan::GFP
CreAΔAcid::GFP
CreAΔConsv::GFP
CreAΔRepr::GFP

Figure 7

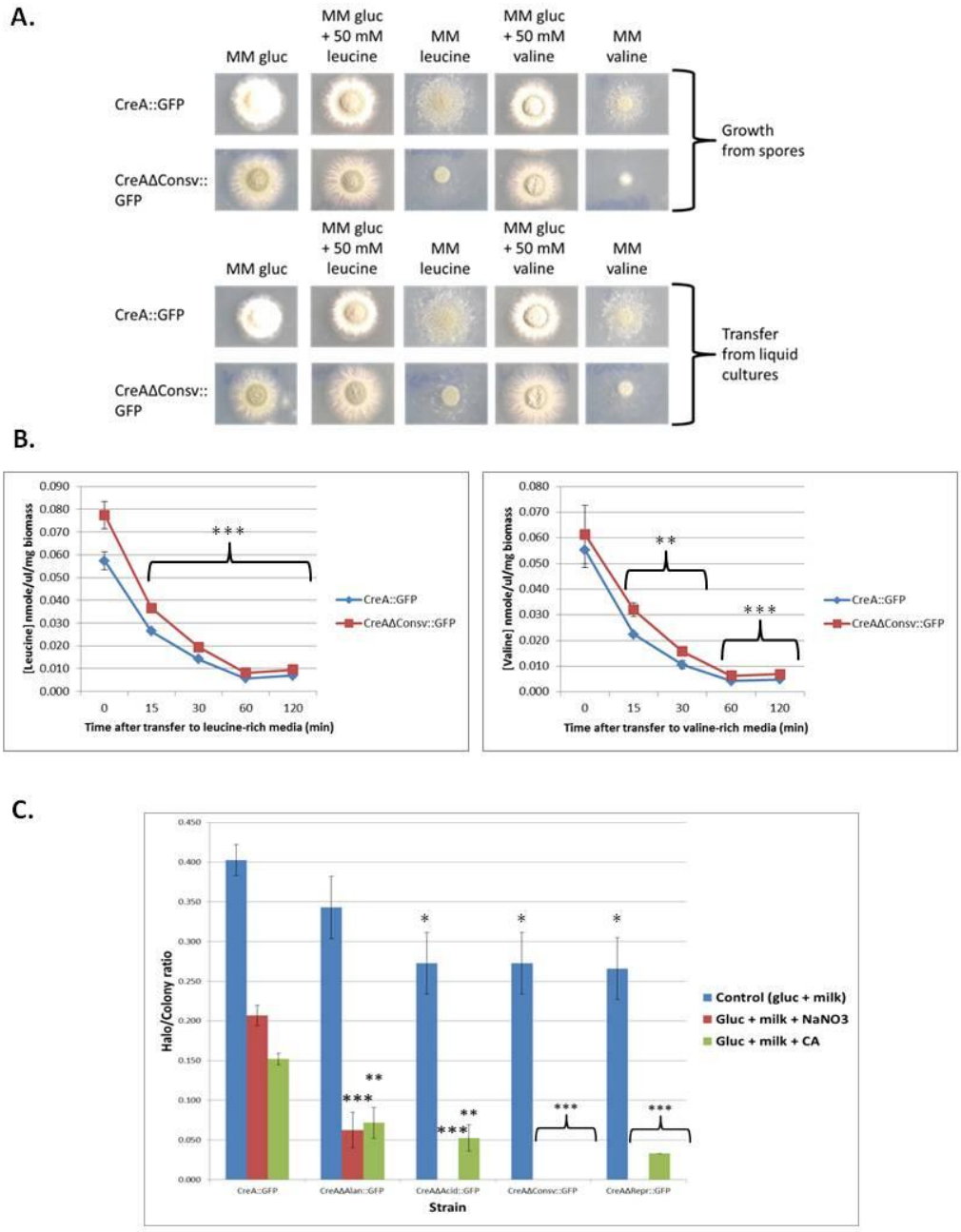

A.
MM gluc
MM gluc + 50 mM leucine
MM leucine
MM gluc + 50 mM valine
MM valine
CreA::GFP
CreAΔConsv:: GFP
Growth from spores
Transfer from liquid cultures
B.
[Leucine] nmole/ul/mg biomass
Time after transfer to leucine-rich media (min)
CreA::GFP
CreAΔConsv::GFP
[Valine] nmole/ul/mg biomass
Time after transfer to valine-rich media (min)
C.
Halo/Colony ratio
Strain
CreA::GFP
CreAΔAlan::GFP
CreAΔAcid::GFP
CreAΔConsv::GFP
CreAΔRepr::GFP
Control (gluc + milk)
Gluc + milk + NaNO3
Gluc + milk + CA

Figure 8