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ORIGINAL ARTICLE

The CreB deubiquitinating enzyme does not directly target the CreA repressor protein in *Aspergillus nidulans*

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Abstract Ubiquitination/deubiquitination pathways are now recognized as key components of gene regulatory mechanisms in eukaryotes. The major transcriptional repressor for carbon catabolite repression in *Aspergillus nidulans* is CreA, and mutational analysis led to the suggestion that a regulatory ubiquitination/deubiquitination pathway is involved. A key unanswered question is if and how this pathway, comprising CreB (deubiquitinating enzyme) and HulA (ubiquitin ligase) and other proteins, is involved in the regulatory mechanism. Previously, missense alleles of *creA* and *creB* were analysed for genetic interactions, and here we extended this to complete loss-of-function alleles of *creA* and *creB*, and compared morphological and biochemical phenotypes, which confirmed genetic interaction between the genes. We investigated whether CreA, or a protein in a complex with it, is a direct target of the CreB deubiquitination enzyme, using co-purifications of CreA and CreB, first using strains that overexpress the proteins and then using strains that express the proteins from their native promoters. The Phos-tag system was used to show that CreA is a phosphorylated protein, but no ubiquitination was detected using anti-ubiquitin antibodies and Western analysis. These findings were confirmed using mass spectrometry, which confirmed that CreA was differentially phosphorylated but not ubiquitinated. Thus, CreA is not a direct target of CreB, and nor are proteins that form part of a stable complex with CreA a target of CreB. These results open up new questions regarding the molecular mechanism of CreA repressing activity, and how the ubiquitination pathway involving CreB interacts with this regulatory network.

Keywords Regulatory deubiquitination · Transcriptional repression · CreA DNA-binding protein · CreB deubiquitinating enzyme · *Aspergillus nidulans* · Carbon catabolite repression

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Introduction

In eukaryotic microorganisms, efficient growth is achieved through the interaction of regulatory networks including those governing carbon and nitrogen metabolism, sulphur, pH balance and salt tolerance (Kelly and Katz 2010; New et al. 2014; Penalva et al. 2008; Pilsyk et al. 2015; Wong et al. 2008). These mechanisms allow the most efficient use of the available nutrients and rapid adaptation to changing environmental conditions. In the case of carbon, carbon catabolite repression (CCR) allows the microorganisms to use favourable carbon sources and simultaneously repress the expression of genes that are required to metabolize less favoured carbon sources. The regulatory networks are not independent, with close interactions between the networks

for carbon and nitrogen repression, and starvation stress. Portney and colleagues reported that some of the transcriptional effect of CCR is via the regulation of transporters and the use of nitrogen substrates (Portnoy et al. 2011). We wish to use the model filamentous fungus, *Aspergillus nidulans,* to dissect and thus understand the molecular mechanism of CCR, which is an important pathway for potential manipulation in biotechnological applications in processes such as heterologous protein expression and ethanol fuel production.

In the filamentous fungus *Aspergillus nidulans*, CreA, a Cys_2-His_2 DNA-binding zinc finger transcriptional repressor protein, is involved in CCR (Dowzer and Kelly 1989, 1991). Missense and frameshift mutations in the *creA* gene result in partial derepression of a range of pathways subject to CCR, and in addition have significant effects on morphology. Complete loss-of-function mutations of the *creA* gene show strong morphological changes and high levels of derepression (Shroff et al. 1996, 1997). Homologues of CreA have been identified in a number of other filamentous fungi. In *Trichoderma reesei* and *Aspergillus oryzae*, strains with a deletion of the *creA* homologous gene also showed a more compact colony morphology than the WT strain (Ichinose et al. 2014; Ilmen et al. 1996). In *A. nidulans*, the activity of CreA is not critically regulated by sub-cellular localization as some CreA was present in the nucleus in both carbon catabolite repressing and derepressing conditions (Roy et al. 2008). Moreover, derepression does not require large scale degradation of CreA (Kamlangdee 2008). Strauss and colleagues reported that the regulation of CreA occurs at both transcriptional and post-transcriptional levels, and speculated that CreA activity could be changed due to modification of the protein which is supported by the presence of a sequence similar to a consensus sequence involved in ubiquitination (Strauss et al. 1999). Very recently, Ries and colleagues (2016) presented some preliminary evidence that CreA is ubiquitinated, based on a smear above the CreA band in a Western blot probed with an anti-ubiquitin antibody which was more intense in conditions that led to less repression.

Screens designed to select mutations that affect CCR also identified the *creB* and *creC* genes. In repressing conditions, mutations in the *creB* and *creC* genes result in expression of some enzymes including alcohol dehydrogenase, acetamidase, and acetyl co-enzyme A synthetase under conditions where they are usually absent due to CCR (Hynes and Kelly 1977; Kelly and Hynes 1977). In addition, these same mutant alleles show a pleiotropic range of phenotypes that are unrelated to CCR, such as poor growth on quinate and proline where these compounds provide the sole source of carbon (Hynes and Kelly 1977). Unlike *creA* mutations, complete and partial loss-of-function alleles of the *creB* gene show only small effects on colony morphology (Hynes and Kelly 1977; Lockington and Kelly 2001). The *creB* gene encodes a deubiquitinating enzyme of the ubiquitin processing protease family (Ubp/Usp) (Lockington and Kelly 2001) and the *creC* gene encodes a protein containing WD-40 repeats (Todd et al. 2000). Co-immunoprecipitation experiments have shown that both proteins are present in a complex and overexpression of CreB can compensate the lack of CreC but not vice versa, suggesting that CreB is the active partner of this complex (Lockington and Kelly 2002).

On the other hand, the *creD* gene was identified as a suppressor of the *creC27* mutant phenotype on medium containing fluoroacetamide in the presence of glucose. This mutation, *creD34*, can also suppress some phenotypic effects of *creB15* and, to a small extent, *creA204* (Hynes and Kelly 1977; Kelly 1980), suggesting that the *creD* gene plays an opposite role to the *creA*, *creB* and *creC* genes in the regulatory network of CCR. The *creD* gene encodes an arrestin motif protein that interacts with a ubiquitin protein ligase, HulA, indicating its involvement in the ubiquitination process in this regulatory network (Boase and Kelly 2004).

Based on the genetic screening results and identification of proteins that are potentially involved in the ubiquitination and deubiquitination pathways, a model was proposed that in derepressing conditions, the CreD/HulA complex might be involved in the ubiquitination of the repressor protein CreA, or a protein that acts in a complex with CreA, modifying its activity and preventing repression. Whereas in the presence of carbon sources that trigger repression, the deubiquitinating enzyme CreB, together with CreC, might remove the ubiquitin molecule or molecules and thus restore the repressing activity of CreA or a CreA complex (Kelly 2004).

CCR has also been studied in the unicellular yeast, *Saccharomyces cerevisiae*, and in a range of filamentous fungi. In *S. cerevisiae*, the repressor Mig1p has some sequence similarities with CreA, notably the DNA-binding domain, and the consensus sequence to which it binds, an acidic region and a small repression domain. The cellular events involved in repression and derepression are understood in *S. cerevisiae*, and important proteins are the Snf1 kinase, required for nuclear export of Mig1p in derepressing conditions, which is necessary for expression of genes required for catabolic pathways to provide glucose, and the Tup1p-Ssn6p corepressor complex which is involved in initiating a repressive chromatin structure in a number of separate repression pathways (Gancedo 1998; Kayikci and Nielsen 2015). In filamentous fungi, the upstream events that trigger repression and derepression, and the role of an Snf1-like kinase remain unclear, and there may be different mechanisms in different filamentous fungi. At least in *A. nidulans, Neurospora crassa* and *Penicillium marneffei,* the

Tup1p homologues are not critically involved (Hicks et al. 2001; Todd et al. 2003; Yamashiro et al. 1996). A SnfA-like kinase is not involved in CCR in *T. reesei* (Cziferszky et al. 2002, 2003) or *Fusarium graminearum* (Yu et al. 2014), and *snfA* mutants have little effect on many carbon catabolite repression phenotypes in *A. nidulans* (Hynes, MJ, Pers. Comm.). Recent work suggests that the nonessential protein kinases SnfA (homologue of Snf1p in *S. cerevisiae*) and SchA might be involved in nuclear export of CreA and hence mediate derepression of at least cellulases in *A. nidulans* (Brown et al. 2013), although the conditions used were more akin to starvation than derepression, which is supported by the elevation of the XprG starvation response transcription factor, and CreA is known to be degraded in starvation conditions (Alam et al., unpublished). *A. nidulans* genes encoding catalytic hexokinases, *frA/hxkA* and *glkA,* have been shown to be involved in CCR, and while mutations in these individual genes do not affect growth on glucose, nor lead to carbon catabolite derepression (Arst et al. 1990; Flipphi et al. 2003), inactivation of both *frA* and *glkA*, which results in neither fructose nor glucose being phosphorylated, results in derepression of genes subject to CCR. This suggests that phosphorylation is required to trigger repression in *A. nidulans*, but HxkA and GlkA must have overlapping function in CCR (Flipphi et al. 2003).

To unravel the regulatory steps in CCR in *A. nidulans*, it is important to investigate the possible interaction between CreA and CreB, as both positively affect repression. Previously, a double mutant strain containing the *creA204*

and *creB15* alleles was used to probe the genetic interaction between them (Hynes and Kelly 1977); however, both alleles were subsequently found to be missense alleles (Dowzer and Kelly 1991; Lockington and Kelly 2001) with only relatively weak phenotypes compared to loss-of-function alleles. In this study, first we describe evidence for genetic interaction in the double null mutant strain containing *creAΔ99* and *creB1937*. In *A. oryzae*, a double null mutant strain for the *creA* and *creB* genes has shown improved production for α-amylase enzymes compared with the *creA* and *creB* single mutants and WT strains (Ichinose et al. 2014). Secondly, we used strains containing tagged versions of CreA and CreB and co-purification, both in strains where the constructs were overexpressed and in strains where the constructs were expressed from their own promoters, to resolve the question as to whether the proteins are directly interacting or in a complex.

Materials and methods

Strains and media

Aspergillus nidulans strains used and their genotypes are shown in Table 1. *Aspergillus* media are based on those described by Cove (1966). Carbon and nitrogen sources were added aseptically to the media to reach the final concentrations as shown. Transformation of *A. nidulans* was based on the procedure of Tilburn et al. (1983).

Table 1 List of strains

Strains	Genotype	References
WT	*biA1; niiA4*	Pateman et al. (1967)
CreB1937	*yA1 pabaA1; creB1937*	Lockington and Kelly (2001)
CreAΔ99	*yA1 pabaA1; creAΔ99*	Shroff et al. (1997)
CreBΔ16	*biA1; creB1937; niiA4;*	Cross WT × CreB1937
CreABΔ	*yA1 pabaA1 creAΔ99; creB1937*	This work
CreA^{cTAP}14	*yA1 creAΔ99::pGEMTeasyCreA^{cTAP}; pyroA4; argB2*	This work
CreB^{FLAG}14	*yA1pabaA1; creB1937::pGEMTeasyCreB^{FLAG}*	This work
CreA^{cTAP}CreB^{FLAG}15	*yA1creAΔ99::pGEMTeasyCreA^{cTAP}; creB1937::pGEMTeasyCreB^{FLAG}; pyroA4*	This work
CreAΔ99nkuAΔ	*yA1 creAΔ99; pyroA2; nkuAΔ*	This work
CreB1937nkuAΔ	*yA1 pabaA1; creB1937; nkuAΔ*	This work
H17A12	*yA1 adE20 suA1adE20; areA217; riboB2*	Hynes (1975)
JKABA3	*yA1 pabaA1; creAΔ99; creB1937; areA217; niiA4*	This work
TA3H2	*yA1 pabaA4 creAΔ::argB$^+$; argB2; niiAx riboB2[pGPD::CreA^{HA2His6};pPL3]*	Kamlangdee (2008)
T3.6	*yA1 pabaA1; creB1937; riboB2::[pPL3]pGPDCreBFLAG*	Lockington and Kelly (2002)
TA3H2/T3.6	Diploid from TA3H2/T3.6	Kamlangdee (2008)
T7	*yA1 pabaA1; riboB2::[pPL3]pGPDCreA^{HAGFP}*	Kamlangdee (2008)
S4	*yA1 pabaA1; riboB2::CreA^{HAGFP}*	(Lockington, Unpublished)
CreB^{cTAP}15	*yA1 pabaA1;creB1937::pGEMTeasyCreB^{cTAP}; pyroA4*	This work
CreB1937 nkuAΔ_1	*yA1 pabaA1; creB1937; pyroA4; nkuAΔ*	This work

Table 2 List of oligonucleotides

Name	Sequence (5′–3′)	Purpose
349	TTGCTGGGAGGGGGCATC	Detection of *creA*Δ99
350	GCAACCAGGATCGTCAGT	
2056	GCGGTCATTACGGCTCTAGG	Detection of *creB1937*
2255	CCTACCAAGTGTTTCCGAACTG	
Tap_GA_2	AGTGCCACCTGACGCGCCCTCAAGCAGCAGGCGATC	5′*creA* fragment (CreA^cTAP construct)
Tap_GA_3	TCTTTTCCATGAACCTCTCAGCCAAGTCAC	
Tap_GA_4	TGAGAGGTTCATGGAAAAGAGAAGATGGAAAAAG	cTAP fragment (CreA^cTAP construct)
Tap_GA_5	TTTGGCCGGATCAGGTTGACTTCCCCGC	
Tap_GA_6	GTCAACCTGATCCGGCCAAAAAACTTCG	3′*creA* fragment (CreA^cTAP construct)
Tap_GA_7	CACCCGCCGCGCTTAATGCGCCGAATAGTAAACTGCTGAGGCGCTTG	
ArgB_F	TCAAGGGCCTGATGCAAT	Detection of single copy *argB* gene in CreA^cTAP14
ArgB_R	TATATTTAGGGACGGAATGATCTC	
cTAP_Test_F	TGAATTTGTTGTCGACTTTCGG	Homologous integration
CreAcTAP Homo	TCGCTCCAGGCTATCACACGC	
CreB_Gene_F	ATGTTCCTGCGATTTGTCTG	*creB* fragment (7 kb)
CreB_Gene_R	TCACTTTACTTCCTCACCTCCTC	
CreB_F	AGAGGTGCAGTCTCAAATCAGC	5′*creB* with FLAG tag
FLAG_CreB_RC_R	GGAAGAAACGGCACAACTCACTTGTCGTCATCGTCTTTGTAGTCTGATGAGAGGATAA	
FLAG_CreB_F	GTTGTGCCGTTTCTTCCTGG	3′*creB* fragment
CreB3′Rev_new	AACCCAGCATTCCACAAGC	
FLAG_test_R	CTTGTCGTCATCGTCTTTGTAGTC	Homologous integration
nkuA_F	GCGAGCGGTGTATGTCAGTA	*nkuA*Δ checking
nkuA_R	CGTTATTAGCGTCCGTCACA	
alcAf2	GAGGCTCTGGACTTCTTCGCT	*alcA* expression (RTqPCR)
alcAr2	GCGATTCTGCCTTGTTCCATA	
tubC3F	TAACCTGCTCAACCCTGTTCC	*tubC* expression (RTqPCR)
tubC3R	CATAGAGCACAGAGCAGTTTGGAC	
5′B5′TAP_RP	TCTTTTCCATTGATGAGAGGATATTAAAGGAC	5′*creB* fragment (CreB^cTAP construct)
TAP 5′ 3′B_FP	CCTCTCATCAATGGAAAAGAGAAGATGGAAAAAG	cTAP fragment (CreB^cTAP construct)
TAP5′3′B_RP	ACGGCACAACTCAGGTTGACTTCCCCGC	
3′B3′TAP_FP	GTCAACCTGAGTTGTGCCGTTTCTTCCTG	3′*creB* fragment (CreB^cTAP construct)

Genetic analysis techniques were based on Todd et al. (2007). The CreABΔ strain (containing both *creA*Δ99 and *creB1937*) was derived by meiotic crossing of CreAΔ99 and CreBΔ16 (Supplementary Fig. 1). DNA was extracted using DNeasy Plant Mini Kit (Qiagen). PCR was performed with gene-specific primers (Table 2) using Phusion HF polymerase (NEB) in a Mastercycler gradient (Eppendorf) PCR cycler. Amplicons were separated by 1% agarose gel electrophoresis, and purified using a MiniElute Gel Extraction kit (Qiagen). Sequencing was performed by the Australian Genomic Research Facility (AGRF) from purified DNA.

A diploid strain (TA3H2/T3.6) was developed by fusing two haploid strains TA3H2 and T3.6 (see Table 1 for genotype) that overexpress His/HA tagged CreA (CreA-^2HAHis) and FLAG tagged CreB (CreB^FLAG) (Lockington and Kelly 2002), respectively. Diploids that express both the proteins were selected for CoIP. The strains were phenotypically wildtype. For Western analysis, strains were incubated in 1% glucose at 37 °C with shaking. CreA was purified using a His-Trap HP column (GE Healthcare) and Anti-HA Matrix (Roche). Detection of proteins was carried out by probing with anti-FLAG peroxidase linked monoclonal antibody (Sigma) for CreB^FLAG and anti-HA

monoclonal antibody conjugated with peroxidase (Roche) for CreA^{2HAHis}.

The construct used to generate the strain CreA^{cTAP}14, containing a TAP-tagged version of CreA, was generated by fusion PCR (Szewczyk et al. 2006) and contains sequences that are 5′ to *creA* including the native promoter, the *creA* coding sequence, a C-terminal TAP-tag and sequences 3′ to *creA* (Supplementary Fig. 3). The fusion product was then ligated into the pGEMTeasy vector (Promega), to produce pGEMTeasy CreA^{cTAP}. The plasmid was transformed into an *nkuA* deletion strain (CreAΔ99nkuAΔ) for homologous integration (Nayak et al. 2006), and colonies that were complemented for CreA were selected. The strains were phenotypically wildtype. Homologous integration was confirmed by genomic PCR using a primer internal to the TAP-tag and another ORF-specific primer, which generated a 3 kb DNA fragment for CreA^{cTAP}14. Plate testing was performed with synthetic media containing allyl alcohol to confirm expression of CreA. For Western analysis, CreA^{cTAP}14 was incubated in 1% glucose (repressing) or 1% arabinose (derepressing) with 0.1% fructose at 37 °C for 20–24 h with shaking. Mycelia were harvested, press dried and lyophilized overnight. CreA^{cTAP} was purified as described by Bayram et al. (2012) and detected by probing with anti-TAP conjugated with peroxidase polyclonal antibody (GeneScript). In the Western analysis, the CreA^{cTAP}(~63 kDa) protein was detected, together with N-terminally processed CreA of lower molecular weight. The processing of the N-terminus of the CreA protein has been detected previously in a strain containing an N-terminal Myc tagged CreA, when CreA could not be detected by anti-Myc antibody in WB analysis (Lockington, unpublished), and smaller CreA variants were detected in previous published work (Roy et al. 2008).

The strains CreB^{FLAG}14 and CreB^{cTAP}15 were generated and tested in an analogous way to CreA^{cTAP}14, except using *creB* sequences and a FLAG-tag or cTAP-tag (Supplementary Figs. 4, 5). Transformants that were fully complemented for CreB were selected, and the expression of CreB in CreB^{FLAG}14 and CreB^{cTAP}15 was confirmed by growth testing on quinate plus ammonium tartrate medium. The growth conditions for Western analysis were the same as for CreA^{cTAP}14. CreB^{FLAG} (~85 kDa) was purified using Anti-FLAG M2 affinity gel matrix (Sigma), and detected by probing with anti-FLAG peroxidase linked monoclonal antibody (Sigma). CreB^{cTAP} (~106 kDa) was purified and detected as for the CreA^{cTAP}14 strain.

A strain was developed (CreA^{cTAP}CreB^{FLAG}15) that contains both CreA^{cTAP} and CreB^{FLAG} by a genetic cross between CreA^{cTAP}14 and CreB^{FLAG}14 (Supplementary Fig. 6). Phenotypically wildtype progeny from the cross were selected and confirmed using PCR and Western analysis.

Enzyme assays

Estimates of β-galactosidase and α-amylase activities were made using plate tests based on the procedures described in Shroff et al. (1997). Enzyme assays for alcohol dehydrogenase (ADH) were performed according the method described in Hynes and Kelly (1977). Strains were incubated in 50 mM ethyl methyl ketone (EMK) and 1% glucose or only 50 mM EMK representing repressed-induced and induced conditions, respectively. 0.1% fructose was added to all media to prevent starvation of strains. After 24 h of incubation at 37 °C with shaking, proteins were extracted and enzyme activity was determined by the ethanol dependent reduction of NAD at 340 nm at 25 °C. Absorbance was measured using a Spectramax M2 spectrophotometer (Molecular Device, USA).

Reverse-transcription quantitative real time PCR (RT-qPCR)

RNA was extracted from the same mycelia that were used for ADH enzyme assay using RNeasy Plant Mini Kit (Qiagen). First strand synthesis of cDNA was performed using the cDNA synthesis kit protocol (Promega). RT-qPCRs were performed with 50 ng cDNA, specific primers and Power SYBR green master mix PCR solution (Applied Biosystem) using an ABI Prism 7000 Sequence Detection System (Applied Biosystem). The PCR cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. In all experiments appropriate negative controls containing no DNA template were used. A standard curve was prepared by serial dilution of cDNA for each experiment. RT-qPCR was performed in triplicate for each sample. Cycle thresholds for each triplicate were averaged and normalized against the expression of *tubC* (β-tubulin), which was used as an endogenous control, as described in Semighini et al. (2002). The relative standard curve method for quantification was used to determine the relative fold change in the expression of experimental samples compared to the endogenous control. Three biological replicates were used for calculation of the expression of the gene of interest.

Detection of phosphorylation

An acryl amide pendant Mn^{2+} Phos-tag ligand system (Wako Pure Chemical Industry, Japan) and phosphate affinity SDS-PAGE was used for detection of phosphorylated proteins indicated by a mobility shift. The dinuclear metal complex acts as a selective phosphate binding-tag molecule in an aqueous solution at a neutral pH. The degree of migration of phosphoproteins is altered compared to those of their nonphosphorylated counterpart, because the

tag molecules trap phosphoproteins reversibly during electrophoresis (Kinoshita et al. 2009a). CreA was immunoprecipitated from T7, expressing CreA^{HAGFP} from the *gpdA* promoter, in repressing and in derepressing conditions. Purified samples were treated with bovine alkaline phosphatase (Sigma) for 60 min according to the manufacturer's protocol, along with β-casein as a positive control. Alkaline phosphatase treated and untreated samples were separated using a 10% SDS-acrylamide gel containing 100 µmol/L Phos-tag solution. Samples were transferred to PVDF membrane, and β-caseins were detected using Coomassie blue staining and CreA^{HAGFP} was detected by probing with anti-HA peroxidase high-affinity antibody (Roche).

Detection of ubiquitination

UbiQapture-Q matrix (Biomol) was used to purify ubiquitinated proteins. The TA3H2 (CreA^{HAHis}) strain that overexpresses *creA* from the *gpdA* promoter was grown overnight in 1% glucose at 37 °C. Three fractions (total extract, unbound fraction, immunoprecipitate) of TA3H2 and WT strains were separated by SDS-PAGE and transferred to PVDF membrane. CreA^{HAHis} was detected by probing with anti-HA peroxidase high-affinity antibody (Roche). An anti-ubiquitin antibody was also used to detect whether purified proteins were ubiquitinated. Two strains, T7 and S4, that express CreA^{HAGFP} from the *gpdA* and native promoter, respectively, were grown in 1% glucose (repressing) and 1% arabinose (derepressing) overnight at 37 °C. CreA^{HAGFP} was purified from the total lysate using anti-HA matrix (Roche). IP samples were loaded in duplicate and probed with anti-HA peroxidase high-affinity antibody (Roche) and anti-ubiquitin conjugated with peroxidase antibody (Abcam). Free ubiquitin was added as a positive control.

Proteomic analysis

CreA and CreB were purified from CreA^{cTAP}14 and CreB^{cTAP}15 using the TAP purification protocol (Bayram et al. 2012) and separated by SDS-PAGE. Proteins were fixed using 10% acetic acid and 40% methanol solution for 2 h at room temperature. Coomassie staining of the gel was performed with CBB G-250 solution for overnight at room temperature.

For CreA, detection of proteins by LC–MS was performed as described in Meding et al. (2013) by The Adelaide Proteomics Center. Raw data files were subjected to the Proteome Discover software (Thermo Scientific) to set up the workflow.

For CreB, Nano-LC–ESI–MS/MS was performed using an Ultimate 3000 RSLC system (Thermo-Fisher Scientific, Waltham, USA) coupled to an Impact HD™ QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) via an Advance CaptiveSpray source (Bruker Daltonics). Peptide samples were pre-concentrated onto a C18 trapping column (Acclaim PepMap100 C18 75 µm × 20 mm, Thermo-Fisher Scientific) at a flow rate of 5 µL/min in 2% (v/v) ACN 0.1% (v/v) FA for 10 min. Peptide separation was performed using a 75 µm ID C18 column (Acclaim PepMap100 C18 75 µm × 50 cm, Thermo-Fisher Scientific) at a flow rate of 0.2 µL/min using a linear gradient from 5 to 45% B (A: 5% (v/v) ACN 0.1% (v/v) FA, B: 80% (v/v) ACN 0.1% (v/v) FA) over 70 min, followed by a 20-min wash with 90% B, and a 20 min equilibration with 5% A. MS scans were acquired in the mass range of 300–2200 *m/z* in a data-dependent fashion using Bruker's Shotgun Instant Expertise™ method. This method uses IDAS (intensity-dependent acquisition speed) to adapt the speed of acquisition depending on the intensity of precursor ions (fixed cycle time), and RT^2 (RealTime Re-Think) to exclude previously selected precursor ions from undergoing re-fragmentation unless the chromatographic peak intensity of the ion has increased by a factor of 5. Singly charged precursor ions were excluded from acquisition. Collision energy ranged from 23 to 65% as determined by the *m/z* of the precursor ion.

Nano-LC–ESI-MS/MS data analysis

All spectra were analysed using the MaxQuant software (version 1.5.2.8) with the Andromeda search engine against the UniProt *Emericella nidulans* database (downloaded on the 10th of January 2016). The standard Bruker QTOF settings in MaxQuant were used with a mass error tolerance of 40 ppm. The variable modifications of oxidation of methionine, phosphorylation of serine/threonine/tyrosine, ubiquitination of lysine, and the fixed modification of carbamidomethyl of cysteines were specified, with the digestion enzyme specified as trypsin. The false discovery rate (FDR) was set to 1% for both proteins and peptides, with a minimum peptide length of 7 amino acids.

Results

The CreABΔ strain; a double null mutant for the *creA* and *creB* genes

Genetic analysis of a double mutant for the *creA* and *creB* genes using the *creA204* and *creB15* alleles has been previously published (Hynes and Kelly 1977). Subsequent molecular analysis revealed that both the alleles contain missense mutations and are only partial loss-of-function alleles showing only intermediate phenotypic effects (Lockington and Kelly 2001; Shroff et al. 1996).

Null alleles of the *creA* gene, such as *creAΔ99* in which the DNA-binding region of the *creA* gene is disrupted by inserting the *argB*[+] gene, were developed using gene replacement techniques. This allele is designed to truncate CreA after the first 45 amino acids, and results in similar phenotypes to the naturally occurring null allele, *creA303*, which theoretically encodes the first 68 amino acids but no zinc finger region or any other putative functional regions (Shroff et al. 1997). On the other hand, a null allele of the *creB* gene, *creB1937,* which was identified during molecular analysis of the gene, contains a G to A mutation which is predicted to result in a truncation of CreB after the first 12 amino acids (Lockington and Kelly 2001). This is the first reported analysis of a strain that is a null mutant for both the *creA* and *creB* genes in *A. nidulans*. To observe the genetic effects due to the functional loss of both the *creA* and *creB* genes, we developed a double null mutant strain, CreABΔ by crossing the CreAΔ99 and CreBΔ16 strains. The double null mutant strain CreABΔ was confirmed using molecular genetic

techniques (see "Materials and methods"; Table 1; Supplementary Fig. 1).

Phenotypic analysis

Phenotypes of the CreABΔ strain were observed in a range of carbon and nitrogen sources (Fig. 1; Supplementary Fig. 2). CreAΔ99 grows as a very compact colony on both complete and synthetic complete media, whereas *creB1937* containing strains (CreB1937 and CreBΔ16) showed only slight effects on colony morphology on these media. However, the double null mutant CreABΔ strain showed an intermediate effect on colony morphology showing a less compact and dense colony, suggesting some suppression of the effects of *creAΔ99* on morphology by *creB1937* (Fig. 1a, c).

Addition of allyl alcohol to synthetic complete medium serves as an indicator of repression or derepression of *alcA*, which encodes alcohol dehydrogenase I, which is subject to carbon catabolite repression in the wildtype strain.
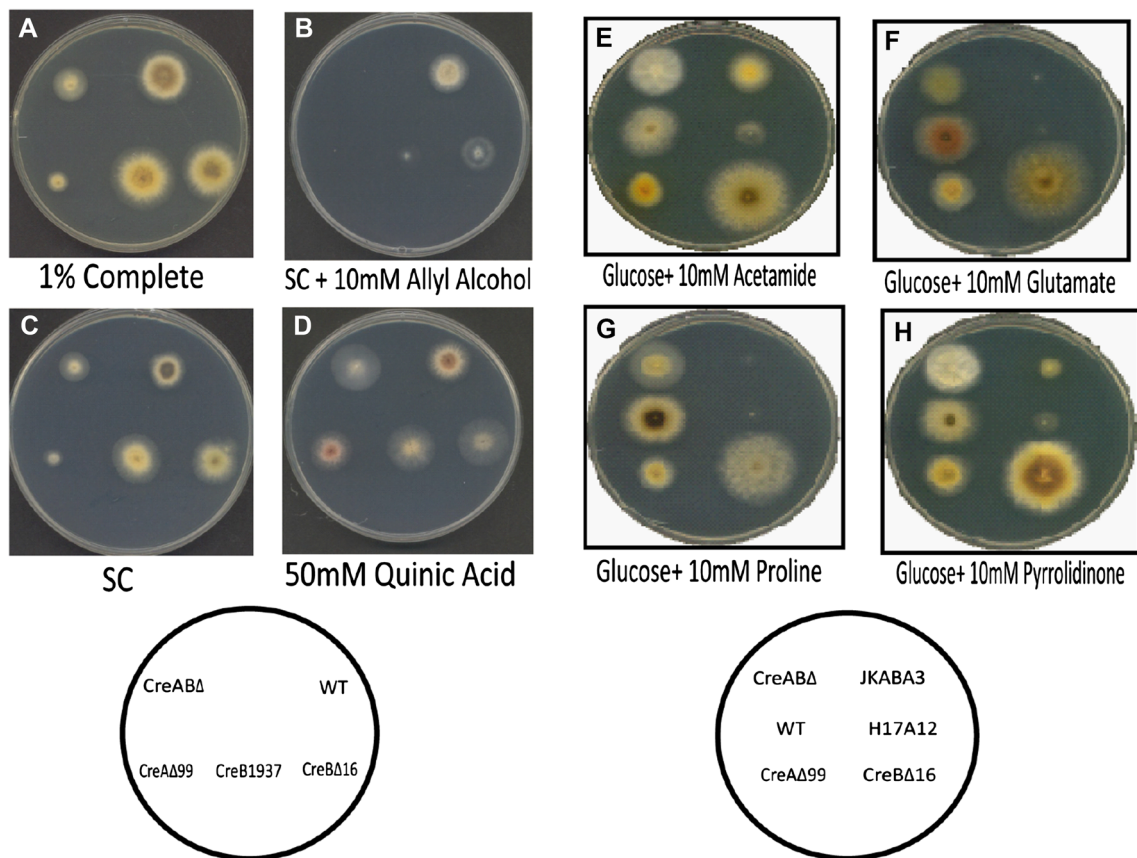

Fig. 1 Phenotypic analysis of strain CreABΔ. Strains were grown on a range of media as indicated at the *bottom of each panel* at 37 °C for 2–3 days panel. In **e**, **f**, **g** and **h** the phenotype of CreABΔ was compared with a triple mutant strain which also contained the *areA217* allele (JKABA3), and scored for suppression of the effects of *areA217*. 10 mM ammonium tartrate was used as nitrogen source in **b**, **c** and **d**, and although tartrate provides a very weak carbon source, it is not a significant carbon source in these tests where carbon starvation is not being tested. Strains keys are at the *bottom of the figure*. H17A12 carries the *areA217* mutation (see Table 1)

CreAΔ99, CreB1937 and CreBΔ16 were sensitive to allyl alcohol, with *creAΔ99* leading to a more extreme phenotype, indicating higher levels of derepression due to the *creAΔ99* mutation. The double null mutant CreABΔ strain was as sensitive as CreAΔ99 in this test (Fig. 1b).

Strains containing *creB1937* (CreB1937 and CreBΔ16) show reduced growth on some sole carbon sources, including quinate and proline, due to failure to express enzymes and permeases that are needed to metabolize those carbon sources, while strains containing *creAΔ* mutations (CreAΔ99) show very little effect in these conditions (Hynes and Kelly 1977). The double null mutant strain, CreABΔ, grew as weakly as CreB1937 and CreBΔ16 on quinate medium. In addition, the effects on colony morphology due to the *creAΔ99* allele are less apparent on this carbon source (Fig. 1d).

The CreABΔ strain was also tested for growth on acetamide, glutamate, proline and pyrrolidinone as a carbon and/or nitrogen source. When these compounds were used as carbon sources, the CreB1937 and CreBΔ16 strains grew more poorly than WT, while the CreAΔ99 strain had little effect on growth. The CreABΔ double mutant strain showed strong growth on acetamide, but showed weak growth like CreB1937 and CreBΔ16 on the other compounds (Supplementary Fig. 2). On the other hand, when these nutrients were used as the sole nitrogen source with glucose provided as a carbon source, CreB1937 and CreBΔ16 showed stronger growth on acetamide and pyrrolidinone and weaker growth on glutamate and proline, than the WT. However, CreAΔ99 showed strong utilization of acetamide, but had little effects on utilization of other nutrient sources. The double null mutant showed strong growth on acetamide like CreAΔ99, but was similar to the CreB1937 and CreBΔ16 phenotypes on other media (Supplementary Fig. 2).

The suppression of the *areA217* mutant phenotype was tested in a strain that was triple mutant for the *creA*, *creB* and *areA* genes. Strains carrying loss-of-function alleles of *areA* grow poorly, in the presence of glucose, on nitrogen sources that also provide carbon sources (Arst and Cove 1973). Mutations affecting carbon catabolite repression can suppress this phenotype. A strain that contained *creAΔ99*, *creB1937* and *areA217* (JKABA3) showed strong suppression of the *areA217* mutant phenotype on glucose plus acetamide medium, but failed to suppress *areA217* on the other nitrogen sources tested (Fig. 1e–h).

creA mutants cause sensitivity to acriflavine and molybdate, while *creB* mutants lead to increased sensitivity to acriflavine but resistance to molybdate (Arst 1981). Phenotypes of the CreABΔ strain were observed in the presence of the toxic compounds acriflavine and molybdate. The CreABΔ strain showed increased sensitivity to acriflavine (Fig. 2a i–ii), but resistance to molybdate like the CreB1937 and CreBΔ16 (Fig. 2a iii–iv).
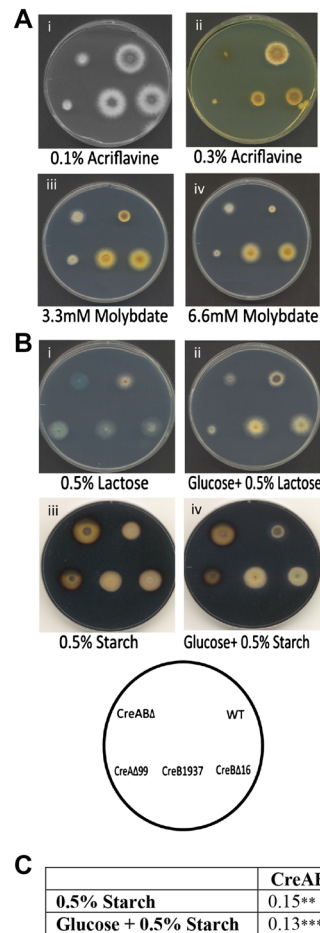

	CreABΔ	CreAΔ99	CreBΔ16	WT
0.5% Starch	0.15**	0.29****	0.03*	0.01
Glucose + 0.5% Starch	0.13****	0.24***	0.00	0.00

Fig. 2 Resistance to toxic compounds; and β galactosidase and α amylase activities of CreABΔ strain. Strains were grown on media containing: for toxicity, the concentrations of acriflavine and molybdate shown (**a**, *i–iv*); for an estimation of β galactosidase activity, 0.5% lactose + X-gal (100 µg/ml) (**b**, *i–ii*); for an estimation of α amylase activity, 0.5% starch (**b**, *iii–iv*). β Galactosidase activity was estimated by the formation of blue precipitate due to break down of X-gal by β galactosidase enzyme. To estimate α amylase activity, the clear zone was measured after 2 days by adding iodine solution to the plates. Estimates were calculated using the distance between the edge of the clear zone and edge of the colony divided by the diameter of the colony. Part **c** shows the average clear zone of three biological replicates. Unpaired *t* test (two-tailed) compared with WT in respective conditions: ****< 0.00005; ***<0.0005; **<0.005; *< 0.05. All plates contain 10 mM ammonium tartrate, and were incubated at 37 °C for 2 days. A strains key is at the *bottom of the figure*

Plate testing as a measure of enzyme activity

β-Galactosidase is required to breakdown lactose into galactose and glucose and the expression is a subject to carbon catabolite repression (Shroff et al. 1997). β-Galactosidase activity can be estimated by growing a colony on a solid agar medium containing X-gal as an indicator, which is broken down by β-galactosidase to produce bright blue colour. Strains were grown in 0.5%

lactose alone and also in the presence of 1% glucose. While the WT strain produced very little blue pigment, and only when glucose was absent, both CreAΔ99, CreB1937 and CreBΔ16 resulted in increased blue pigment whether glucose was present or absent. The CreABΔ strain produced higher blue pigment than either of the parents in both the presence and absence of glucose (Fig. 2b i–ii). This result indicates that higher levels of expression were achieved when both the *creA* and *creB* genes were mutated whether glucose was present in the media or not.

Aspergillus nidulans secrets α-amylases into the media surrounding a colony when starch is present. Total α-amylase activity can be estimated by adding iodine solution to the medium which interacts with starch to produce a deep blue colour. In contrast, the area where starch has been digested by α-amylases appears as a nonstained clear zone. The distance between the edge of the clear zone and edge of the colony divided by the diameter of the colony allows an estimate of the amount of α-amylase secreted by the colony (Shroff et al. 1997). In the presence of glucose, the WT strain does not express α-amylases as these enzymes are subject to carbon catabolite repression. To estimate the amount of α-amylases secretion, strains were incubated with 0.5% starch and/or 1% glucose, and after 2 days incubation, the clear zone was measured. CreAΔ99 produced the biggest clear zone in both the growing conditions, whereas CreBΔ1937 and CreBΔ16 produced little clear zone and only in the absence of glucose. In the presence of glucose, CreBΔ1937 and CreBΔ16 formed no clear zone like the WT strain. The double null mutant strain had less α-amylases activity than the CreAΔ99, but more than CreBΔ1937 and CreBΔ16 (Fig. 2b iii–iv). Figure 2c shows the average ratio of clear zone produced by the strains in three biological replicates.

Alcohol dehydrogenase activity (enzyme assay and RTqPCR)

The extent of derepression in the CreABΔ strain was also investigated by assaying alcohol dehydrogenase activity (ADH). Alcohol dehydrogenase I (ADHI) is encoded by the *alcA* gene and it is required to breakdown ethanol to acetaldehyde (Lockington et al. 1985; Pateman et al. 1983). Expression of *alcA* is regulated both by specific induction and carbon catabolite repression. A co-inducer, such as ethanol or ethyl methyl ketone (EMK), is required for expression, but when glucose is also present carbon catabolite repression mediated by CreA prevents the transcription of both the structural gene *alcA* and the regulatory gene required for induction *alcR* (Kulmburg et al. 1992, 1993). Strains were incubated in 1% glucose plus 50 mM EMK medium, or in 50 mM EMK medium alone, representing repressed-induced and induced conditions, respectively.

0.1% fructose and 10 mM ammonium tartrate were added to all media. Enzyme activity was determined after 24 h incubation [see "Materials and methods"; (Hynes and Kelly 1977)].

In repressed-induced conditions there was undetectable enzyme activity in the WT, whereas CreAΔ99 showed activity in these conditions which was as expected, and CreB1937 showed marginal activity (which is less than previously published results with the *creB15* allele (Hynes and Kelly 1977)). The double null mutant showed higher levels of enzyme activity than either of the parent strains. In inducing conditions, CreAΔ99, CreB1937 and the double null strain showed similar activity to the WT strain (Fig. 3a).

The CreB deubiquitinating enzyme is thought to play an important role in carbon catabolite repression of *A. nidulans,* possibly by removing a ubiquitin molecule from CreA, or a protein complexed with CreA. Experimental evidence suggests that CreB also has other targets including QutD (Kamlangdee 2008). Poly-ubiquitination/deubiquitination plays a role in degradation by the proteasome, but ubiquitination/deubiquitination is also known to play a number of other regulatory roles in the cell [reviewed in (Kaiser and Huang 2005)]. Due to the possible involvement of an ubiquitination–deubiquitination system that causes protein degradation, RT-qPCR was performed to confirm whether the differences in alcohol dehydrogenase activity were reflected at the mRNA level.

RNA was extracted from the same mycelia that were used for ADH enzyme assay. In repressed-induced conditions, there was undetectable *alcA* mRNA in the WT, whereas CreAΔ99 showed *alcA* mRNA in these conditions which was as expected. However, *alcA* mRNA was not detectable in CreB1937, although previous experiments using the *creB1937* allele did show some *alcA* mRNA (Hunter et al. 2013). The double null mutant strain gave indication of higher *alcA* mRNA than either CreAΔ99 or CreB1937 in all replicates, but this was not statistically significant overall due to the variation between replicates (Fig. 3b).

Are CreB and CreA present in the cell in a complex?

Since the plate tests and enzyme assays confirmed a genetic interaction between *creA* and *creB*, co-immunoprecipitation experiments were performed to investigate whether the products of these two genes are present in a complex. Since there are no antibodies available to CreA, a diploid strain (TA3H2/T3.6) was constructed by fusing two haploid strains TA3H2 and T3.6, that express His/HA tagged CreA (CreA[2HAHis]) and FLAG tagged CreB (CreB[FLAG]), respectively, from the constitutively expressing *gpdA* promoter [(Lockington et al. 2002; Roy et al. 2008); Table 1]. The diploid strain and the haploid parent strains were grown in
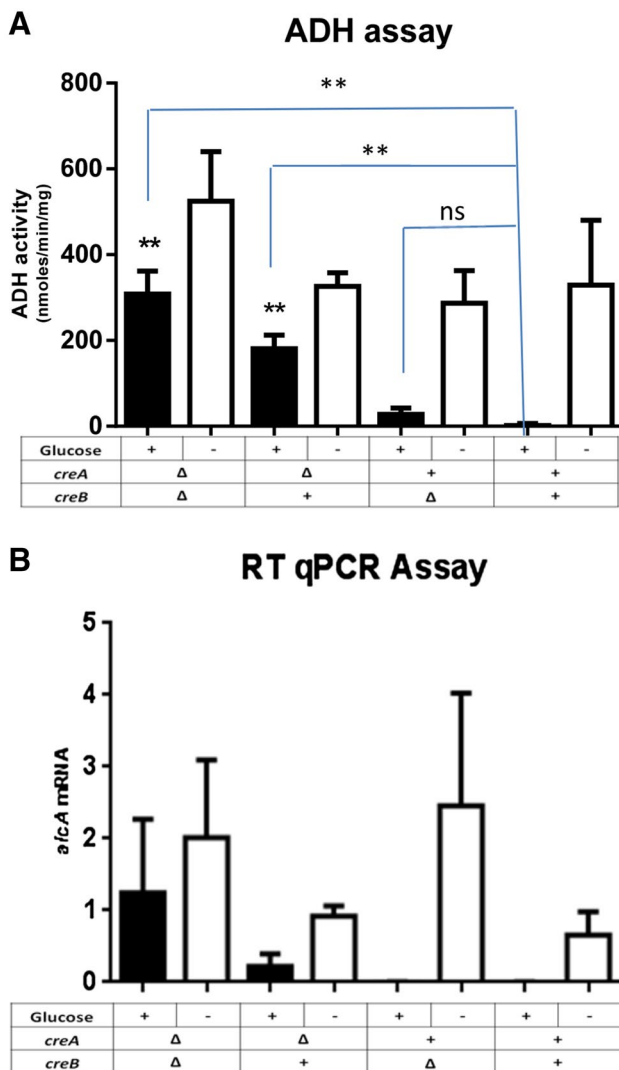
A   ADH assay

Glucose	+	–	+	–	+	–	+	–
creA	Δ		Δ		+		+	
creB	Δ		+		Δ		+	

B   RT qPCR Assay

Glucose	+	–	+	–	+	–	+	–
creA	Δ		Δ		+		+	
creB	Δ		+		Δ		+	

Fig. 3 Alcohol dehydrogenase activity and *alcA* expression in CreABΔ. Strains were grown in media containing 0.1% fructose + 50 mM EMK + 10 mM ammonium tartrate with and without 1% glucose for 24 h at 37 °C with continuous shaking. The alcohol dehydrogenase reaction was initiated by 2.8 ml of assay buffer with NAD, ethanol and 0.1 ml of crude extract. ADH activity was determined by ethanol dependent reduction of NAD at 340 nm at 25 °C. This graph represents the average ADH activity of three biological replicates. Unpaired *t* tests (two-tailed) compared with WT in respective conditions were calculated, and *p* values are shown for repressed-induced conditions (**<0.005); No significant *p* values were detected between relevant samples from induced mycelia (**a**). RNA extracted from the same mycelia were used to generate cDNA and qPCR was performed using SYBR green as detector. Cycle thresholds for each triplicate were averaged and normalized against the expression of *tubC*. This graph represents average *alcA* mRNA of three biological replicates. Unpaired *t* tests (two-tailed) compared with WT in respective conditions were calculated, and no significant *p values* were detected between the relevant samples. In the induced conditions, this probably indicates no significant difference, but the variation is large. In repressed conditions, although not statistically significant, the graph is included to indicate a possible trend in the double null strain (**b**). *Error bars* represent the standard errors of the means

glucose medium, and proteins were extracted and initial Westerns showed that CreA[2HAHis] and CreB[FLAG] could be detected as expected using anti-HA and anti-FLAG antibodies, respectively. CreA was purified from the lysate using a two-step procedure, firstly by using a His-trap column, and then the eluate was subjected to a further round of purification using anti-HA matrix. The purified proteins were analysed by Western transfer. When probed with anti-FLAG antibody, after two rounds of purification for CreA-[2HAHis], CreB[FLAG] was also co-purified in the diploid strain, but not in the haploid strain containing CreB[FLAG] (Fig. 4). This indicates that in this strain, at least some CreB and CreA may exist in a complex in vivo in *A. nidulans*. However, when CreB[FLAG] was purified from the lysate using anti-FLAG matrix, no CreA[2HAHis] was detected in the diploid strain (results not shown), possibly indicating that CreB has a range of substrates, and any one interaction was below the range of detection.

These experiments were designed using overexpressed *creA* and *creB*, in order to allow detection. However, native *creA* transcription is autoregulated, and furthermore, overproduction of proteins can lead to misfolding and processing via the proteasome, a particularly important consideration since CreB is a deubiquitinating enzyme. In addition, strains that are both mutant for *creB* and overexpressing *creA* show normal levels of CCR, so overexpression of *creA* partially overrides the requirement for *creB* for repression (Lockington, RA, personal communication). Since indications of interaction were detected in the overexpressing strains, experiments using strains that express the proteins from their native promoters were undertaken. New strains containing CreA[cTAP] (CreA[cTAP]14) and CreB-[FLAG] (CreB[FLAG]14) were constructed in which the fusion genes are expressed from their native promoters (Supplementary Fig. 4), and a strain containing both constructs (CreA[cTAP]CreB[FLAG]15) was derived from a meiotic cross. CreA[cTAP]CreB[FLAG]15 was incubated in both repressing and derepressing conditions, and proteins were purified using the TAP protocol for CreA[cTAP] or the anti-FLAG matrix for CreB[FLAG]. The WT strain was used as a negative control in these experiments. The purified samples were loaded in duplicate, and the membranes were probed with the antibody specific to the tag. CreA[cTAP] purified by the TAP purification protocol was probed with anti-FLAG peroxidase linked (Sigma) that detected CreB[FLAG] in the positive control CreB[FLAG]14, but no CreB[FLAG] was detected for CreA[cTAP]CreB[FLAG]15 in either repressing or derepressing conditions (Fig. 5a). CreA[cTAP] purified by the TAP purification protocol was also probed with anti-TAP conjugated with peroxidase (GeneScript) and CreA[cTAP] was detected in both repressing and derepressing conditions (Fig. 5b). Similarly, CreB[FLAG] purified by FLAG purification was
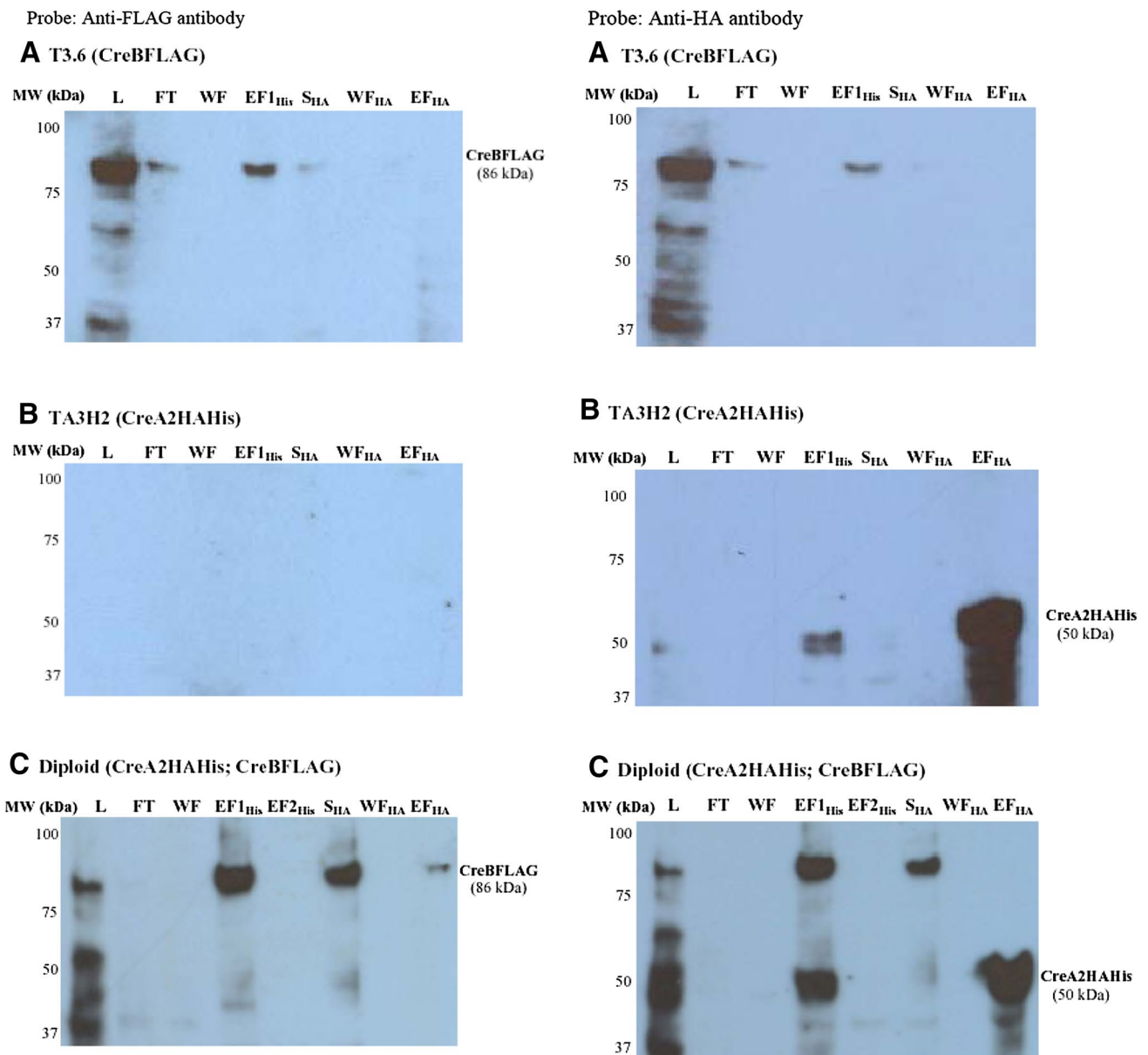
Probe: Anti-FLAG antibody

A T3.6 (CreBFLAG)

B TA3H2 (CreA2HAHis)

C Diploid (CreA2HAHis; CreBFLAG)

Probe: Anti-HA antibody

A T3.6 (CreBFLAG)

B TA3H2 (CreA2HAHis)

C Diploid (CreA2HAHis; CreBFLAG)

Fig. 4 CreB is present in a complex with CreA in overexpressing strains. Total proteins were extracted from three strains, T3.6 (Part **a**), TA3H2 (Part **b**), and TA3H2/T3.6 (Part **c**), that were grown in 1% glucose for 18 h. CreA was purified from total protein using a His-Trap HP column (GE Healthcare) and further purified using anti-HA matrix (Roche). Total protein lysate (L); flow through from His-Trap column (FT); Wash fraction from His-Trap column (WF); eluted fractions from His-Trap column (EF1$_{His}$ and EF2$_{His}$); supernatant of HA-matrix (S$_{HA}$); wash fraction of HA-matrix (WF$_{HA}$); eluted fraction of HA-matrix (EF$_{HA}$). Samples were separated by SDS-PAGE, transferred to PVDF membrane and probed firstly with anti-FLAG monoclonal antibody conjugated with peroxidase (Sigma; LHS) and then

further probed with anti-HA monoclonal antibody conjugated with peroxidase (Roche: RHS). In part A (LHS; probed with anti-FLAG) there is some CreB^{FLAG} signal carried over in the T3.6 haploid after a single purification of CreA using a His-trap column, which is the reason that CreA needed to be purified in a two-step process, first using the His column, followed by anti-HA matrix, to ensure that there were no carry-over proteins. Note that the stripping of the Anti-FLAG probe from the membranes in the LHS of the figure was partial, and the signal is carried over to the Anti-HA probed analysis in the RHS of the figure. Because the proteins are different molecular weights, this does not affect the interpretation of the experiment

probed with anti-TAP conjugated with peroxidase (Gene-Script) and anti-FLAG peroxidase linked (Sigma), respectively. Probing with anti-TAP antibody detected CreA^{cTAP} in the positive control, CreA^{cTAP}14; however, CreA was

not detected in CreA^{cTAP}CreB^{FLAG}15 in either of the two conditions (Fig. 5c), whereas CreB^{FLAG} was detected for CreA^{cTAP}CreB^{FLAG}15 in both the lysate and purified fractions (Fig. 5d). These results indicate that CreA and CreB
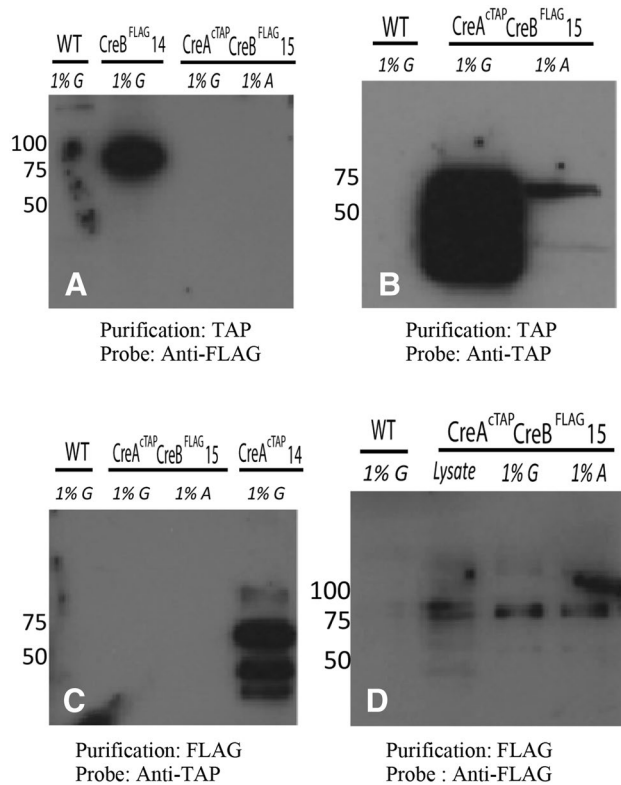
Fig. 5 CreA^cTAP and CreB^FLAG are not co-purified when expressed from their native promoters. CreA^cTAP CreB^FLAG15 was incubated in 1% glucose and 1% arabinose representing repressing and derepressing conditions, respectively. Proteins were purified using TAP (**a**, **b**) or FLAG (**c**, **d**) protocols. IP samples were loaded in duplicate and separated by SDS-PAGE, transferred to PVDF membrane and probed with specific antibody. For TAP purified proteins, one half of the membrane was probed with anti-FLAG peroxidase linked (Sigma) that detected CreB^FLAG in the positive control CreB^FLAG14 but not in CreA^cTAP CreB^FLAG15 (**a**); and the other half of the membrane was probed with anti-TAP conjugated with peroxidase (GeneScript) that detected CreAcTAP in CreA^cTAP CreB^FLAG15 in both conditions (**b**). For FLAG-purified proteins, one half of the membrane was probed with anti-TAP conjugated with peroxidase (GeneScript) that detected CreA^cTAP in CreA^cTAP14, which acts as a positive control, but CreA^cTAP was not detected in CreA^cTAP CreB^FLAG15 (**c**). The other half of the membrane was probed with anti-FLAG peroxidase linked (Sigma), and CreB^FLAG was detected for CreA^cTAP CreB^FLAG15 in both IP samples and the lysate (**d**). The WT strain was used as a negative control in these experiments

are not present in the same complex, at least at levels that are detectable using these procedures.

Post-translational modifications: is CreA a phosphorylated or ubiquitinated protein?

The absence of interaction between CreA and CreB in co-purification experiments led us to investigate the phosphorylation and ubiquitination status of the CreA protein directly. The interaction between phosphorylation and ubiquitination, two of the major post-translational modifications, can play an important role in eukaryotic regulation, and phosphorylation of protein can be a marker to trigger subsequent ubiquitination (reviewed in (Nguyen et al. 2013). The Phos-tag system (Wako Pure Chemical Industries, Japan; M&M) that binds with phosphorylated proteins and alters their mobility was used to study the phosphorylation status of CreA. CreA was immunoprecipitated from T7 grown in conditions that were either repressing or derepressing, and samples were treated with bovine alkaline phosphatase (AP), and separated using phosphate affinity SDS-PAGE, with treated and untreated β-casein as the positive control. β-Casein was detected by staining with Coomassie blue and CreA^HAGFP was detected by probing a Western blot with anti-HA antibody peroxidase high-affinity antibody (Roche). β-Casein was detected in both AP treated and untreated samples, with dephosphorylation detected as the AP-treated sample showed altered mobility (Fig. 6a). CreA^HAGFP was detected in both repressing and derepressing conditions, with clear evidence of altered mobility in AP-treated protein from repressed conditions, but little change in protein from derepressed conditions (Fig. 6b). In the Phos-tag system, dephosphorylated proteins are detected by altered mobility compared to the phosphoprotein counterparts, usually increased mobility. However, dephosphorylation of some phosphoproteins can result in slower mobility, for example the loss of a phosphate group during dephosphorylation favours the self-association of β-casein (Yoshikawa et al. 1974), which is consistent with the lower mobility band in the control track. Lower mobility bands in Phos-tag SDS-PAGE have been detected during partial dephosphorylation of β-casein (Kinoshita et al. 2009b), and the decreased mobility of CreA^HAGFP may be due to partial dephosphorylation. Altered mobility bands were clearly detected for CreA extracted from repressing conditions, and this indication that CreA is a phosphorylated protein was confirmed using mass spectrometry (see below).

To determine the ubiquitination status of CreA, proteins were extracted from TA3H2, which overexpressed HA- and His6- tagged CreA, and ubiquitinated proteins were purified using UbiCapture-Q matrix (Enzo life Sciences; M&M). In Western blots, CreA was strongly detected in both the total lysate and unbound fractions and weakly in the fraction purified for ubiquitinated proteins. As expected no band was detected for WT in all three sample fractions. This result suggests that at least a fraction of CreA is ubiquitinated in a strain that overexpressed CreA (Data not shown). In this experiment, *creA* was overexpressed in order to allow detection. However, since native *creA* transcription is autoregulated, and furthermore, overproduction of proteins can lead to misfolding and processing via the proteasome as ubiquitinated protein, these indications
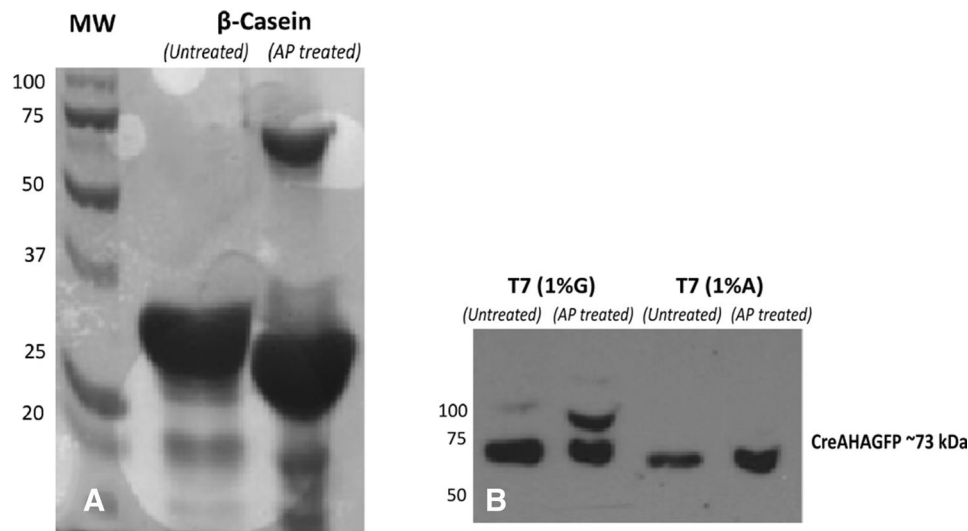
Fig. 6 Phosphorylation status of CreA. Strain T7 was grown in 1% glucose for 18 h with shaking at 37 °C and then treated for 4 h at 37 °C with shaking in two different conditions 1% glucose (G) and 1% arabinose (A) that represent repressing and derepressing conditions, respectively. CreA was purified from total proteins by immunoprecipitation using anti-HA matrix (Roche). IP samples were treated with bovine alkaline phosphatase (Sigma) for 60 min and separated by SDS-PAGE using 'Phos tag' (Wako Pure Chemical Industries, Japan). β-Casein (1 µg/µl) was used as positive control and incubated in Coomassie blue stain overnight (**a**). The blot was probed using anti-HA peroxidase high-affinity antibody (Roche) (**b**)

of ubiquitination were followed up using a strain that expressed the protein from the native promoter.

Proteins were extracted from T7 (*creA* overexpressed) and S4 (*creA* expressed from native promoter) after growth in glucose or arabinose, and CreA^{HAGFP} was immunoprecipitated (Fig. 7). When probed with an anti-ubiquitin antibody, no ubiquitinated CreA was detected for either T7 or S4 in either repressing or derepressing conditions (Fig. 7a, d). As expected, CreA^{HAGFP} was detected for both strains in both conditions when the membranes (Fig. 7b, c) were probed with an anti-HA antibody. These results indicate that CreA detected in these experiments is not ubiquitinated.

In Western blots, the sensitivity of the antibody is critical for detection of proteins of interest, and the sensitivity of the anti-ubiquitin antibody used is apparently low as a high concentration of free ubiquitin (positive control) was required for detection (results not shown). To obtain further confirmation regarding the post-translational modifications as well as physical interactions between CreA and CreB, mass spectrometry analysis of CreA and CreB proteins was undertaken. CreA^{cTAP}14 and CreB^{cTAP}15 were incubated in either repressing (1% G) or derepressing (1% A) conditions, along with the WT strain in the repressing (1% G) condition. Both CreA^{cTAP} and CreB^{cTAP} were separately purified using the TAP purification protocol, separated by SDS-PAGE, and stained with Coomassie blue (M&M). Only a few very weak nonspecific bands were detected in the WT sample. For CreA^{cTAP}14, 6 bands were analysed from the sample grown in repressing conditions and 7 bands were analysed from the sample grown in derepressing conditions (Fig. 8a), while for CreB^{cTAP}15, 3 bands were analysed from the sample grown in repressing conditions and 4 bands were analysed from the sample grown in derepressing conditions (Fig. 8b). These bands were digested with trypsin (Promega) and peptide fragments were analysed using LC–MS (Adelaide Proteomic Centre). Using a MaxQuant search, CreA and CreB proteins were identified and analysed for post-translational modifications including ubiquitination, phosphorylation and oxidation. To identify phosphorylated peptide/s in CreA the data were searched with modified variables for phosphorylation (ST and/or Y). A SRPNSPNSTAPSSPTF-SHDSLSPTPDHTPLATPAHSPR (aa 277–314) peptide with three phosphorylated STY sites (SRPNSPNSTAPS$^{(p)}$S$^{(p)}$PTFSHDSLSPTPDHTPLATPAHS$^{(p)}$PR) was present only in repressed conditions, whereas the peptide with two phosphorylated STY sites (S$^{(p)}$RPNSPNSTAPS$^{(p)}$SPTFSHDSLSPTPDHTPLATPAHSPR) was detected in both conditions. The LKPLS$^{(p)}$PSELHLPSIR (aa 315–329) peptide was identified as STY phosphorylated only in repressed conditions. The SHS$^{(p)}$HEDEDSYASHR (aa 260–273) STY phosphorylated peptide was detected in both conditions (Table 3). To identify ubiquitinated peptide/s in CreA, the data were searched again with modified variables for ubiquitination (CKST). However, no ubiquitinated peptide was detected for CreA in either repressed or derepressed conditions.
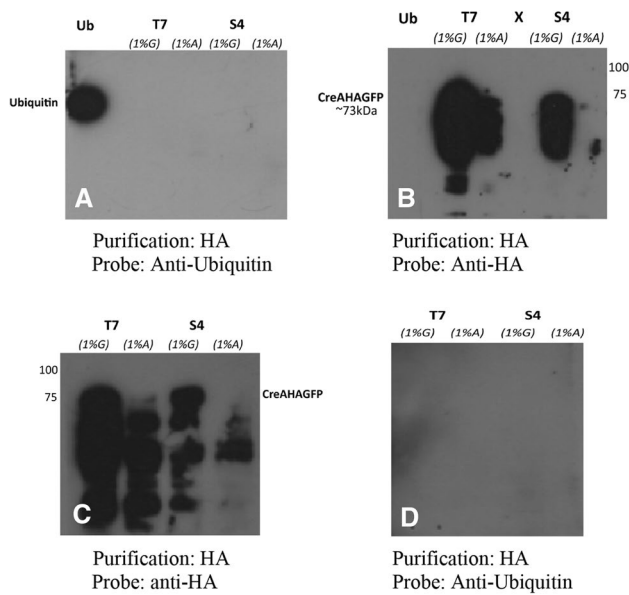
Fig. 7 Ubiquitination status of CreA. Two strains T7 (*creA* expressed from the *gpdA* promoter) and S4 (*creA* expressed from the native promoter) were grown in 1% glucose for 18 h with shaking at 37 °C and then treated for 4 h at 37 °C with shaking in two different conditions 1% glucose (G) and 1% arabinose (A) that represent repressing and derepressing states, respectively. CreA was purified from total proteins by immunoprecipitation using anti-HA affinity matrix (Roche). IP samples, loaded in duplicate, were separated by SDS-PAGE, transferred to PVDF membrane and divided into two for specific antibody probing. The first half was probed with anti-ubiquitin conjugated with peroxidase antibody (Abcam) that detected free ubiquitin as the positive control (due to the small MW of ubiquitin, Ub was loaded only for the final 20 min of the electrophoresis) (**a**), and this same membrane was stripped and probed with anti-HA peroxidase high-affinity antibody (Roche) for detection of CreA (**b**). The other half of the membrane was first probed with anti-HA peroxidase high-affinity antibody (Roche) for the detection of CreA (**c**), and then this same membrane was stripped and probed with anti-ubiquitin conjugated with peroxidase antibody (Abcam) (**d**)

Furthermore, no CreB was detected among the co-purified proteins with CreA, and CreA was not present among proteins that were co-purified with CreB in repressed and derepressed conditions (results not shown). However, CreC was detected with CreB in both experimental conditions which serves as an excellent internal positive control (Lockington and Kelly 2001). Taken together, both Western blots and MS analysis confirmed that CreA is differentially phosphorylated, but is not an ubiquitinated protein, and CreA and CreB are not present in a stable complex.

Discussion

In *A. nidulans*, it has been proposed that ubiquitination and deubiquitination systems, involving products of the *creD, hulA, creB* and *creC* genes, are involved in the carbon

catabolite repression mechanism (Kelly 2004). Although mutations in the *creB* gene confirmed the association of CreB with the CCR regulatory network, the role of the CreB deubiquitinating enzyme is not fully understood at the molecular level. The effects of *creB* missense mutations in a *creA* missense mutant background have been studied (Hynes and Kelly 1977); however, the effects of a null *creB* mutant in a null *creA* background had not previously been investigated.

Mutations in *creA* and *creB* cause morphological effects on solid media. Mutations in the *creA* gene produce the same range of morphological characteristics across the alleles that include slower growth rate and smaller colony size than the wildtype strain (Shroff et al. 1996). Null alleles of *creA* are viable, but they show extreme effects on colony morphology in all growth conditions tested (Shroff et al. 1997). Conversely, both partial and complete loss-of-function alleles of the *creB* gene cause only a small effect on colony morphology (Hynes and Kelly 1977; Lockington and Kelly 2001). A strain that contains partial loss-of-function alleles *creA204* and *creB15* was morphologically similar to a strain containing *creA204* alone (Hynes and Kelly 1977). A strain that was a complete loss-of-function for both the *creA* and *creB* genes was developed. In this strain, the extreme colony morphology of *creAΔ* was suppressed to some extent in that it showed a less compact and less dense colony than a strain containing *creAΔ* alone, indicating that the total loss of both proteins leads to fewer effects on morphology than the loss of CreA alone. This is consistent with the finding of *creB* alleles among suppressors of the effects of *creA204* on morphology on complete medium (JMK and Lockington, RA, unpublished). Thus in *A. nidulans*, the effects of the mutations on morphology indicate a genetic interaction. How *creA* mutations result in morphological effects is not known at the molecular level, but the partial suppression of the morphological changes by *creB* mutations implies that the complete failure to deubiquitinate one or more unknown target proteins (not CreA) relieves this effect. In *A. oryzae*, a strain containing a deletion of both the *creA* and *creB* genes retained the phenotype of *creAΔ* in minimal media (Ichinose et al. 2014).

Mutations in *creA* and *creB* cause reduced CCR for some enzymes and permeases. It was reported previously that in the presence of carbon sources such as glucose and sucrose, mutations in the *creA* and *creB* gene can lead to derepression of alcohol dehydrogenase I, encoded by the *alcA* gene (Bailey and Arst 1975; Hynes and Kelly 1977; Shroff et al. 1996, 1997). Like the single *creAΔ* strain, the *creAΔ creBΔ* double null mutant also led to high levels of *alcA* expression in the presence of glucose as detected using a plate test monitoring toxicity of allyl alcohol, whereas strains containing the *creBΔ* mutant showed at least partial relief of CCR. Enzyme assays of
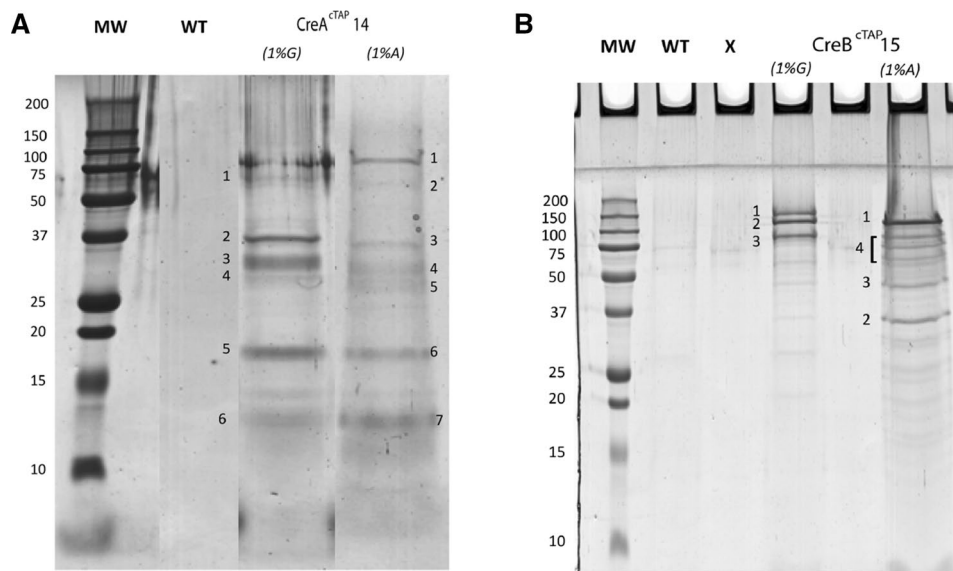
Fig. 8 Bands purified using the TAP purification protocol from Cre-A^cTAP^14 and CreB^cTAP^15. CreA^cTAP^14 and CreB^cTAP^15 were incubated in both repressing (1% G) and derepressing (1% A) conditions. Both CreA^cTAP^ and CreB^cTAP^ were purified by using TAP purification protocol. Purified samples, including WT as a negative control, were separated by two individual SDS-PAGE. Proteins were fixed for 2 h and Coomassie stained overnight at room temperature. In WT, no band was detected. For CreA^cTAP^14 6 and 7 bands (**a**), and for CreB^cTAP^15 (**b**) 3 and 4 bands were selected in repressing and derepressing conditions, respectively. All bands were processed for proteomic analysis at Adelaide Proteomic Center

Table 3 Peptides identified in repressed and derepressed conditions by LCMS with modified variables

Peptides	Modifications	PEP	Repressed	Derepressed
ASSGMDINLLATAASQVER	Unmodified	6.5595E−76	Detected	Not detected
ASSGMDINLLATAASQVER	Oxidation (M)	4.7078E−62	Detected	Not detected
SHSHEDEDSYASHR	Unmodified	8.9241E−32	Detected	Detected
VAVQDMLNPSGFTSVSSSTANSVAGGDLAER	Unmodified	1.9348E−27	Detected	Detected
VAVQDMLNPSGFTSVSSSTANSVAGGDLAER	Oxidation (M)	2.1195E−21	Detected	Detected
GLPSLSAYAISHSMSR	Unmodified	8.2313E−20	Detected	Not detected
KLPIPQVPK	Unmodified	2.5919E−19	Detected	Detected
GLPSLSAYAISHSMSR	Oxidation (M)	1.02E−13	Detected	Not detected
LKPLSPSELHLPSIR	Unmodified	1.75E−12	Detected	Detected
LKPLS(ph)PSELHLPSIR	Phospho (STY)	5.28E−12	Detected	Not detected
SHS(ph)HEDEDSYASHR	Phospho (STY)	7.61E−12	Detected	Detected
ASSGMDINLLATAASQVERDESFGFR	Oxidation (M)	4.16E−08	Detected	Not detected
DESFGFR	Unmodified	3.00E−07	Detected	Not detected
LPIPQVPK	Unmodified	8.97E−06	Detected	Detected
SNLSPYSR	Unmodified	1.37E−05	Detected	Not detected
S(ph)RPNSPNSTAPS(ph)SPTFSHDSLSPTPDHTPLATPAHSPR	2 Phospho (STY)	4.39E−05	Detected	Detected
SHHMYGPR	Unmodified	1.51E−04	Detected	Not detected
SRPNSPNSTAPS(ph)S(ph)PTFSHDSLSPTPDHTPLATPAHS(ph)PR	3 Phospho (STY)	9.87E−03	Detected	Not detected

PEP posterior error probability

alcohol dehydrogenase I activity showed that the double null mutant strain had the highest level of derepression and elevation in both repressed-induced and induced conditions, indicating a synergistic effect. Using RTqPCR, synergy was confirmed at the transcript level. Higher *alcA* expression was detected for the double null mutant than either of the single null mutants in repressed-induced conditions, again indicating genetic interaction. Though some

partial relief of CCR was observed for the *creBΔ* mutant in the plate tests and enzyme assays, no significant derepression was detected for *alcA* transcription, whereas Hunter et al. (2013) reported that the *creB1937* allele showed more *alcA* expression than a wildtype strain in these conditions, and the reason for this difference is unknown. The extent of derepression achieved by the double null mutant was also determined using plate assays for β-galactosidase and α-amylase that are required for the breakdown of lactose and starch, respectively. The expression of β-galactosidase was highest in the double null mutant in both the presence and absence of glucose, indicating elevation and a synergistic interaction. For α-amylase, the double null mutation led to less expression in either the presence or absence of glucose than the *creAΔ* null mutant alone, but more than the *creBΔ* and the wildtype strains. Thus in *A. nidulans*, the effects of the mutations on CCR indicate a genetic interaction.

In *A. nidulans*, mutations in the *creB* gene produce a pleiotropic range of phenotypes which are unrelated to CCR; for example, reduced growth on quinate as a sole carbon source (Hynes and Kelly 1977). These are likely to be due to effects on permeases, and the quinate permease QutD, required for the induction of quinate dehydrogenase, is one of the direct targets of deubiquitinating enzyme, CreB (Kamlangdee 2008). Mutations in the *creA* gene have little to no effect for quinate utilization. The double null mutant strain showed as poor growth on quinate as a sole carbon source as the single *creBΔ* mutant strain. These findings support the suggestion that the role of CreB in conditions that do not lead to CCR, such as the direct effects on permeases, is independent of its role in CCR.

The finding of a clear genetic interaction between *creA* and *creB* mutant alleles led us to investigate whether the CreA and CreB proteins directly interact or form part of a complex. In other systems, substrates of deubiquitinating proteins have been identified using protein co-purification methods. For example, liquid facets was shown to be a substrate of the fat facets deubiquitinating enzyme in *Drosophila* (Chen et al. 2002). In addition, FANCD2 and p53 were co-immunoprecipitated as a substrate with USP1 and USP7 in HEK293 and H460 cell lysate, respectively (Li et al. 2002; Nijman et al. 2005). Thus, we used co-purification methods to identify whether CreA was a substrate for CreB. In preliminary experiments, strains containing CreA tagged with HA and His6 and CreB tagged with FLAG were constructed, with the tagged constructs expressed from the constitutive *gpdA* promoter (Punt et al. 1990). When CreA was purified, some CreB was copurified, indicating that in this strain, at least some CreB and CreA may exist in a complex in vivo. However, when CreB was purified, no CreA was detected, which may suggest that CreB has a range of substrates, and any one interaction

was below the range of detection. However, these preliminary experiments do not confirm that the interaction occurs physiologically when the proteins are expressed from their own promoters. Thus strains were constructed that express the proteins from their native promoters at their native loci. This was particularly important as in the native state transcription of *creA* is autoregulated (Shroff et al. 1996), and moreover, overexpression of CreA can partially override the requirement of CreB for repression (Lockington, RA, personal communication), and further, inappropriate expression of proteins can lead to misfolding and processing via the proteasome. The possibility of processing misfolded and mis-expressed proteins via the proteasome is of vital importance because of the involvement of ubiquitination and deubiquitination pathways in proteasomal degradation (Goldberg 2003; Kraft et al. 2010). Co-purifications were performed in a strain that expresses CreA and CreB from their native promoters, and when CreA was purified no CreB was co-purified, and when CreB was purified no CreA was co-purified, and thus CreA and CreB do not physically interact, and nor are they part of a single stable complex that can be purified via these means.

Previously, two separate experiments concluded that CreA requires post-translational modifications to become an active repressor (Roy et al. 2008; Strauss, et al. 1999). Post-translational modifications in terms of phosphorylation and ubiquitination and their interactions has played important roles in cell signalling pathways and phosphorylation of protein can often be a marker to trigger subsequent ubiquitination [reviewed in (Nguyen et al. 2013)]. Both Western blots and MS analysis showed that CreA is phosphorylated in both repressing and derepressing conditions, and the numbers of phosphopeptides and phosphorylation sites were varied between these two conditions. Mass spectrometry analysis showed that a tri-phosphorylated form of the aa 277–314 peptide was only detected in repressed conditions, whereas a di-phosphorylated form was present in both repressed and derepressed conditions. In addition, mono-phosphorylated peptide aa 315–329, LKPLS(p)PSELHLPSIR, was only identified in repressed conditions. Whether either of these differential phosphorylations has regulatory significance requires further experimentation. The role of phosphorylation in the repressor activity of CreA and its homolog has been investigated previously. In *T. reesei*, phosphorylation of the Ser^{241} residue of Cre1 is required for DNA binding. Replacing of Ser^{241} by glutamic acid mimics phosphorylation, but replacing by alanine allows binding without phosphorylation, with the latter leading to permanent carbon catabolite repression for cellobiohydrolase I expression (Cziferszky et al. 2002). On the other hand, substitution of Glu^{244} by valine results in no phosphorylation, binding or repression (Cziferszky et al. 2002). In *S. sclerotiorum*, Ser^{266} (equivalent to Ser^{241}

in *T. reesei*) substitution by alanine leads to derepression (Vautard-Mey and Fevre 2000). In *A. nidulans*, deletion of amino acid region SHED[262–265] of CreA (equivalent to SNDE[241–244] of Cre1 in *T. reesei* and SHEE[266–269] of CRE1 in *S. sclerotiorum*) showed little effect on carbon catabolite repression (Roy et al. 2008). The amino acid region aa 277–314 of CreA is conserved in several ascomycetes and in *T. reesei*, although deletion of this amino acid region did not affect phosphorylation of the Cre1 protein (Cziferszky et al. 2002). In *S. cerevisiae*, phosphorylation of Mig1p by Snf1 kinase is essential to exert repression (DeVit and Johnston 1999; DeVit et al. 1997), although no Snf1 kinase-like target motif was identified in Cre1 of *T. reesei*, where casein kinase II is involved in the phosphorylation of Ser[241] of Cre1 (Cziferszky et al. 2002). Recently, Brown et al. (2013) suggested that *A. nidulans* SnfA was required for CreA derepression of cellulase enzymes through alteration of CreA nuclear localization, however mycelia were incubated in conditions that are more similar to starvation than derepression and the elevation of the XprG starvation response transcription factor supports that starvation occurred, and CreA is known to be degraded in starvation conditions (Alam, et al. unpublished). CreA nuclear localization was reduced but not fully abolished in glycerol (31%) which is considered as a condition in which derepression occurs (Ruijter and Visser 1997) compared to glucose (100%) (Brown et al. 2013). Incubation in MM with 1% Avicel for 3–5 days could lead to starvation as incubation in Avicel for 5 h showed similar CreA nuclear localization as incubation without a carbon source for the same length of time (Brown et al. 2013). Microarray analysis also indicated a starvation response. In *A. nidulans*, carbon starvation can induce complex changes in transcriptomes and involves regulation of biological processes including programmed cell death, secondary metabolite production, and the production of extracellular hydrolases and proteases (Szilagyi et al. 2013). Genes that are involved in glucose utilization and carbohydrate synthesis were down-regulated though genes that are involved in carbohydrate hydrolysis were up-regulated (Szilagyi et al. 2013). In addition, genes involved in protein synthesis, autophagy and nucleases and peptidases were upregulated, indicating that CreA might be degraded in starvation conditions. This observation is in agreement with Western analysis that showed CreA was not detected in strains incubated in starvation conditions (Alam et al., unpublished). However, although there was clear evidence for phosphorylation of CreA, our Western blot and MS analyses provided no evidence that CreA is ubiquitinated. Recently, Ries et al. (2016) used an anti-ubiquitin antibody to probe purified CreA::GFP, and a smear was identified between 60 and 100 kDa, which led them to conclude that CreA may be ubiquitinated; however, there was no indication of a ladder of CreA due to differently ubiquitinated species, and our

MS evidence makes that very unlikely. Moreover, CreA was not present in the ubiquitinated sites in the *A. nidulans* proteome analysis of Chu et al. (2016).

Our finding that the CreB deubiquitinating enzyme does not act directly on CreA or proteins in a complex with CreA raises a number of pressing questions, the first being how is CreB involved in CCR. Chu et al. (2016), analysed the ubiquitinated sites in the *A. nidulans* proteome, and identified 1913 ubiquitinated proteins. Proteins that were ubiquitinated were very different in *S. cerevisiae* compared to *A. nidulans*, and the *A. nidulans*–specific proteins are enriched for those that are involved in transcription and protein–protein interactions. CreB itself was present among the proteins identified, and it has a potential ubiquitination site, KEPQPLPMTPLEK. Also present were CreC (Todd et al. 2000), HulA (Boase and Kelly 2004) and AcrB (Boase et al. 2003), all implicated by mutational analysis to be involved in CCR. It has been suggested that all the phenotypes of *creB* mutant strains can be accounted for by effects on permeases and transporters, and it is clear that CreB does directly affect a range of permeases and transporters (Arst 1981; Hynes and Kelly 1977; Kamlangdee 2008), but even though extensive studies have been undertaken, only very mild effects on glucose uptake have been demonstrated (Espeso et al. 1995; Strauss et al. 1999). In addition, alcohol dehydrogenase activity is derepressed in *creB* mutant strains, but some other enzymes normally subjected to CCR are not, and if the effect was via effects on a glucose permease then a wider range of enzyme activities might be expected to be affected. The inducer, ethanol, does not require active transport into the cell, which argues against a role for CreB in the stability of permeases affecting inducer availability.

The second pressing question is how CreA is regulated to allow it to act as a repressor. CCR is well studied in the unicellular yeast, *S. cerevisiae*, and the main proteins involved in regulating the Mig1p repressor are the Snf1 kinase which is necessary for expression of genes required for catabolic pathways to provide glucose, and the Tup1p-Ssn6p co-repressor complex which is involved in initiating a repressive chromatin structure in a number of separate repression pathways (Gancedo 1998; Kayikci and Nielsen 2015). The Tup1p and Ssn6p homologues in *A. nidulans* are not centrally involved in the CCR mechanism, implying that the regulation of the repressor protein is quite different in the two species (Hicks et al. 2001), which is consistent with the ubiquitome study showing that yeast and *A. nidulans* have different ubiquitinated protein patterns in proteins involved in transcription (Chu et al. 2016). In *A. nidulans*, CreA was reported to be involved in the rearrangement of nucleosome positioning in carbon catabolite repressible promoters *alcA*, *alcR* and *prnD-prnB,* although the rearrangements did not correlate with derepression

(Garcia et al. 2004; Mathieu et al. 2005; Reyes-Dominguez et al. 2008). A similar result was observed with Cre1, a CreA homolog in *T. reesei*, in *cbh1* coding regions under repressing condition (Ries et al. 2014), though the molecular mechanism of nucleosome rearrangement remains unclear.

In eukaryotes, transcriptional activation and repression involves chromatin rearrangement and modifications of histone tails, including acetylation, phosphorylation, sumoylation, ubiquitination, and methylation (Brosch et al. 2008). In *S. cerevisiae*, the co-repressor complex Tup1p-Ssn6p mediates repression through the organization of chromatin and directly interacts with the amino terminal of H3 and H4 histones (Edmondson et al. 1996). In addition, repression of genes by Tup1p involves the utilization of H3/H2B specific histone deacetylase 1 (HDA1) in a number of promoters including *ENA1, GAL10, SUC2* (Wu et al. 2001). In *A. nidulans* the Tup1p homologue, RcoA, plays no crucial role in CCR (Hicks et al. 2001) although deletion of this gene showed rearrangement of nucleosome positioning in *alcR* and *prnD-prnB* promoters (Garcia et al. 2008).

Both ubiquitination and deubiquitination have been shown to be involved in transcriptional regulation. In yeast, histone H2B monoubiquitination by Rad6 precedes methylation and the recruitment of the SAGA complex, when Ubp8, a component of the SAGA histone acetyltransferase, deubiquitinates the monoubiquitinated histone H2B to allow transcriptional initiation (Reyes-Turcu et al. 2009, Shilatifard 2006). There are apparent differences between the DUB modules of the Yeast and *A. nidulans* SAGA complexes; in *S. cerevisiae* the DUB module consists of Sgf11p, Sus1p, and Ubp8p, whereas in *A. nidulans* there are no convincing homologues of Sgf11p and Sus1p in the genome, and the DUB module did not copurify with the complex (Georgakopoulos et al. 2012, 2013). Deletion of the *A. nidulans* SAGA components AcdX (Spt8p) and SptC (Spt3p) did not lead to carbon catabolite derepression, but there was some elevated expression (Georgakopoulos et al. 2012). Unlike Ubp8, deubiquitination of ubiquitinated H2B by Ubp10 results in gene silencing in the telomere region and at the rDNA locus (Emre et al. 2005).

Ubiquitination/deubiquitination pathways have multiple effects on gene transcription. We show here that the involvement of the CreB/HulA/CreD ubiquitinating/deubiquitinating pathway in CCR in *A. nidulans* does not involve direct interaction with the CreA protein, or proteins complexed with it. It is possible that the ubiquitination/deubiquitinating pathway affecting an aspect of nucleosome arrangement and chromatin structure interacts with the regulation by CreA. Further studies are required to reveal the role of the pathway in *A. nidulans*.

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