Identifying target proteins of the CreB deubiquitination enzyme in the fungus *Aspergillus nidulans*

By

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Abstract

Carbon catabolite repression in *A. nidulans* is a regulatory system which allows the organism to utilize the most preferable carbon source by repressing the expression of genes encoding enzymes utilizing alternative carbon sources. A ubiquitination pathway was shown to be one of the key mechanisms which regulate carbon source utilization, when *creB* was found to encode a deubiquitinating enzyme. Strains containing mutations in *creB* show loss of repression for some metabolic pathways in carbon catabolite repressing conditions, and also grow very poorly on several sole carbon sources such as quinate and proline, suggesting CreB plays multiple roles in the cell.

This work describes the analysis of the interaction of CreB with CreA, and with PrnB and QutD. Various epitope-tagged versions of CreA were expressed in *A. nidulans*, and an internally located HA-epitope tag was found to allow detection of CreA using Western analysis. A diploid strain was constructed between strains containing HA-tagged CreA and FLAG-tagged CreB. When CreB was immunoprecipitated, HA-tagged CreA was also precipitated in the diploid, indicating that CreA and CreB are present in a complex *in vivo*. To determine whether CreA is a ubiquitinated protein, a version of CreA that was tagged with both an HA epitope and a His-tag was expressed in *A. nidulans*, and protein extracts were precipitated with an UbiQapture™-Q matrix. Western analysis was used to show that CreA was present in the precipitate. These findings suggest that CreA is a ubiquitinated protein, and a target of the CreB deubiquitination enzyme.

To determine whether the proline permease (PrnB) is a direct substrate of CreB, plasmids to express epitope-tagged versions of PrnB were constructed and introduced into the *prnB* mutant strain. No tagged protein could be detected by Western analysis, even when these constructs were over-expressed from the *gpdA* promoter. However, a construct to express an HA epitope tagged version of quinate permease (QutD) fully complemented the *qutD* mutant strain, and HA-tagged QutD could be easily detected in Western analysis when probed with the anti-HA monoclonal antibody. A diploid strain was made between a complementing transformant and a strain expressing a FLAG-tagged CreB construct. When QutDHA was immunoprecipitated, CreBFLAG was detected in the immunoprecipitate of the diploid. A proportion of QutDHA was also co-precipitated in the diploid when CreBFLAG was immunoprecipitated. Thus, CreB is

present in a complex with QutD *in vivo*. Further results showed that the concentration of QutD in the cell is lower in a *creB* null mutant background than in the wild-type background, indicating that deubiquitination is required to prevent protein turnover. Northern analysis of mRNA showed that the failure of *creB* mutant strains to grow on quinate medium was not due to a failure of transcriptional induction of *qutD*, as the amount of mRNA was not lower in a *creB1937* mutant background compared to the wild-type. Furthermore, experiments were undertaken that showed that QutD is a ubiquitinated protein. These findings suggest that quinate permease is regulated through deubiquitination involving the CreB deubiquitination protein in *A. nidulans*.

In addition to the candidate protein approach asking whether CreA is a substrate of CreB, a proteomics approach was also used to identify proteins that interact with CreA. However, no clear interacting proteins were identified using this approach.

Declaration

This work contains no material which has been accepted for the award of any other degrees or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

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List of Abbreviations

ANM	*Aspergillus* nitrogen free media
A	Adenine
ANGIS	Australian National Genomic Information Service
[α^{-32}P]dATP	alpha-labelled deoxyadenosine triphosphate
BLAST	basic local alignment search tool
bp, kb	base pairs, kilobase pairs
BSA	bovine serum albumin
C	cytosine
^{0}C	degree Celsius
cDNA	deoxyribonucleic acid complementary to ribonucleic acid
CCR	carbon catabolite repression
C-terminal	carboxy-terminal
Da, kDa	Dalton(s), kiloDaltons
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide-5'-triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraaminoacetic acid
G	guanine
g, mg, µg, ng	gram(s), milligram(s), microgram(s), nanogram(s)
GFP	green fluorescent protein
His	histidine
IPTG	isopropylthio-β-D-galactosidase (or 1-isopropyl-β-D-1-thiogalactopyranoside
KOP	potassium orthophosphate
L, ml, µl	litre(s), millilitre(s), microlitre(s)
M, mM	mole per litre, millimole per litre
mCi	microCurie
min, hr	minute(s), hour(s)
mRNA	messenger ribonucleic acid
NAD	*β*-nicotinamide adenine dinucleotide
NCBI	National Center of Biotechnology Information
N-terminal	amino-terminal
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
®	registered
RNA	ribonucleic acid
rpm	revolutions per minute
RSP	reverse sequencing primer
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	saline sodium citrate buffer
SSPE	saline sodium phosphate-EDTA buffer

STC	Sorbital-TrisHCL-$CaCl_2$ buffer
™	Trademark
T	thymidine
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris[hydroxymethyl] aminomethane
U	unite(s) of enzyme
UAS	Upstream activation sequence
Ub	ubiquitin
X-gal	5'-bromo-4-chloro-3-idoyl-β-D-galactopyranoside
(%) v/v	percent volume per volume
(%) w/v	percent weight per volume

Chemical Formula

$CaCl_2$	calcium chloride
CH_3COONa	sodium acetate
$CHCl_3$	chloroform
HCl	hydrochloric acid
KCl	potassium acetate
KH_2PO_4	potassium dihydrogen phosphate (monopotassium phosphate)
$MgSO_4$	Magnesium sulfate
MOPS	3-(N-Morpholino)propanesulfonic acid
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
Na_2HPO_4	sisodium hydrogen phosphate
$Na_3C_6H_5O_7.2H_2O$	sodium citrate dihydrate
NaOH	sodium hydroxide

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Microbial cells contain compounds that are similar in number and complexity to those present in plants and animals. Therefore, despite their small size, these microbes have a complex physiology and metabolism, deriving energy from substrates and synthesizing DNA, RNA, proteins, polysaccharides, vitamins and other metabolites. Microorganisms exhibit great metabolic diversity and tolerance of marked environmental differences. To achieve this, despite having genetic information for a diversity of biochemical pathways, only enzymes utilizing the preferred nutrient are synthesized. These enzymes are expressed if, firstly, the nutrient is present, and secondly, more preferable nutrients are absent. From the cellular physiology viewpoint, this is beneficial for two reasons: the energetically most favorable carbon source is used, and no energy is wasted on the synthesis of enzymes required for other catabolic pathways. Many microorganisms are able to adapt their metabolism for optimal utilization of the carbon sources available in the environment. This adaptation is often made at the level of specific induction and repression of genes encoding specific enzymes.

The regulation of gene expression is an essential function in all living organisms. To effect this control, cells must be able to detect stimuli and transmit a signal to the regulatory mechanisms acting at the DNA level, which respond by activating or repressing target gene(s). Among the mechanisms employed to regulate gene expressions are those targeting transcriptional or translational initiation, elongation or termination, as well as post transcriptional and post translational modifications. One of the most commonly employed methods of regulation occurs at the level of initiation of gene transcription, and genes are not transcribed unless required. Repression may occur not only at the promoters of structural genes but also at the promoters of positively acting regulatory genes to ensure tight control. Activation of transcription also occurs at several levels and often requires induction, activation and either relief from repression or overriding of repression mechanisms. In the case of carbon source utilization, the selection of carbon source is made by the two mechanisms called induction and carbon catabolite repression (CCR).

CCR, or glucose repression, is a wide domain regulatory system that responds to the unique nutritional environment to regulate carbon metabolism. The choice of the carbon source to be utilized is made at the level of specific induction of transcription. Repression of genes for the utilization of alternative potential carbon sources present in the local environment ensures that only a minimum set of genes is expressed. By these mechanisms, microorganisms are able to establish a hierarchy of carbon source utilization. In the presence of the preferred carbon source such as glucose, the structural genes that encode enzymes and permeases for utilizing alternative sources of carbon such as ethanol, proline, cellulose and starch are repressed. Upon depletion of glucose these pathway specific enzymes are expressed to utilize the specific alternative carbon sources present in those environments. Hence the organism conserves energy by only producing proteins as they are required.

Studies on the regulation of gene expression have a broad range of applications in the fields of medicine, pharmacy, food production, and biotechnology. Optimised expression can be achieved in a number of ways with thoughtful consideration to growth condition such as fixed- or cell-free suspension and use of favorable nutrients, strain selection, selected promoters employed for high gene expression, protein fusions, and heterologous protein stabilization (Agger *et al.*, 2002; Davies, 1994; Gouka *et al.*, 1997).

1.2 *Aspergillus nidulans* as a model organism

The filamentous fungus *A. nidulans* is a homothallic ascomycete that has proven to be a very successful experimental organism in which to study gene regulation, in particularly carbon catabolite repression and nitrogen repression, and development. *A. nidulans* has minimal nutritional requirements and can grow as compact colonies on simple defined solid media to form visible colonies within 48 hours at 37 ^{o}C; as well as in submerged culture with a variety of carbon and nitrogen sources. *A. nidulans* has a short asexual (mitotic) life cycle, and a sexual (meiotic) life cycle that can be manipulated in the laboratory, plus a parasexual cycle that allows haplodization analysis of diploid strains (Figure 1.1). The asexual cycle consists of conidiophores growing from mycelia, elongating into the air to produce conidia, which disperse, and subsequently the cycle starts again. Asexual spores can be easily mutagenised and there are a variety of markers available to follow the segregation of the genes of interest. The sexual cycle as utilized using laboratory strains involves two different strains with

different nutritional requirements coming together and being forced to form a heterokaryon in order to survive on minimal medium. The heterokayon produces cleistothecia, or fruiting bodies, which form from a single nuclear fusion event and each cleistothecia contains a number of asci. The parasexual cycle entails the formation of a balanced heterokaryon, and when two vegetative nuclei fuse, a diploid strain is formed. The parasexual cycle is useful for complementation, dominance and linkage analysis, including mapping by mitotic recombination and haploidization (Martinelli, 1994). Precise phenotypic analysis of variation in morphology and growth is an advantage when using *A. nidulans*, as characterization is not limited to the presence or absence of growth.

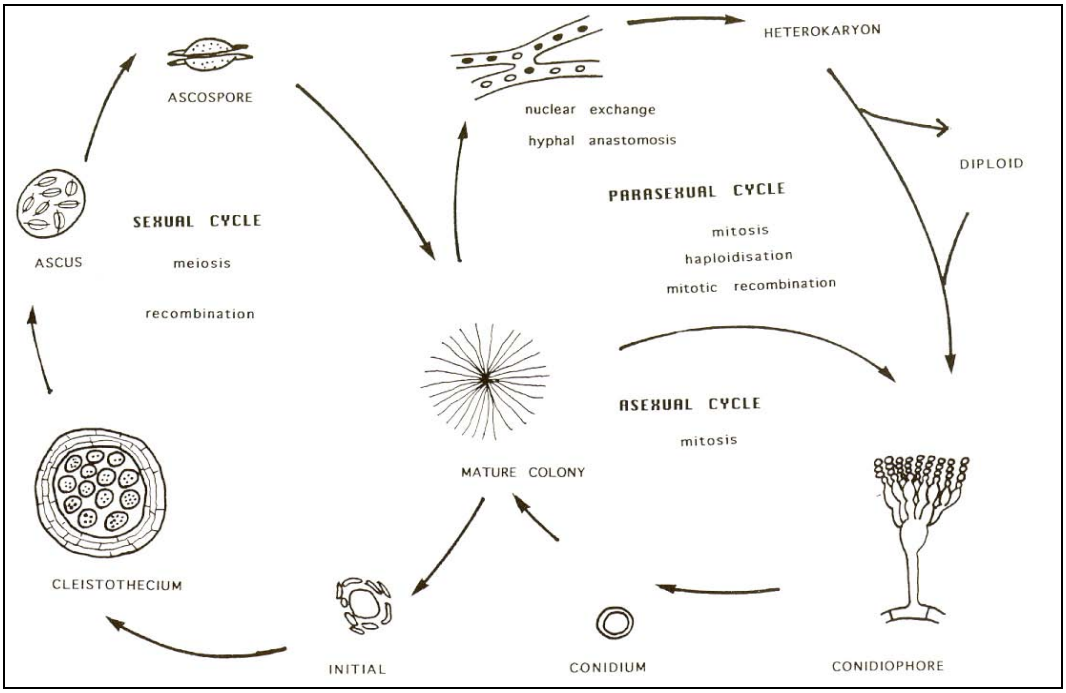


Figure 1.1 Lifecycle of *A. nidulans* showing the sexual, asexual and parasexual cycles. Figure is taken from Figure 4.1, Martinelli (1994).

1.3 Carbon Catabolite Repression

1.3.1 Introduction

Carbon catabolite repression (CCR) is the mechanism whereby energetically favorable repressing carbon sources are used preferentially to less readily metabolized carbon sources. CCR has been studied in both prokaryotic and eukaryotic organisms, and has been most extensively investigated in the bacterium *Escherichia coli*. Glucose repression of the lactose operon in *E. coli* is a classical example of transcriptional regulation in a prokaryote. Studies of CCR in eukaryotes have mainly focused on the yeast *Saccharomyces cerevisiae*, and the filamentous fungi *Neurospora crassa* and *A. nidulans*. The elements of glucose repression in *S. cerevisiae* and *A. nidulans* are discussed in detail in this chapter. Information for other fungal species such as *Aspergillus niger*, *N. crassa*, and *Trichoderma reesei* has been added to provide an overview of the regulatory events which occur in response to the presence of glucose in the cell. The sections to follow will delve into these microorganisms, giving particular importance to the major transcriptional regulator involved in each group.

1.3.2 Carbon catabolite repression in bacteria

Carbon catabolite repression in bacteria is a regulatory mechanism to ensure the sequential utilization of carbohydrates. The most striking difference in transport processes in bacteria is the entry of carbohydrates either in phosphorylated or non-phosphorylated form (Postma *et al.*, 1993). The enzymes involved in sugar transport and phosphorylation play an essential role in signal generation, leading through different transduction mechanisms, to catabolite repression in most bacteria. The actual mechanisms of regulation are substantially different in various bacteria (Stulke and Hillen, 1999). In Gram positive bacteria, many carbohydrates are transported into the cell using the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (Postma *et al.*, 1993). This requires two general phosphotransfer proteins, enzyme I (EI) and phosphorcarrier histidine-containing protein (HPr), and a multitude of sugar-specific permeases (EII). The specific permeases are composed of up to four protein domains (EIIA, B, C and D) at least one of which is membrane bound. The PTS phosphoryl transfer starts with EI and PEP, proceeds via HPr, EIIA and EIIB to sugars, which are transported by EIIC. The PTS is also a major generator of signals for regulatory events. Components of the PTS can influence induction of catabolic operons, nitrogen metabolism, chemotaxis, competence and CCR. The classical example for CCR and gene regulation in gram negative bacteria has been the

mechanism to achieve the glucose-lactose diauxic growth of *E. coli* on glucose and lactose media (Inada *et al.*, 1996). Glucose permeases in *E. coli* are composed of the membrane protein $EIICB^{glc}$ and the cytoplasmic $EIIA^{glc}$. The glucose-specific EIIA ($EIIA^{Glc}$) of the PTS encoded by the *crr* gene is central for glucose repression of the *lac* operon (Postma *et al.*, 1993). Phosphorylated $EIIA^{Glc}$ stimulates adenylate cyclase and cyclic AMP (cAMP) to bind to catabolite activator protein (CAP) when glucose is absent, which activates transcription from the *lac* promoter. On the other hand, the formation of the intracellular inducer allolactose is prevented if the lactose permease is inhibited by non-phosphorylated $EIIA^{Glc}$ in the presence of glucose.

In contrast to the enteric bacterium *E. coli*, the majority of CCR signals in Gram-positive bacteria with low GC content depend on the PTS phosphocarrier protein HPr. In low GC Gram-positive bacteria, catabolite control protein A (CcpA) is of central importance for global transcriptional control in CCR (Henkin, 1996). CcpA functions mainly as a repressor of transcription, but activation is also documented. For example, HPr from *Bacillus subtilis* can be phosphorylated at two different sites: at His-15 by PTS Enzyme I, and at Ser-46 by an ATP-dependent metabolite-activated HPr kinase (Darbon *et al.*, 2002; Sondej *et al.*, 1999). CCR in *B. subtilis* functions in two modes: first, repression of catabolic genes and operons by CcpA, and second, induction of catabolic operons due to HPr-directed phosphorylation control of enzymes generating an inducer, or of transcriptional activators (Stulke *et al.*, 1998). Comparatively little is known about the molecular mechanisms of CCR in *Streptomyces* and other Gram-positive bacteria with high GC content that lack the HPr kinases/phosphates (HPrK/P) dependent regulatory system (Bruckner and Titgemeyer, 2002). The mode of control of the carbohydrate specific reaction in those bacteria depends on a specialized duplicated protein domain, the PTS regulation domain (PRD) (Stulke *et al.*, 1998). PRD proteins mediate sugar-specific induction mostly of PTS carbohydrate catabolism operons. A functional PTS was exhibited in *S. coelicolor* and *S. lividans*, and the genes encoding EI and HPr have been sequenced, but no implication for CCR has been presented in this report (Titgemeyer *et al.*, 1995).

1.3.3 Carbon catabolite repression in *Saccharomyces cerevisiae*

Amongst lower eukaryotes, carbon repression has been studied extensively in the yeast *S. cerevisiae* and the filamentous fungus *A. nidulans*. In the yeast *S. cerevisiae*, CCR acts at the transcriptional level as mRNA levels for genes subject to repression are

reduced in the presence of glucose (Carlson and Botstein, 1982). Many of the genes which are involved in CCR in *S. cerevisiae* have been identified as mutations which lead to either derepression in the presence of glucose, or failure to derepress when glucose concentrations are low.

1.3.3.1 *MIG1*

MIG1 encodes the repressor protein, Mig1p, which was first identified as a multi copy inhibitor of the *GAL1* gene in derepressing conditions (Nehlin and Ronne, 1990). Strains carrying a deletion of *MIG1*, mutated *mig1* or mutated Mig1p binding sites showed relief of glucose repression (Hu *et al.*, 1995; Lundin *et al.*, 1994; Nehlin and Ronne, 1990). These results indicated that Mig1p is a negatively acting control protein in yeast. Mig1p is a Cys_2-His_2 zinc-finger DNA protein that is able to bind to promoters of a variety of genes repressed by glucose in *S. cerevisiae*. The zinc-finger of Mig1p in the amino acid terminal region binds with target DNA at the consensus sequence (5'-WWWWWNSYGGGG-3') found in carbon catabolite repressible promoters (Lundin *et al.*, 1994). Glucose regulates Mig1p function by affecting its phosphorylation, which is catalyzed by the Snf1p protein kinase. Phosphorylation alters the subcellular localization of Mig1p, which is shuttled between the nucleus and cytoplasm (Nehlin and Ronne, 1990; Ostling and Ronne, 1998). But binding by Mig1p alone does not cause repression as was shown when LexA-Mig1p fusion proteins repressed target genes bearing LexA operators in an Ssn6p-Tup1p dependant manner (Tzamarias and Struhl, 1994). Yeast two-hybrid screens showed a direct interaction between Ssn6p and Mig1p and indicated that Ssn6p provides the link between Tup1p and Mig1p (Treitel and Carlson, 1995).

Strains containing *mig1* deletions have also been analysed for their ability to repress target promoters in the presence and absence of glucose, with the result that three other functional domains have been identified. The repression domain is comprised of the last 24 amino acids of the carboxy terminus and it is necessary for repression to occur, and two internal regulatory domains, R1 and R2, are essential for relief from repression. A potential nuclear localization domain, which is very similar to the nuclear localization signal of yeast transcriptional factor Swi5p, has been identified in the region following the zinc fingers and called the B (basic) domain (Cassart *et al.*, 1995). However, the region required for nuclear localization of Mig1p in response to glucose levels is thought to occur between amino acids 261-400 rather than in the B domain

(Ostling *et al.*, 1996). Experiments using a Mig1::GFP fusion protein have shown that Mig1p is located in the nucleus when glucose is present and in the cytoplasm when glucose is depleted (DeVit *et al.*, 1997). Immunodetection of Western blots with antibodies against Mig1p has shown that Mig1p is phosphorylated in the absence of glucose but not when glucose is present. It suggests a role for phosphorylation in the regulation of the Mig1p response to glucose, and correlates with the fact that Snf1p kinase is required for release of glucose repression by Mig1p. Mig1p is constitutively located in the nucleus of *snf1* mutants (DeVit *et al.*, 1997; Vallier and Carlson, 1994). This subcellular localization of Mig1p is regulated by the Snf1p kinase (detail in section 1.3.3.3) and Msn5p, a β-importin family nuclear transport receptor, which mediates the nuclear export of Mig1p after its phophorylation. In addition, glucose control of *SUC2* expression is also mediated by the Snf1-Mig1 pathway (Elbing *et al.*, 2004). However, it has been shown that Mig1p remains nuclear and *GAL1* transcription is normal in *S. cerevisiae* mutants lacking of Msn5p, suggesting that Mig1p translocation may not be the key step in this regulation (DeVit and Johnston, 1999).

Genes similar to *MIG1* have been cloned in the yeasts *Kluyveromyces lactis* (Cassart *et al.*, 1995), *K. marxianus* (Cassart *et al.*, 1997), *Schizosaccharomyces pombe* (Tanaka *et al.*, 1998), and *Candida albicans* (Gancedo, 1998) and in ascomycetes fungi including *A. nidulans* (Dowzer and Kelly, 1991). In addition, two further proteins with zinc-fingers similar to those of Mig1p have been identified in *S. cerevisiae* (Lutfiyya and Johnston, 1996). One of these, Mig2p, binds to the promoter of *SUC2* and contributes to its repression by glucose but has little affinity for the *GAL1* promoter.

1.3.3.2 *SSN6* and *TUP1*

The screening of mutants with defects in CCR identified two further genes, *SSN6* (also called *CYC8*) and *TUP1*, which appeared to be functionally related because mutations in either one caused some similar phenotypes including constitutive expression of glucose repressible enzyme, sporulation and mating defects, abnormal cell morphology and flocculence (Rothstein and Sherman, 1980; Trumbly, 1992). Co-immunoprecipitation experiments have shown that these proteins associate to function (Williams *et al.*, 1991). Analysis of the high molecular weight Tup1p/Ssn6p complex revealed that 1 unit of Ssn6p associates with 4 units of Tup1p (Varanasi *et al.*, 1996). The complex also represses the expression of genes regulated by a variety of signals other than glucose, including mating type, DNA damage and oxygen (Keleher *et al.*, 1992;

Komachi *et al.*, 1994; Treitel and Carlson, 1995; Zhou and Elledge, 1992). It has been proposed that the complex is recruited to promoters by specific DNA binding protein, and neither Tup1p nor Ssn6p appear to bind directly to DNA. There is strong evidence that Mig1p exerts its repression effect by recruiting a complex which contains Tup1p and Ssn6p (Tzamarias and Struhl, 1994). For example, Mig1p has been shown to bind directly to Ssn6p and recruit Ssn6p-Tup1p to the UAS (upstream activating sequence) of *SUC2* which encodes invertase (Keleher *et al.*, 1992). It had been proposed that the relocalization of Mig1p to the cytoplasm results in dissociation of the Ssn6p-Tup1p complex from the promoter of target genes (Smith and Johnson, 2000). However, this hypothesis is not consistent with current data where the Ssn6p-Tup1p complex was shown to be present at the *GAL* promoter both under repressing and activating conditions (Papamichos-Chronakis *et al.*, 2002), and the localization of Mig1p may not be important in this regulation.

The caboxy terminus of Tup1p contains 7 copies of a WD40 repeat sequence (Williams and Trumbly, 1990) and Ssn6p contains 10 copies of a 34 amino acid TPR (tetratrico-peptide repeat) motif (Schultz *et al.*, 1990). The first 72 amino acids at the N-terminus of Tup1p is the site of interaction with Ssn6p (Tzamarias and Struhl, 1994). Both WD40 and TPR motifs have been implicated in protein-protein interactions. The specificity of the Ssn6p/Tup1p repressor complex for the structurally dissimilar DNA binding proteins via which it is recruited to different promoters may be permitted by various combinations of these WD and TPR repeat sequences (Tzamarias and Struhl, 1995).

1.3.3.3 *SNF1*

The Snf1 protein kinase is a central component of the signaling pathway for glucose repression and is required for transcription in reponse to glucose limitation in yeast. *SNF1* was identified in several different screens when mutants were unable to grow on glycerol or maltose, on ethanol, or on sucrose or raffinose (Ciriacy, 1977; Entian and Zimmermann, 1982; Neigeborn and Carlson, 1984; Zimmermann and Scheel, 1977). In yeast, Snfi1p is found associated with other proteins such as Snf4p, Sip1p, Sip2p, and Gal83p (reviewed in Gencedo, 1998). Snf1p is highly conserved among eukaryotes (Celenza and Carlson, 1986, 1989; Celenza *et al.*, 1989). The mammalian homologue of Snf1p, AMP-activated protein kinase (AMPK), has also been identified and reported (Carling *et al.*, 1994). Its activity is also repressed in the presence of glucose, which is

involved in the control of repression and activation of glucose-repressed genes in *S. cerevisiae*. Snf1p has a carboxy terminal regulatory domain (RD) and an amino terminal catalytic domain (KD) which are proposed to bind to each other to inhibit Snf1p function in high glucose level conditions by a conformational change of the kinase complex. In a low glucose environment, an activating subunit, Snf4p, binds to the regulatory domain of Snf1p to activated the protein (Jiang and Carlson, 1996). But the deletion of the regulatory domain to bypass the requirement for Snf4p does not abolish the repression by glucose, and therefore Snf4p is not the only target of the glucose signal (Celenza and Carlson, 1989). Jiang and Carson (1996) reported that there is an equilibrium between the active and inactive forms of Snf1 complex, and the bridging protein (Brp) maintains the Snf1p and Snf4p complex.

The Snf1 kinase regulates the activity of the Mig1 DNA-binding repressor protein (Carlson, 1999). The Snf1p binds with two serine residues in Mig1p, and experiments using a Mig1p-VP16 fusion protein have shown that Snf1p can no longer phosphorylate Mig1p in low glucose condition when these (serine) sites are mutated (Ostling and Ronne, 1998), and Mig1p is constitutively located in the nucleus (DeVit *et al.*, 1997). But the translocation of Mig1p as a result of phosphorylation by Snf1p does not directly control glucose repression in yeast. The study on the coordinated repression of Mig1p and Ssn6p-Tup1p complex on *GAL1* transcription showed the phosphorylation of Mig1p by the Snf1p kinase alleviates its interaction with Ssn6p-Tup1p complex, and it is proposed that this interaction is the key molecular switch controlling transcriptional repression/derepression mechanism in yeast (Papamichos-Chronakis *et al.*, 2004).

Snf1 kinase activity is required not only for transcription of glucose-repressed genes when glucose is limiting but also for sporulation, glycogen storage, peroxisome biogenesis, and thermotolerance (Hardie *et al.*, 1998). Snf1p also regulates both the expression and the function of two activators, Sip4p and Cat8p, which control the transcription of gluconeogenic genes. It is now apparent the Snf1p can regulate a particular gene at multiple levels via regulation of the expression and function of its repressors and/or activators (Carlson, 1999).

1.3.3.4 *SNF4*

SNF4 encodes the transcriptional activator protein, Snf4p, which forms a high molecular mass complex with Sip1/Sip2/Gal83 subunits. Snf1 kinase is found associated with these activating subunit and scaffolding proteins, and Snf4p (Cat3p) is required for Snf1p activity (Carlson, 1999). Studies show that Gal83p, a member of a family of proteins related to Snf1p, also mediates interaction between the Snf1 kinase and the transcriptional activator Snf4p. The proteins Sip1/Sip2/Gal83 appear to optimize the regulatory response to glucose starvation but are not essential for function of the Snf1 kinase pathway (Jiang and Carlson, 1997).

When glucose is limiting, the Snf4p activating sub-unit binds the Snf1p regulatory domain and counteracts the autoinhibitory interaction. Thus, the direct interaction between Snf1p and Snf4p within the kinase complex is regulated by the glucose signal (Jiang and Carlson, 1996). Glucose must be transported into the cell to cause repression, however none of the major hexose transporters serve a sensing function for glucose repression. The signal for glucose repression is related to the glucose concentration rather than the glucose flux (Meijer *et al.*, 1998), and evidence that cells have significant concentrations of intracellular glucose suggests that glucose itself could be a signaling molecule (Teusink *et al.*, 1998).

1.3.3.5 *REG1*, *GLC7* and *GRR1*

REG1 and *GRR1* were identified as 2-deoxy-D-glucose resistant mutants and are also known as *HEX2* and *CAT80*, respectively (Entian and Zimmermann, 1982; Niederacher and Entian, 1987; Zimmermann and Scheel, 1977). Invertase, maltase and malate dehydrogenase were expressed in the presence of glucose in these mutants. However, none of the proteins bind directly to promoters to repress transcription. A yeast two hybrid screen has shown that Reg1p interacts with Glc7p (which forms the catalytic subunit of protein phosphatase type 1) and these two proteins can be co-immunoprecipitated (Tu and Carlson, 1995). A proposed role for Reg1p/Glc7p complex is to dephosphorylate Mig1p in high glucose conditions and therefore enable Mig1p mediated repression to occur (DeVit *et al.*, 1997). Reg1p has also been shown to bind to the kinase domain of Snf1p to effect inactivation by recruiting Glc7p (Ludin *et al.*, 1998).

It has been reported that Reg1p interacts with the yeast regulatory protein Grr1p, and it has been proposed that Grr1p may recognize PEST sequences, a potential signal for protein degradation of Reg1p, resulting in inactivation of the Reg1p/Glc1p complex (Li and Johnston, 1997). As a result, Snf1p would be in its active form and Mig1p would be in a phosphorylated form and be located in the cytoplasm and not active as a repressor. Mutations in *GRR1* have glucose transport defects and are unable to activate the *HXT1-4* (hexose transporter genes). Grr1p also has a role in the regulation of Rgt1p, which is involved in glucose transportation in yeast cells. Mutants lacking Reg1p, Glc7p and Grr1p lack the ability to dephosphorylate Hxk2p monomers (detail in section 1.3.3.7), which suggests that they are involved in the phosphorylation of this protein (Randez-Gil *et al.*, 1998).

1.3.3.6 *SNF3*, *RGT2* and *RGR1*

Among the large number of hexose transporter (*HXT*) genes identified in *S. cerevisiae*, *SNF3 and RGT2* are two unusual members of the hexose transporter gene family. These genes are expressed at very low levels when compared to most *HXT* genes, and they play a specific role (Neigeborn *et al.*, 1986; Ozcan *et al.*, 1996a). *SNF3* is repressed by a high glucose concentration and required for high-affinity glucose transport (Bisson *et al.*, 1987), while *RGT2* is expressed constitutively. Snf3p is likely to function as a sensor for low glucose level and is required for the induction, by low levels of glucose, of some hexose transporter genes such as *HXT2*. Rgt2p would be a sensor for high glucose level and required for induction of *HXT1* (Ozcan *et al.*, 1996a). Snf3p and Rgt2p have long cytoplasmic domains at the C-terminus, and it has been considered that the binding of glucose to these proteins causes a conformational change affecting a C-terminal signal domain (Celenza *et al.*, 1988; Ozcan *et al.*, 1996a; Ozcan *et al.*, 1996b).

The involvement of *RGR1* in glucose repression was shown when mutations in the gene led to overexpression of a reporter gene under the control of the *SUC2* (encodes enzyme invertase) promoter (Sakai *et al.*, 1988). Mutations in *RGR1* cause derepression of a number of genes subject to glucose repression, and produce an abnormal cell morphology. Rgr1p acts as a negative transcriptional regulator of *HO* and *IME1*, but is also required for the full transcriptional activation of other genes such as *HIS4* and *CTS1* (Jiang and Stillman, 1992). Rgr1p also interacts with the mediator and RNA polymerase II holoenzyme as part of a sub-complex which is proposed to control

transcription by RNA polymerase. This would explain why Rgr1p has a role in both activating and repressing conditions (Li *et al.*, 1995). Sin4p regulates the transcription of *HIS4*, *CTS1*, *HO*, and *IME1* in a similar way to Rgr1p, and Sin4p and Rgr1p have been shown to associate *in vivo* (Jiang *et al.*, 1995). These two proteins appear to function to regulate the transcription of a range of genes and are not exclusively concerned with CCR.

1.3.3.7 *HXK* and their role in glucose signaling in *S. cerevisiae*

HXK genes encode the hexokinases which catalyze glucose to hexose-6 phosphate, and play an important role in glucose sinsing (Frommer *et al.*, 2003). In *S. cerevisiae*, there are three hexokinases; hexokinase PI (Hxk1p) and hexokinase PII (Hxk2p), and glucokinase (Glkp), which can catalyze this reaction (Albig and Entian, 1988; Entian and Frohlich, 1984; Entian *et al.*, 1984; Frohlich *et al.*, 1984, 1985). Hxk1p is found in cells grown on ethanol (Muratsubaki and Katsume, 1979), but Hxk2p is the main activity for phosphorylating glucose (Gancedo *et al.*, 1977; Rodriguez *et al.*, 2001), whereas Glkp appears to be constitutive (Muratsubaki and Katsume, 1979). However, the level of *GLK* transcript varied, with higher levels in non-fermentable carbon sources, indicating that regulation of expression could occur at the transcriptional level, and the expression level of *HXK1* and *HXK2* was also dependent on the carbon source (Herrero *et al.*, 1995). These observations could be related to the roles they might play in glucose repression.

Two different regions in the Hxk2p are involved in glucose phosphorylation; the ATP-binding site and the glucose-binding site (Wilson, 1995). Site-directed mutagenesis indicated that Ser158 was important for the catalytic activity of the protein (Arora *et al.*, 1991) and this residue was reported to interact with glucose (Kuser *et al.*, 2000). Yeast Hxk2p has protein kinase activity (Fernandez *et al.*, 1988; Herrero *et al.*, 1989), and this domain is important for glucose phosphorylation (Kuser *et al.*, 2000; Ma *et al.*, 1989). The phosphorylation of Hxk2p is essential for signal transduction (Randez-Gil *et al.*, 1998), and exists in dimeric-monomeric equilibrium that is dependent on phosphorylation, where the monomeric protein is dephosphorylated and an increase in the unphosphorylated dimer is seen upon addition of glucose (Randez-Gil *et al.*, 1998). Induction of yeast *HXT* genes is mediated by Hxk2p, and appears also to be dependent on the phosphorylated state of Hxk2p (Ozcan and Johnston, 1995; Randez-Gil *et al.*, 1998). Dephosphorylation of Hxk2p is though to be mediated by Reg1p, which targets

the ubiquitous protein phosphatase 1 (Alms *et al.*, 1999). It is possible that the phosphorylation of Hxk2p could provide an intracellular signal of glucose status (Behlke *et al.*, 1998; Rose *et al.*, 1991).

The role of the glucose-sensing process in *S. cerevisiae* has been ascribed to Hxk2p phosphorylation of glucose though a number of genes, which have been shown to be involved in one way or another (Gancedo, 1998; Kruckeberg *et al.*, 1998). *HXK2* lesions were shown to cause failures in glucose repression (Entian *et al.*, 1977; Ma and Botstein, 1986). Interestingly, about 14% of Hxk2 is nuclear in glucose grown cells (Santangelo, 2006). This suggests an alternative explanation for the regulatory involvement of hexokinase in the glucose response. The yeast Hxk2p has also been shown to stimulate binding and/or phosphorylation of Reg1p or inhibits dephosphorylation of Reg1p by Glc7p (Sanz *et al.*, 2000a; Sanz *et al.*, 2000b). Furthermore, Hxk2p was shown to participate in the regulatory DNA-protein complex with *cis*-acting regulatory elements of the *SUC2* gene promoter (Herrero *et al.*, 1998).

S. cerevisiae is able to detect the extracellular glucose levels and generate intracellular signals that result in an adequate cellular response to variations in the glucose medium composition (Moreno *et al.*, 2005). The majority of these alterations occur at the level of mRNA transcription of many genes that encode enzymes for carbon metabolism (Gancedo, 1998; Johnston, 1987; Ozcan *et al.*, 1998). The possible mechanisms for glucose sensing which include 1) direct sensing by a membrane-bound protein, or 2) sensing of glucose or metabolite by a hexose-phosphorylating protein that which has involved in regulatory role such as that involved in regulatory (Rolland *et al.*, 2002). Glucose binds to the glucose receptors, Rgt2p/Snf3p, on the membrane and initiates signals that activate a pathway that allows hexose transporter gene expression by repressing Rgt1p function (Ozcan *et al.*, 1996b). An additional pathway that involves transcriptional change in response to glucose is the stimulation of adenylyl cyclase by G-protein coupled receptor (Gpr1p) and two G-proteins (Gpa1p and Gpa2p), resulting in the activation of cAMP-dependent protein kinase A (PKA) (Kraakman *et al.*, 1999). Glucose also activates another pathway inside the cell, involving the repression of genes not needed during growth on glucose. Moreno *et al.* (2005) demonstrated that Hxk2p interacts directly with Mig1p *in vivo* and *in vitro* and that the ten amino acids between K6 and M15 are required for their interaction to bind with *SUC2* promoter. This finding shows that the main role of Hxk2p in the glucose signaling pathway is the

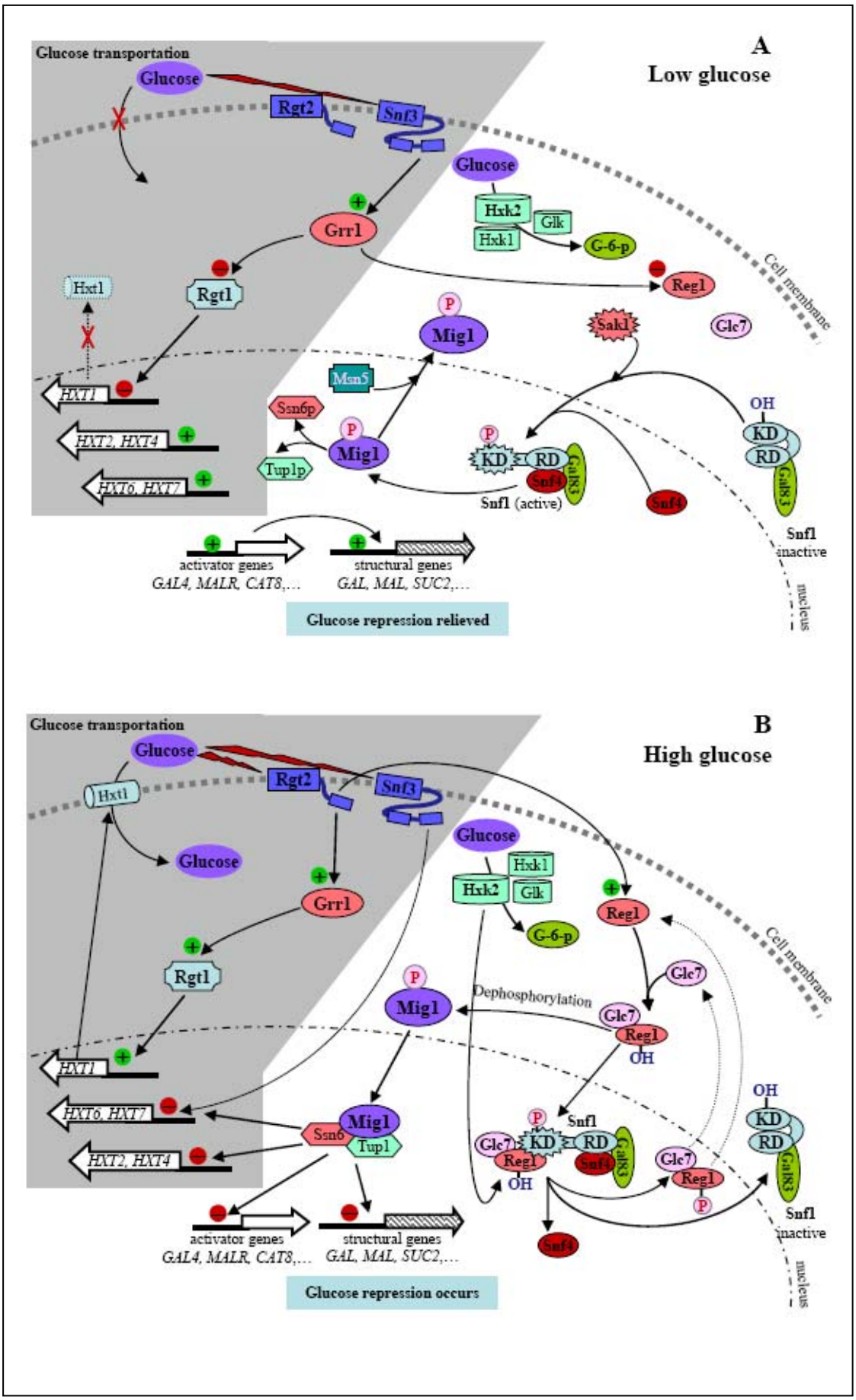
interaction with Mig1p to generate a repressor complex located in the nucleus of the cell (Moreno *et al.*, 2005; Palomino *et al.*, 2005)

Figure 1.2 Pathway of glucose signaling elements and the regulation of genes in *S. cerevisiae* leading to glucose repression or derepression.

A. In low glucose conditions, the membrane bound low-glucose sensor, Snf3p, transmits an intracellular signal, through its C-terminal tail region, to Grr1p which inactivates Reg1p and thus inactivates the Reg1p/Glc7p complex. The Snf1p kinase is activated by Sak1 and translocated into nucleus, allowing the kinase domain of Snf1p to be phosphorylated and remain as an active kinase by interaction with other factors, Snf4 and Gal83. This active complex phosphorylates Mig1p which results in disassociation of Ssn6p and Tup1p from Mig1p, and finally carbon catabolite repression is relieved. At the same glucose concentration, Grr1p inhibits Rgt1p in the hexose transport system of the cell, resulting in the inhibition of the genes involved in high level glucose transportation, such as *HXT1*.

B. In high glucose conditions, Rgt2p, which has a long C-terminal tail, transmits a glucose signal to activate Grr1p in the glucose transport system, and Reg1p in the regulatory system. Reg1p changes to the active form and recruits Glc7p to form a complex. The active Glc7/Reg1 complex can dephosphorylate Snf1p, which changes the Snf1 kinase to an inactive form and exports it to the cytoplasm. The unphosphorylated Mig1p forms a complex with Ssn6p and Tup1p and becomes an active repressor in the nucleus, leading to glucose repression, including repression of genes involved in the hexose transport system. In high glucose conditions, Snf3p is also involved in inhibitory some high affinity hexose transporter genes, such as *HXT2*, *HXT4* and *HXT6*.

Drawn from: (Ahuatzi *et al.*, 2004; Ahuatzi *et al.*, 2007; DeVit *et al.*, 1997; Gancedo, 1998; Moreno *et al.*, 2005; Ostling and Ronne, 1998; Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a; Ozcan *et al.*, 1998; Santangelo, 2006)


A
Low glucose
Glucose transportation
Glucose
Rgt2
Snf3
Grr1
Rgt1
Hxt1
HXT1
HXT2, HXT4
HXT6, HXT7
Glucose
Hxk2
Hxk1
Glk
G-6-p
Reg1
Glc7
Sak1
Mig1
Mig1
Ssn6p
Tup1p
Mss5
KD
RD
Gal83
Snf4
Snf1 (active)
Snf1
inactive
Cell membrane
nucleus
activator genes
GAL4, MALR, CAT8,...
structural genes
GAL, MAL, SUC2,...
Glucose repression relieved
B
High glucose
Glucose transportation
Glucose
Rgt2
Snf3
Hxt1
Grr1
Rgt1
HXT1
HXT6, HXT7
HXT2, HXT4
Glucose
Hxk1
Hxk2
Glk
G-6-p
Reg1
Glc7
Dephosphorylation
Mig1
Mig1
Ssn6
Tup1
Snf1
inactive
Cell membrane
nucleus
activator genes
GAL4, MALR, CAT8,...
structural genes
GAL, MAL, SUC2,...
Glucose repression occurs

1.3.4 Carbon catabolite repression in *Aspergillus nidulans*

Yeast is highly adapted for growth in high sugar concentrations and prefers fermentation of glucose to ethanol rather than the oxidation of glucose. Filamentous fungi utilize glucose via aerobic oxidation like most other eukaryotic cells. Carbon repression in filamentous fungi usually results in lower levels of activity of an enzyme after growth on a mixture of an inducing plus a repressing carbon source compared with the growth on the inducing carbon source alone. Systems that are regulated by carbon repression in fungi can be divided into three groups on the basis of their metabolic function. The first group comprises genes encoding enzymes required for catabolism of less preferred carbon sources. The second group includes genes encoding gluconeogenic and glyoxylate cycle enzymes, although it is unclear whether gluconeogenic genes are repressed at all. It has been reported that phosphoenolpyruvate carboxykinase is probably inducible and only weakly regulated by carbon repression (Kelly and Hynes, 1981). The third group of genes subject to carbon repression is those involved in secondary metabolism. The best characterized system to date is penicillin production by *A. nidulans*. It has been shown that transcription of *ipn*A, encoding isopenicillin *N*-synthetase, is regulated by carbon repression, but the physiological mechanism of carbon control on penicillin biosynthesis is not completely clear (Espeso *et al.*, 1993).

Studies on carbon repression in *Aspergilli* are abundant and in *A. nidulans* and *A. niger* the repressor protein CreA plays a major role in carbon repression (Bailey and Arst, 1975; Kelly, 1994). *creA* was first indentified as a suppressor mutation in a strain bearing the *areA* loss of function (Arst and Cove, 1973). Mutations in the *creA* gene have been selected in a number of screens, and they lead to reduced repression of many systems normally subject to CCR (Arst and Cove, 1973; Dowzer and Kelly, 1991; Kelly, 2004). This evidence indicates that *creA* encodes a wide domain, negatively acting repressor protein. CreA inhibits transcription of many target genes by binding to specific sequences in the promoter of these genes. Various *creA* alleles show no clear hierarchy in the level of derepression of various enzymes in the presence of glucose, which further indicates a broad regulatory role for CreA, and also implies that most of the alleles have not completely lost all CreA functions.

1.3.4.1 Identification of *creA* as a suppressor of *areA* loss-of-function mutation

The *A. nidulans creA* gene was discovered among *areA* loss-of-function suppressor mutations selected for growth on glucose medium containing mitrogen sources that can also provide carbon sources (Arst and Cove, 1973; Bailey and Arst, 1975). AreA is a wide-domain transcriptional activating protein required for expression of genes required for utilization of nitrogen sources other than ammonium and glutamine (Arst and Cove, 1969). Loss of function *areA* alleles lead to an inability to grow on medium containing D-glucose and any nitrogen source other than ammonium or glutamine (Arst and MacDonald, 1973). Some alternative carbon sources such as proline and acetamide can provide both a nitrogen and carbon source for the cell. The enzymes required for their utilization are thus regulated by both carbon catabolite repression and nitrogen metabolite repression, and the relief of either leads to expression. Therefore, in the absence of glucose, *areA* null mutants can grow on the medium containing acetamide or proline as the sole carbon and nitrogen sources. Arst and Cove (1973) exploited this interaction to identify mutations that suppress the effects of loss of function *areA* mutations such that the enzymes for acetamide or proline utilization are produced even in the presence of glucose. This genetic screen identified the *creA*, *creB* and *creC* genes as involved in carbon repression (Arst and MacDonald, 1973; Bailey and Arst, 1975; Hynes and Kelly, 1977).

Other strategies have also been used to isolate *creA* mutants, for example, using *A. nidulans pyc*A and *pdh*A mutants that lack the pyruvate carboxylase and pyruvate dehydrogenase complex. Lack of function mutations in the pyruvate carboxylase and pyruvate dehydrogenase encoding genes lead to the inability of the strain to produce acetyl-CoA from pyruvate, and these strains require an alternative source of acetyl-CoA. Mutations in the *creA* gene have been found among phenotypic suppressors of *pycA* or *pdhA* mutant strains selected on D-glucose media containing ethanol or acetamide (Bailey and Arst, 1975). The *areA* and *pdhA* mutants have enabled the classification of the effectiveness of carbon sources to stimulate carbon repression. This classification is based on the extent to which various carbon sources allow utilization of proline in an *areA* background and ethanol in a *pdhA* background (Arst and Cove, 1973).

creA mutants have also been isolated in *A. niger* using a strategy based on selection of pseudorevertants of *areA*-1 on medium containing 4-aminobutyric acid (GABA) and

D-glucose. Most of the mutations also resulted in derepression of proline and alanine utilization in the presence of D-glucose, and these mutations could be substituted by the *A. niger creA* gene (Ruijter and Visser, 1997).

1.3.4.2 Molecular analysis of *creA*

The *creA* gene from *A. nidulans* was cloned by complementation by transformation of the *creA204* mutant phenotype on sucrose and allyl alcohol media (Dowzer and Kelly, 1989, 1991). Without a functional CreA repressor, the derepressed *creA204* mutation allows the cell to metabolise allyl alcohol to produce the toxic end-product, acrolein, due to the derepression of the *alcA* gene encoding alcohol dehydrogenase I. The complemented transformants were resistant to the presence of allyl alcohol in the medium.

creA encodes a protein of 416 amino acids containing two DNA binding zinc-fingers of the Cys_2-His_2 class (Dowzer and Kelly, 1991), an alanine-rich region, and frequent S(T)PXX motifs which are commonly found in regulatory proteins (Shroff *et al.*, 1996; Shroff *et al.*, 1997). The zinc-finger region was found to have a high level of sequence similarity (84%) with the zinc-finger region of Mig1 protein in *S. cerevisiae* (Nehlin and Ronne, 1990). The consensus sequence recognized by the CreA zinc finger domain in the 5' region of several genes is 5'-SYGGRG-3', and the presence of an AT-rich sequence 5' to the consensus sequence can affect the binding at some the sequences that fit this consensus (Cubero and Scazzocchio, 1994; Kulmburg *et al.*, 1993; Panozzo *et al.*, 1998). These genes include the *alc* regulon, *ipnA*, the *prn* cluster, *amdS* and *facB* (Chamalaun-Hussey, 1996; Cubero and Scazzocchio, 1994; Espeso and Penalva, 1992). Binding of CreA at this consensus sequence, found within the upstream sequences of those genes, prevents activation of transcription. Regulation of transcription by CreA can be through direct competition with other regulatory proteins for binding at the promoter regions, or indirectly, such as via the recruitment of specific transcriptional repressors to the promoter region (Kelly, 2004).

A range of *creA* alleles have been selected and analysed. They can be grouped into two categories, missense mutations in the zinc-finger DNA binding region, and frameshift or nonsense mutations that result in a truncated CreA peptide (Shroff *et al.*, 1997). All of these mutations in *creA* result in various degrees of deregulated expression of a wide range of genes that would be repressed in the presence of glucose. Many mutations in

creA lead to elevated levels of gene expression in both carbon catabolite repressing and derepressing conditions, indicating roles for *creA* in growth conditions generally regarded as repressing (Kelly, 2004). The mRNA analyses from mycelium grown in glucose (repressing) or arabinose (derepressing) cultures, and mutational analyses indicated that the *creA* gene is autoregulated (Arst *et al.*, 1990), and it contains a number of CreA binding site in its promoter region (Shroff *et al.*, 1996). Two of these CreA binding sites were shown to be required for the autoregulation of the *creA* transcript (Strauss *et al.*, 1999). Mutations in *cre*A also affect colony morphology on complete medium, with extreme alleles such as *creA*30, *creA*306 and *creA*Δ leading to very small and compact morphology.

Analysis of the *creA* truncated mutants has indicated that the region required for repression is located within eight amino acids at the C- terminal end of CreA (Shroff *et al.*, 1997). There is some similarity in the C-terminus sequences of the CreA protein and the Mig1p repression domain in *S. cerevisiae* (Ostling *et al.*, 1996). Also toward the C-terminus of CreA are 40 amino acids that are completely conserved in a number of filamentous fungi and show weak sequence similarity to the Rgr1p repressor protein in *S. cerevisiae* (Drysdale *et al.*, 1993), but when this region is replaced with the corresponding region of Rgr1p the hybrid *creA* gene did not appears to function (Shroff *et al.*, 1997). The mutations in the zinc-finger region are predicted to have an altered affinity for their binding site but most of these mutants are still predicted to bind DNA, and since they do not have an identical phenotype they cannot all be loss-of-function alleles (Shroff *et al.*, 1997). Three alleles, *creA303*, *creA304* and *creA306* are predicted to have mutations in amino acids identified as critical for binding, or produce proteins which are truncated before or within the zinc-finger. The *creA*306 allele is the only allele predicted to produce a full-length protein where DNA binding is completely abolished, and shows an extreme phenotype which could be due to the CreA306 protein titrating other proteins that interact with CreA (Shroff *et al.*, 1997). The *creA303* mutation is a truncation mutation resulting in a predicted protein with only 68 (out of 415) amino acids, which has no known functional region (Shroff *et al.*, 1997), and the *creA303* and *creA304* alleles have the same phenotype as a deletion mutation (Shroff *et al.*, 1997) and can be regarded as complete loss of function mutations.

1.3.4.3 The homologues of CreA in other eukaryotes

The *creA* gene has been identified in a large number of fungi, including *A. oryzae, A. niger, Metarhizium anisopilae, N. crassa, Sclerotinia sclerotiorum* and *Trichoderma reesei* (Drysdale *et al.*, 1993; Ilmen *et al.*, 1996a; Ilmen *et al.*, 1996b; Reymond-Cotton *et al.*, 1996; Screen *et al.*, 1997). The zinc-finger region of CreA shows strong sequence similarity to the zinc-finger region of *S. cerevisiae* Mig1p repressor protein (Nehlin and Ronne, 1990), the mammalian C_2H_2 zinc-binding family Krox/Egr/Zif268, and Wilms'tumor suppressor protein (as cited in Kulmberg *et al.*, 1993). CreA1 from *S. sclerotiorum* is functionally related to CreA of *A. nidulans*, but cannot complement *MIG1* and *MIG2* deletions in *S. cerevisiae* (Vautard *et al.*, 1999). The fusion protein of *S. sclerotiorum* GST-CreA1 is localized in the nuclei of glucose-grown hyphae, and in the cytoplasm when glucose is removed from the culture, similar to the glucose dependent localization of Mig1p in *S. cerevisiae* (Vautard *et al.*, 1999). Phosphorylation has also been implicated in the DNA binding of Cre1 from *Hypocrea jecorina*/*T. reesei*, with the phosphorylation of Ser241 required to allow a DNA binding conformation of Cre1. The phosphorylation independent DNA binding mutation (S241A) resulted in the permanent carbon catabolite repression of cellobiohydrolase-1 expression (Cziferszky *et al.*, 2002). Thus Cre1 phosphorylation results in the ability to effect repression via DNA binding, whereas it prevents Mig1p dependent repression by signaling export from the nucleus in *S. cerevisiae*. The *cre1* genes from *T. reesei* and *T. harzianum* contain *cre1* consensus binding sites in their promoters similar to *creA*, indicating that *cre1* may be auto regulated (Ilmen *et al.*, 1996a; Ilmen *et al.*, 1996b).

1.3.4.4 *creB* and *creC*

Strains containing the *creB15* and *creC27* mutations were identified as suppressors of the phenotype due to *areA217* on glucose and acetamide medium, in the same screen that produced *creA* alleles. Genetic and phenotypic analysis mapped the genes to linkage group II and they are not linked to each other (Hynes and Kelly, 1977). The *creB* and *creC* mutants share an almost identical phenotype and have pleiotropic effects on carbon metabolism, resulting in derepression of some genes, such as those encoding acetamidase and alcohol dehydrogenase that are usually subject to CCR, but the pathways affected are only a subset of those systems affected by mutations in *creA* (Hynes and Kelly, 1977). In addition, *creB* and *creC* mutants fail to derepress other genes such as those for the utilization of L-proline and D-quinate, even in the absence

of a repressing carbon source, and thus proteins encoded by the *creB* and *creC* genes clearly play a role or roles in conditions that are carbon catabolite repressing and derepressing. In addition, the *creB* and *creC* mutants show resistance to molybdate and sensitivity to acriflavine in complete medium. Alleles of *creB* and *creC* result in the same range of mutant phenotypes, and these effects are not additive in strains containing mutations in both *creB* and *creC*, indicating that the proteins act as steps in the same pathway, or as a protein complex (Hynes and Kelly, 1977). Double mutant strains containing *creB*15 or *creC*27 alleles with *creA*204 have the compact morphology of a strain containing *creA204*, and they show the amount of derepression of a *creA204* strain implying that *creA204* is epistatic to *creB15*/*creC27* for derepression. However, the presence of *creA204* in the double mutant strains with either *creB* or *creC* cannot repair the poor growth on L-proline and D-quinate in *creB15* or *creC27* containing strains. *creB* has previously been described as the *molB* mutation that conferred resistance to toxic concentrations of molybdate (Arst and Cove, 1970; Arst *et al.*, 1970; Arst *et al.*, 1981).

The *creB* gene was cloned by genome walking from the *acoB* gene (26 map units away) and by complementation and found to encode a deubiquitinating enzyme of 767 amino acids containing 6 deubiquitination (DUB) homology domains, indicative of members of the ubiquitin processing protease (ubp) family, and a coiled-coil region that may be involved in substrate recognition (Lockington and Kelly, 2001). CreB has four highly significant PEST sequences beginning at amino acid 240, 385, 473 and 538. PEST sequences are hydrophilic polypeptide enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) and serve as proteolytic signal targeting proteins for ubiquitination and rapid destruction (Rogers *et al.*, 1986). Homologues of CreB have been found in a variety of higher organisms, for example in human; UBH1, *Arabidopsis thalania*; UBP3, *Pichia anomala*; UBP1, *Caenorhabditis elegans*; CAB54286, and *Drosophila melanogaster*; AAF56066, but no highly similar sequence was identified in *S. cerevisiae*.

The *creC* gene was cloned by genome walking and complementation utilizing its proximity to the *glnA* gene (Todd *et al.*, 2000). The *creC* gene encodes a 630 amino acid polypeptide containing a proline-rich region near the N-terminus, a putative nuclear localization region, and five WD40 repeat motifs at the C-terminus (Todd *et al.*, 2000), indicating a probable regulatory role. WD40 repeat regions form a propeller-

like structure which facilitates protein-protein interaction (Neer *et al.*, 1994). Using deletion constructs of the plasmid containing the *creC* gene, it was shown that the most C-terminal WD40 motif is absolutely required for function (Todd *et al.*, 2000). Highly conserved proteins are found in humans; DMR-N9, mouse; DMR-N9, *C. elegans*; C08B6.7, *A. thaliana*; T2N18.8, and *S. pombe*; Yde3, but there is no closely conserved CreC peptide sequence in *S. cerevisiae* although the WD40 region shows a weak similarity with Tup1p. However, the RcoA protein, which is involved in asexual development and sterigmatocystin production in *A. nidulans* shows a higher similarity to Tup1p than does CreC, and deletion of *rcoA* does not affect CCR (Hicks *et al.*, 2001). DMR-N9 in humans and mouse is associated with the myotonic dystrophy region (Mahadevan *et al.*, 1993; Shaw *et al.*, 1993). Interestingly, the presence of CreB and CreC homologues in humans and mouse but not in *S. cerevisiae*, supports the suggestion that the regulatory mechanism CreB and CreC are involved in is conserved among multicellular eukaryotes (Todd *et al.*, 2000) but may have been lost in yeast.

That CreB and CreC proteins are present in a complex *in vivo* under both repressing and derepressing conditions was shown by co-immunoprecipitation experiments, and it has been proposed that the CreB-CreC deubiquitination complex removes ubiquitin moieties from CreA and other substrates, thus modifying or stabilizing these proteins (Lockington and Kelly, 2002). Overexpression of the CreB deubiquitinating protein can compensate for the lack of CreC, but not *vice vesa*, indicating that CreB is the active partner. The role of CreC may be to stabilize or activate the CreB deubiquitinating protein, perhaps by the CreC masking the PEST region of CreB to stabilize or alter substrate recognition for 26S proteasome (Lockington and Kelly, 2002). The presence of proteins in a range of organisms strongly conserved with the CreB and CreC proteins in *A. nidulans* implies that these proteins may be a component of a regulatory network that is present in most eukaryotes.

1.3.4.5 *creD*

The *creD34* mutation was identified as a suppressor of the *creC27* mutant phenotype on glucose medium containing fluoroacetamide, and was also found to suppress the *creB15* mutant phenotyes on this medium, suggesting a role in the regulatory network (Kelly and Hynes, 1977). Genetic analyses have indicated that *creD* is closely linked to *creC* on chromosome II and that *creD34* is recessive to $creD^{+}$ in a diploid strain (Kelly and Hynes, 1977). The *creD* gene was cloned by complementation and physically

analysed, and it encodes a protein of 597 amino acids that contains an arrestin_N and arrestin_C domain, a PPXY motif and two PXY motifs (Boase and Kelly, 2004). The PPXY and PXY motifs are proline rich and basic sequences, respectively, those are found in transcriptional factors and are implicated in protein-protein binding (Chen and Sudol, 1995; Chen *et al.*, 1997; Sudol *et al.*, 1995). Arrestin domains bind proteins which have been phosphorylated (Palczewski, 1994). The *creD34* mutation suppressed other aspects of the *creB* and *creC* mutant phenotype, such as the derepression of the *facA* and *alcA* genes as analyzed through the fluoroacetate and allyl alcohol sensitivity found in *creB15* and *creC27* strains, but not in combination with *creA204* (Kelly and Hynes, 1977). However, enzyme assay data revealed some increased repression of alcohol dehydrogenase-1 in the *creD34;creA204* double mutant strain when compared with the *creA204* containing strain. A *creD34* mutant strain is more resistant than wild-type on glucose and fluoroacetamide medium, suggesting that the *creD34* mutation leads to tighter repression of enzymes subject to CCR. The suppression of the *creB* and *creC* mutant phenotypes by *creD34* implies that *creD* is involved in the reverse process to the deubiquitination role of the CreB/CreC protein complex, such as the ubiquitination of the same target proteins (Boase and Kelly, 2004). CreD is similar to the Rod1p and Rog3p proteins in *S. cerevisiae*. Rod1p and Rog3p interact with ubiquitin ligase Rsp5p in *S. cerevisiae* in the HECT (homologous to the E6-Associated Protein Carboxyl Terminus) domain. The homologue of *rsp5* in *A. nidilans* is *hulA* (HECT ubiquitin ligase) and CreD interacted with HulA in the bacterial 2-hybrid system (Boase and Kelly, 2004). The CreD protein is predicted to be involved in the ubiquitination aspect of the regulatory network involving CreA, CreB, and AcrB from the analysis of the epistatic interactions of the various mutation combinations (Boase *et al.*, 2003; Kelly and Hynes, 1977).

1.3.4.6 *acrB*

The *acrB2* mutation was isolated in the *A. nidulans* as a spontaneous resistant sector on an acriflavin containing medium (Roper and Kafer, 1957). The *acrB* gene encodes a novel protein that contains three putative transmembrane domains and a coiled-coil region (Boase *et al.*, 2003). A strain containing the *acrB2* mutation showed resistance to some dyes, such as crystal violet and malachite green, and *acrB2* is recessive to the wild-type allele (Arst, 1981). The *acrB2* mutant showed reduced growth on a rang of sole carbon sources, including fructose, cellobiose, and starch, and reduced utilization of GABA and alanine, as sole carbon and nitrogen sources (Boase *et al.*, 2003). The

phenotype of *acrB2* is similar to *creD34*, and it also suppresses the phenotypic effects of mutations in the *creB* and *creC* genes. Thus, AcrB interacts with a regulatory network controlling carbon source utilization that involves ubiquitination and deubiquitination, and may play a role in the ubiquitination aspect of this regulatory network.

1.3.5 Hexokinases and glucose signaling in filamentous fungi

Hexokinases catalyze the first step in glucose metabolism and play an important role in glucose phosphorylation in fungi. Conservation of residues believed to be important for ATP-binding and glucose-binding is found across a wide range of organisms (Bork *et al.*, 1992, 1993). The presence of a number of isoenzymes in these organisms indicates a role other than catalytic phosphorylation (Frommer *et al.*, 2003). Hexokinases have been shown to be involved in glucose sensing and glucose repression (Rolland *et al.*, 2002). The hexokinase genes in filamentous fungi have been reported in *Aspergillus parasiticus* (Davidson, 1960), *A. niger* (Panneman *et al.*, 1996; Panneman *et al.*, 1998; Steinbock *et al.*, 1994), *A. nidulans* (Bernardo *et al.*, 2007; Flipphi *et al.*, 2003; Ruijter *et al.*, 1996), and *N. crassa* (Lagos and Ureta, 1980).

In *A. nidulans*, glucokinase (*glkA*) was identified by Panneman *et al.* (1996), encoding a 50 kD protein that is able to phosphorylate glucose, 2-deoxyglucose, mannose, and glucosamine. Several consensus CreA binding sequences were found in the promoter region of this gene, although it was not tested weather the CreA protein actually binds to these sequences (Panneman *et al.*, 1996). A hexokinase gene, *hxkA*, has also been cloned and characterized, and encoded a protein that has significant amino acid similarity to the hexokinase and glucokinase of *S. cerevisiae* (Panneman *et al.*, 1998). The mRNA level of both *hxkA* and *glkA* rapidly decreased when the carbon source becomes exhausted implying that expression of these genes requires the presence of a carbon source (Panneman *et al.*, 1998). The studies of the role of hexokinases in filamentous fungi are limited. The *xprF* (*hxkD*) gene was first isolated and sequenced from *A. nidulans*, and codes for a putative hexokinase as shown by sequence comparison with other hexokinases (Katz *et al.*, 2000). This gene was identified in the isolation of mutant strains with altered levels of extracellular protease expression (Katz *et al.*, 1996). The *xprF1* and *xprF2* mutations affect the levels of extracellular protease and the utilization of certain nitrogen sources. These mutants showed increased levels of extracellular protease activity, when compared with wild-type, in medium containing

milk as carbon source and impaired growth on medium containing hypoxanthine or uric aid as nitrogen source (Katz *et al.*, 1996). However, the production of extracellular protease occurs only in the absence of a carbon source and hence appears to be regulated in response to a carbon starvation mechanism (Katz *et al.*, 2000).

Flipphi *et al.*, (2003) investigated the role of hexose phosphorylating enzymes in the signaling of CCR in *A. nidulans*. It appears that transcriptional repression by glucose was fully retained in strains lacking either hexokinase (*hxkA1*) or glucokinase (*glkA4*), but the double hexose kinase mutants (*hxkA1/glkA4*) showed transcriptional derepression in the presence of glucose and a complete absence of glucose phosphorylating activity. The *hxkA* mutants grow normally on glucose and this mutant also produces glucokinase activity in the presence of either fructose or glucose (Flipphi *et al.*, 2003). It is in contrast to the regulation in *S. cerevisiae* where Hxk2p is essential for glucose repression, indicating these two glucose-phosphorylating enzymes in *A. nidulans* can functionally substitute each other. These fundamental differences might be relevant to the different mechanism between Mig1p and CreA.

Two other non-catalytic hexokinase genes, *hxkD*, and *hxkC* were haracterized (Bernardo *et al.*, 2007). The *hxkD* deletion strain showed increased extracellular protease levels in response to carbon starvation and this mutant is not completely recessive, indicating that the level of the gene product is critical. The *hxkC* deletion mutant exhibits a phenotype similar, but not identical, to *hxkD* mutants. As with *hxkD*, mutations in *hxkC* are suppressed by loss-of-function mutations in *xprG*, which encodes a putative transcriptional activator involved in the response to nutrient limitation (Bernardo *et al.*, 2007). Fluorescence microscopy showed that HxkD-GFP was found only in the nucleus suggesting a regulatory role of this protein, whereas the HxkC-GFP was associated with mitochondria (Bernardo *et al.*, 2007).

1.4 Ubiquitination and deubiquitination systems

The correct regulation of gene expression is a demanding but vitally important process. It is not surprising that cells have evolved elaborate mechanisms to regulate the first step in gene expression-transcription. The transcription proteins themselves have to be present at the right place, at the right time and in the correct amounts to produce levels of transcription that are appropriate for each gene. In recent years it has become evident

that one of the ways for cells to meet this regulatory challenge is to make extensive use of the "ubiquitin(Ub)-proteasome" system (Muratani and Tansey, 2003).

1.4.1 Ubiquitination system

Ubiquitination is the process whereby ubiquitin is conjugated to the substrate protein. Ubiquitin (Ub) is a highly-conserved 76 amino acid protein that is found only in eukaryotic cells. It is found throughout the cell and can exist either in free form or linked covalently via the glycine at the C-terminal end of UB to the side chain of lysine residues in target proteins, and often signals their destruction (Hicke and Dunn, 2003). Ub functions to regulate protein turnover in a cell by closely regulating the degradation of specific proteins. Ubiquitination functions in an ATP-dependent fashion. Ubiquitin is involved in many cell processes. For example, Ub is conjugated to the protein cyclin during the G1 phase of mitosis and thus plays an important role in regulating the cell cycle. Ub conjugation is also involved in DNA repair, embryogenesis, the regulation of transcription, and apoptosis of cells. Ub does not itself degrade proteins and instead merely tags proteins for degradation by the proteasome (Wilkinson, 1999).

The ubiquitinating enzyme mechanism requires three enzyme complexes: E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (Ub protein ligase) (Hicke and Dunn, 2003). There are two classes of E3 enzymes, the RING E3s and the HECT E3s. HECT E3s are mechanistically difference form RING E3S in that they interact directly in the chemistry of protein ubiquitination (Scheffner *et al.*, 1995). The E3 complex is of interest as it thought to select a protein for ubiquitination through an interaction with the substrate's N-terminal residue. Proteins can be modified by a single-Ub moiety or by poly-Ub chains. At least three lysines within Ub, Lys29, Lys 48, and Lys63, are used to form a polyubiquitin chain (Arnason and Ellison, 1994). For example, Lys48-linked polyubiquitin chains regulate numerous nuclear, cytosolic, and Endoplasmic reticulum (ER) membrane proteins by targeting them for degradation by the 26S proteasome (Chau *et al.*, 1989; Thrower *et al.*, 2000). Generally, substrates with four or more Ub moieties attached are targeted for degradation by the proteasome (Figure 1.3), whereas less than four moieties may modify the surface of the target protein thus affecting the protein function (Chau *et al.*, 1989; Hoege *et al.*, 2002).

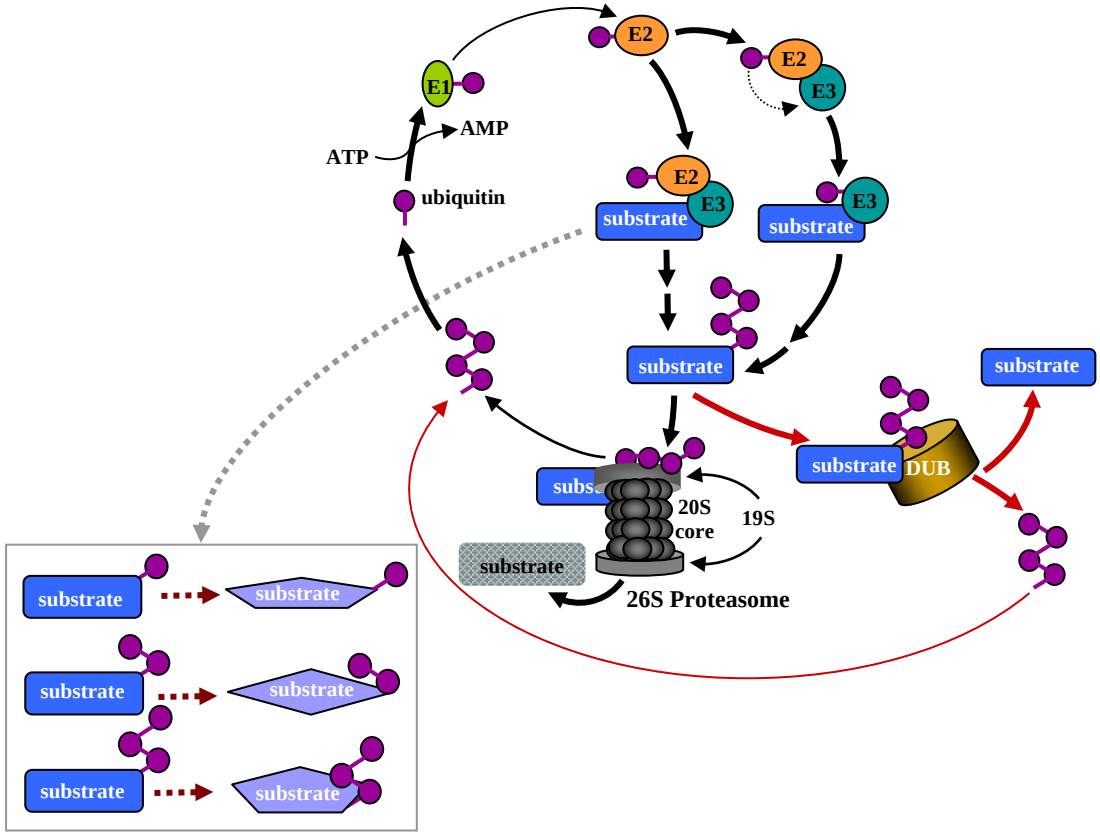


Figure 1.3 Diagram showing the ubiquitination (black arrow) and deubiquitination (red arrow) systems. Ubiquitin (Ub) is activated for conjugation by ATP-dependent Ub-activating enzyme (E1). The Ub linked to E1 is then moved via a trans-esterification process to a Cys residue on E2. E3 (ubiquitin ligase) selects a protein for degradation via an interaction with its degradation signal and then recruits the E2 complex to transfer its Ub moiety to Lys on the substrate. This process can be repeated to form poly-ubiquitinated proteins which are finally targeted to the 26S proteasome for degradation. On the other hand, addition of less than 4 ubiquitin molecules can cause conformational changes to the substrate (in gray square). The debubiquitinating enzyme (DUB) prevents the Ub-substrate complex from degradation by removing the ubiquitin chain from the substrate. (Picture drawn from Hicke and Dunn, 2003; Hoege *et al.*, 2002; Muratani and Tansey, 2003 and Wilkinson, 1999).

In *S. cerevisiae*, Rod1p and Rog3p proteins have been shown to interact via their PY motifs with the WW domains of the E3 ubiquitin-pritein ligase Rsp5p (Andoh *et al.*, 2002). WW is a protein module, 40 amino acids in length, that contain two highly conserved tryptophans about 20-23 amino acids apart ((Bork and Sudol, 1994). The Rsp5p is one of five HECT (Homologous to the E6-associated protein carboxyl terminus) ubiquitin ligases that catalyses ubiquitin transferase activity and three WW domains. Rsp5p is involved in protein degradation and cellular functions such as transcriptional regulation and transcriptional-coupled repair (Chang *et al.*, 2000), and essential for yeast under normal growth conditions (Wang *et al.*, 1999). Rsp5p ubiquitinates several integral plasma membrane proteins, including Fur4p (Galan *et al.*, 1996), Gap1p (Hein *et al.*, 1995), and Ste2p (Dunn and Hicke, 2001), and targeting them for ubiquitin-mediated endocytosis, and also direct ubiquitin-mediated trafficking of proteins from trans-Golgi network to the vacuole (Helliwell *et al.*, 2001). In *A. nidulans*, *creD* encodes a protein that contains an arrestin_N and an arrestin_C domains and PY motifs. Rod1p and Rog3p from *S. cerevisiae* are the two most hightly similar characterized proteins to CreD and ApyA (Boase and Kelly, 2004). The CreD protein is predicted to be involved in the ubiquitination aspect of the regulatory network involving CreA, CreB, CreC and AcrB from the analysis of epistatic interactions of the various mutation combinations (Boase *et al.*, 2003; Boase and Kelly, 2004). The interaction between Rod1p and Rog3p with E3 ubiquitin-protin ligase Rsp5p in *S. cerevisiae*, and the presence of two homologous proteins, CreD and ApyA, in *A. nidulans* would suggest that there might also be interaction between CreD and ApyA, and ApyA and HulA in *A. nidulans*. Bacterial two-hybrid assays for protein interaction of CreD and ApyA with the WW domainsof HulA from *A. nidulans* indicated that ApyA and the WW domains of HulA interact strongly, and also that CreD and HulA interact in this system (Boase and Kelly, 2004), however strain containing an *apyA* gene disruption had on obvious phenotype (Denton JA., personal communication).

1.4.2 Deubiquitination system

Ubiquitination can be reversed by the hydrolyzing activity of deubiquitinating enzymes (DUBs) (Hicke and Dunn, 2003). DUBs are the proteins that cleave the bond of the ubiquitin (Ub)-protein complex. DUBs can be grouped on the basis of sequence homology into two classes; ubiquitin carboxy-terminal hydrolases (UCH), and ubiquitin processing proteases (UBPs) (also referred to ubiquitin specific protease (USPs)) (Wing, 2003). *S. cerevisiae* has 1 UCH and 16 UBPs/USPs, and there are 27

UBPs/USPs in the plant *Arabidopsis* (Yan *et al.*, 2000), and at least 4 and 63 distinctive gene encoding UCHs and UBPs/USPs respectively in the human genome (Wing, 2003). The main function of DUBs is to remove Ub or Ub-chains from specific protein targets, and they act as substrate-specific regulators of ubiquitination and proteolysis. However, DUBs can process the ubiquitin precursor proteins to generate mature ubiquitin and reverse the polyubiquitination of substrate proteins (Chen *et al.*, 2002; Li *et al.*, 2002). These enzymes are also removing ubiquitin from cellular adducts, and keeping the 26S free of inhibitory ubiquitin chains (Amerik and Hochstrasser, 2004) Several DUBs have been noted that could potentially perform such regulatory roles in a variety of biological process.

A well-studied system in multicellular eukaryotes is the regulation by ubiquitination and deubiquitination of substrate proteins involved in *Drosophila* eye development. E3 ubiquitin ligases have a number of roles during eye development in *Drosophila*, including control of cell proliferation, specification, differentiation and death (Wu *et al.*, 1999). Genetic analysis has revealed that the substrate of Fat facets, a DUB, in the eye is Liquid facets (Cadavid *et al.*, 2000). The ubiquitination of epsin (encoded by the *liquid facets* gene) results in this protein being degraded and leads the cell adopting a photoreceptor fate. The deubiquitination enzyme Faf (encoded by the *fat facets*) deubiquitinates epsin, thereby stabilizing epsin and promoting endocytosis, preventing the misspecification of photoreceptor cells in the developing eye (Chen and Fischer, 2000; Chen *et al.*, 2002).

Doa4p is a yeast DUB that recycles ubiquitin from proteolytic intermediates(Hicke and Dunn, 2003). A screen for suppressors of *doa4* identified several genes encoding vacuolar protein sorting involved in multivesicular bodies (MVB) formation. Doa4p localizes to a late endosome compartment in a *vps* mutant defective in MVB vesicle formation (Amerik and Hochstrasser, 2004) and associates with BroIp to mediates its recruitment to endosomes (Richter *et al.*, 2007), and ubiquitin-modified cargo destined for the vacuole accumulates in Doa4p-deficient cells (Katzmann *et al.*, 2001; Losko *et al.*, 2001). This information indicates that a major cellular activity of this DUB is to remove ubiquitin from transmembrane proteins prior to their transport into the vacuole lumen (Hicke and Dunn, 2003). Kee *et al.* (2005) found that the Rsp5p was mediated by the Ubp2p, a prominent Rsp5p-associated DUB, and Rup1p, a ubiquitin-associated domain-containing protein, both *in vivo* and *in vitro*. The Ubp2p could copurified with

the epitope-tagged Rsp5p, indicating that at least a fraction of Rsp5p exists in a complex with Ubp2p, and that the Ubp2p/Rup1p complex serves to anagonize, and potentially regulate, Rsp5p *in vivo* in *S. cerevisiae* (Kee *et al.*, 2005).

In *A. nidulans*, the CreB protein is the first deubiquitinating enzyme shown to be involved in CCR (Lockington and Kelly, 2001) and it is known that CreB forms a complex with the WD40 motif containing a protein encoded by *creC* (Lockington and Kelly, 2002). Mutations in these genes lead to altered carbon source utilization and the *creD*34 mutation suppresses the phenotypic effect of mutation in *creC* and *creB* (Lockington and Kelly, 2002). Characterization and subsequent analysis of *cre*B has shown that it encodes a ubiquitin carboxy-terminal hydrolase (UCH), which is a functional member of a novel subfamily of the ubiquitin-processing protease family (Lockington and Kelly, 2001).

1.5 Roles of CreA, CreB, CreC and CreD in carbon catabolite represssion in *Aspergillus nidulans*

CreA is a DNA binding protein that is required for the repression of a wide range of carbon catabolites. The evidence is clear that CreA is required for repression in carbon catabolite repressing conditions, but there is also clear evidence that CreA plays a role or roles in conditions considered to be carbon catabolite derepressing. The real physiological significance of the role of CreA in experimentally de-repressing conditions is not clear, as it is experimentally impossible to produce conditions that are completely carbon catabolite derepressing, but which do not also lead to starvation and a cessation of growth. Experiments by Arst and Cove (1973), and Bailey and Arst (1975) demonstrated that the various growth conditions they used form a continuum with respect to repression. There is also evidence that protein modification and stability could be an important component of the carbon catabolite repression mechanism (Lockington and Kelly, 2002). CreA protein might be ubiquitinated by the CreD-HulA ubiquitin ligase complex, resulting in a conformational change or targeting to the proteasome for degradation (Boase and Kelly, 2004). It is clear that CreB and CreC play roles in both carbon catabolite repressing and carbon catabolite derepressing conditions, and that CreB and CreC form part of a high molecular weight complex in a regulatory deubiquitination network (Lockington and Kelly, 2002). Whether such a

CreB/CreC network acts directly on CreA, or exerts its effects on carbon metabolism independently of CreA, has yet to be demonstrated.

A model can be put forward that is compatible with the existing data in which a regulatory deubiquitination network involved in the CreB/CreC complex acts directly on CreA such that the effect of CreB and CreC on carbon catabolite repression is via an effect on the stability or activity of CreA (Figure 1.4) (Lockington and Kelly, 2002). In this model, CreA is directly recognized as a substrate by the coiled-coil region of the CreB deubiquitinating enzyme. Ubiquitin chains are removed from CreA reducing its degradation via the proteasome or altering its activity, and thus CreA is present to repress the transcription of genes subject to CreA-regulated carbon catabolite repression (Figure 1.4A). The CreB deubiquitinating enzyme is subject to a degree of proteolysis via the PEST-mediated pathway in carbon catabolite derepressing conditions, leading to reduced deubiquitination of CreA. Therefore, lower concentration of CreA leads to relief of carbon catabolite repression. There are a number of open questions in this model including whether other proteins also interact with and/or modify CreA activity, and whether other proteins are present in the CreB/CreC complex. In addition, this model can be used to investigate the interactions of the CreB/CreC regulatory deubiquitination complex with various permeases and transporters. The effects on carbon catabolite repression may be the consequence of altered concentrations or cellular localizations of signaling molecules or by stabilizing membrane proteins involved in glucose sensing.

Ubiquitination regulatory networks involving ubiquitin ligases are the counterbalance to deubiquitinating networks, like that involving CreB and CreC which involve the removal and recycling of ubiquitin moieties (Kelly, 2004). It is possible that the CreB/CreC complex is required to stabilize permeases and transporters by the removal of ubiquitin (Figure 1.4B), and affects intracellular concentrations or locations of glucose or other effecter molecules, or stabilizes a membrane protein involved in glucose sensing. There are numerous complex examples of relationships between carbon and nitrogen regulation, including an increase in the number of cases where the CreA status of the cell has consequences for the regulation of compounds that only provide a nitrogen source to the cell (Lockington and Kelly, 2002). A complete understanding of all the events involved, from carbon and nitrogen sensing to gene expression outcomes, is still some way from being achieved.

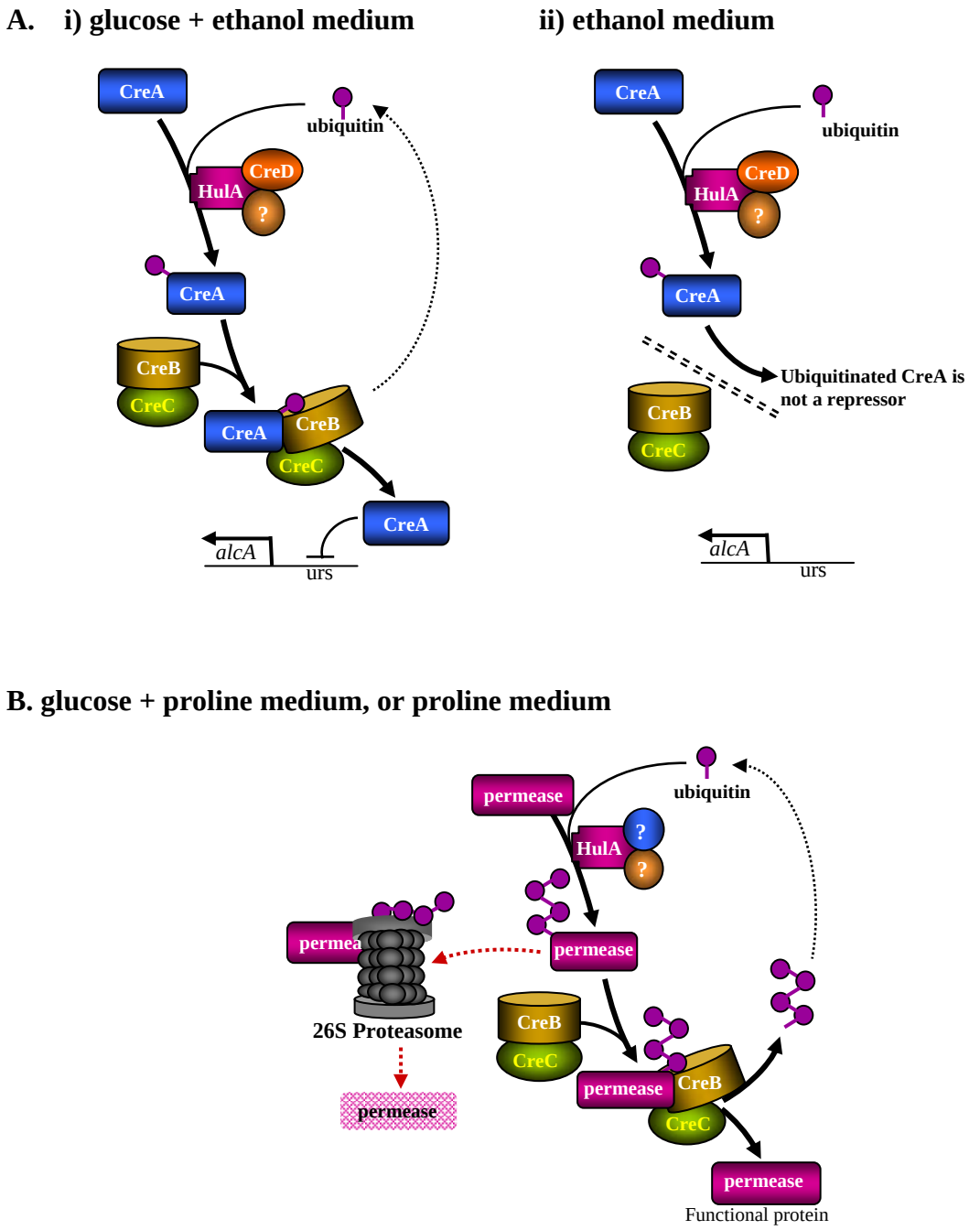


Figure 1.4 Model for interaction of CreB, CreC, CreD, HulA with CreA and permeases in repressing (Ai and B) or derepressing (Aii) conditions. For genes that are derepressed in creB mutant strains such as *alcA* (in Ai and Aii), Ub moieties are added by a complex that includes CreD and HulA, and in the presence of glucose, CreB is required to remove the Ub to allow CreA to function as a repressor. In the absence of glucose, CreA activity is not required. For genes that fail to express in *creB* mutant strain (B) such as proline and quinate permeases, whether glucose is present or not, a different complex targets a Ub chain to the relevant permease, and CreB is required to remove the Ub chain to prevent degradation via the protesome (Boase and Kelly, 2004; Kelly, 2004; Lockington and Kelly, 2001, 2002).

1.6 Permease enzymes in fungi

A number of transporters or permeases mediating amino acid uptake have been identified and studied genetically and physiologically in *S. cerevisiae*, *A. nidulans* and *N. crassa*. Most of them are specific for one or a few related L-amino acids and exhibit different properties with respect to substrate affinity, specificity, capacity and regulation (Sophianopoulou and Scazzocchio, 1989; Sophianopoulou and Diallinas, 1995; Wiame *et al.*, 1985). *S. cerevisiae* imports amino acids from the surrounding medium through a set of amino acid permeases. Activities of these amino acid permeases are regulated by the quantity of available amino acids as well as the quality of the nitrogen source. Molecular data indicates that fungal amino acid permeases belong to a single and unique family of transporters, highly regulated transcriptionally and post-transcriptionally, and follow a very specific translocation pathway to localize in the plasma membrane. An example of an intensively studied permease is the proline permease, PrnB, in *A. nidulans*, which is presented in the following section.

1.6.1 Proline permease of *A. nidulans*

A. nidulans is able to utilize proline as either a carbon or nitrogen source for growth, and the genes required are subject to pathway specific induction by proline. Induction of the *prn* genes is dependent on efficient accumulation of proline by the product of the *prnB* gene which encodes the permease (Sophianopoulou and Diallinas, 1995). Efficiency of *prnB* transcription is itself highly dependent on the presence of other nitrogen and carbon substrates in the growth medium (Sophianopoulou *et al.*, 1993). *prnB* transcription is very low when both repressing nitrogen (ammonia, glutamine or asparagine) and carbon (glucose or sucrose) sources are present in the growth medium (Bailey and Arst, 1975; Sophianopoulou *et al.*, 1993).

PrnB is the L-proline transporter of *A. nidulans*, encoded by the *prnB* gene (Sophianopoulou and Scazzocchio, 1989; Sophianopoulou and Diallinas, 1995). It belongs to the Amino acid Polyamine Organocation (APC) transporter family which is conserved in prokaryotes and eukaryotes (Tavoularis *et al.*, 2003). The transportation of PrnB to the plasma membrane has been shown to be facilitated by ER chaperons (Erpapazoglou *et al.*, 2006). The biochemical evidence and *in silico* analysis suggest that the PrnB protein comprises 12 transmembrane segments (TMS) connected short cytoplasmic loops and both N- and C-terminal domains towards the cytoplasm (Kafasla *et al.*, 2007; Tavoularis *et al.*, 2003). Tavoularis *et al.*, (2003) reported that the

transmembrane segment-6 (TSM-6) has an important role in proline uptake, and clearly affects PrnB uptake kinetics. While some *prnB* mutations affect proper translocation of PrnB in the membrane, at least two mutants (K245E and F248L) exhibit physiological cellular expression of PrnB and, thus, the corresponding mutations can be classified as mutations directly affecting proline binding and/or transport (Kafasla *et al.*, 2007).

1.6.2 Quinate permease in *A. nidulans*

A. nidulans is able to utilize quinic acid as a carbon source for growth, and 6 genes required for quinate catabolism; *qutA, qutB, qutC, qutD, qutE* and *qutF* have been characterised (Hawkins *et al.*, 1982; Hawkins *et al.*, 1985; Lamb *et al.*, 1990). *qutD* codes for the quinate permease, however, there is not much information on its function or mechanism of action in the fungal cell. The deduced protein sequence of the *qutD* gene reveals the presence of 8 highly hydrophobic sequence motifs that have the potential to insert into the lipid bi-layer, and there is no discernible signal peptide and the carboxy terminus is highly hydrophilic (Hawkins *et al.*, 1988). These observations are consistent with conclusion that the *qutD* gene encodes a permease.

1.7. Conclusion

The molecular analysis of the genes involved in CCR in *A. nidulans* has revealed that the molecular mechanism for carbon catabolite repression is very different to that described in *S. cerevisiae*. Although the transcriptional repressors CreA and Mig1p share some similarity in amino acid sequences in the DNA binding and repression domains, there is no evidence to indicate that CreA recruits a co-repressor complex in a manner similar to the recruitment of the Tup1p/Ssn6p by Mig1p, and CreA and Mig1p do not have homologous functions. Similarly, the *creB* and *creC* gene products indicate that ubiquitination and deubiquitination play an important role in aspects of the repression mechanism in *A. nidulans*, but not in yeast (Kelly, 2004). The precise details of how the transcriptional repressor, CreA, is activated or deactivated in response to changing nutritional conditions remain unclear. It is reasonable given the evidence to suggest that sensing the carbon status of the fungal cell, and transmitting the signal, will involve both phosphorylation and ubiquitination signaling components. The research interest in the area of the regulatory response to carbon starvation and the components of this regulatory mechanism will no doubt interact with components of the carbon catabolite repression mechanism. Once these systems become clearer,

perhaps a complete understanding of the complex interaction between carbon and nitrogen repression in organisms may be aimed for.

1.8 Aims of the study

In *A. nidulans*, several genes involved in the carbon catabolite repression system have previously been investigated. Three genes in this group were characterized, *creA*, *creB* and *creC*. CreB and CreC form a complex *in vivo* and it has been proposed that this complex acts to stabilize the CreA repressor protein by removing ubiquitin moieties that either target CreA for destruction via the 26S protesome or alter it function, however direct evidence to prove the interaction between CreA and the CreB/CreC complex has not been available. The CreB/CreC complex may also play a role in the turnover of permease enzymes, such as those for proline and quinate uptake, in both the presence and absence of carbon catabolite repression, as *cre*B and *cre*C mutations lead to the failure to express enzymes for the utilization of L-proline in both the presence and absence of glucose (Lockington and Kelly, 2001). Regulation of the proline and quinate utilization clusters including the proline permease, PrnB, and quinate permease, QutD, would suggest that this action is likely to be directly on the permeases or enzymes themselves, rather than via the pathway specific regulatory proteins involved in induction.

This work will experimentally test the proposed interaction between CreA and CreB, PrnB and CreB, and QutD and CreB. These likely targets will be analysed by assessing the stability of these permeases, and whether they are ubiquitinated *in vivo* to help define the role of CreB/CreC in their regulation. These experiments will generate direct evidence to confirm or reject whether there is an interaction between CreA and the CreB/CreC complex, and between PrnB and QutD and the CreB/CreC complex, and thus the model will be able to be confirmed or modified. In addition, this investigation may reveal other proteins that may be involved in carbon catabolite repression in *A. nidulans*.

The main objective of this project is to further elucidate the deubiquitination and/or ubiqiutination network regulating carbon catabolite repression in *A. nidulans*. In particular, the direct substrates of the CreB/CreC complex will be determined. There are three aims for the study of the substrates of the CreB/CreC complex involved in CCR in *A. nidulans*.

1.8.1 CreA: a candidate protein target for the CreB/CreC complex

In the model proposed by Lockington and Kelly (2002), the CreB/CreC complex acts directly on CreA to affect the stability or activity of CreA, and also effects carbon catabolite repression in fungal cells. In order to experimentally test this model, the first aim will be a study of the candidate protein target of CreB/CreC complex, CreA. Whether CreA is a target substrate will be determined by using strains that express epitope tagged CreA and CreB proteins in western blot and immunoprecipitation and co-immunoprecipitation experiments, using specific antibodies directed against the epitope tags and ubiquitin (in Chapter 3).

1.8.2 Proline permease: a candidate protein target for the CreB/CreC complex

The second aim is to determine whether the proline permease (PrnB) is a direct target substrate of the CreB/CreC complex. The CreB/CreC protein complex will be tested for the ability to interact with the permease proteins. In this experiment, initially PrnB-epitope tagged strains will be constructed. These strains will be crossed to the CreB-epitope tagged strains, and strains containing both proteins will be used to determine whether PrnB is a target, by using immunoprecipitation and co-immunoprecipitation experiments using specific antibodies directed against the epitope tags (in Chapter 4).

1.8.3 Quinate permease: candidate protein target for the CreB/CreC complex

The third aim is to determine whether the quinate permease (QutD) is a direct target substrate of the CreB/CreC complex. The CreB/CreC protein complex will be tested for the ability to interact with the permeaese protein. In this experiment, initially QutD-epitope tagged strains will be constructed. The positive strains will be crossed to the CreB-epitope tagged strains, and strains containing both proteins will be used to determine whether QutD is a target, by using immunoprecipitation and co-immunoprecipitation experiments using specific antibodies directed against the epitope tags (in Chapter 5).

1.8.4 Proteomic approach to identifying other proteins that interact with CreA

Investigation of other proteins which interact with the CreA protein in the fungal cell will be undertaken using a proteomics approach. Total protein from wild type and a strain containing an epitope-tagged CreA will be extracted and precipitated with specific antibodies against the tagged CreA. The protein fractions will be separated by

SDS-PAGE, and proteins will be identified using proteomin techniques at Adelaide Proteomic Center (in Chapter 6).

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

General laboratory chemicals and constituents of growth media were of analytical grade and were purchased from a range of commercial manufacturers.

2.1.1 Antibodies

- Anti-FLAG::M2® Monoclonal Antibody-Peroxidase Conjugate, SIGMA-ALDRICH Gmbh, Steinheim, Germany. #104K6251.

- Anti-HA-Peroxidase High Affinity (3F10) Rat Monoclonal Antibody, Roche Diagnostics Gmbh, Mannheim, Germany. #2013819.

- Anti-[C-myc]-peroxidase (9E10) mouse monoclonal antibody, Roche Diagnostic, Indianapolis, IN. #11814150001.

- Peroxidase Conjugated Affinity Purified Anti-MOUSE IgG (H&L) (DONKEY) (Min X Bv Ch Gt GP Ham Hs Hu Rb Rt & Sh Serum Proteins), Rockland Immunochemicals for Research Inc., Gilbertsville, PA, SA. #610-703-124.

- Ubiquitin-conjugate specific antibody, BIOMOL, Exeter, UK. #KW8805 (For use with IgM pr pan-Ig secondary antibody).

2.1.2 Enzymes

Restriction enzymes (and corresponding 10X buffers) were purchased from Boehringer-Mannheim, New England Biolabs (NEB) and Promega. Other enzymes were obtained from the following manufacturers;

Klenow fragment	Geneworks, Adelaide, Australia	#KF-1
T4 DNA ligase	Boehringer-Mannheim, Germany	#481220
Taq DNA polymerase	Genework, Adelaide, Australia	#BTQ-1
PfuTurbo® DNA polymerase	Stratagene, LA Jolla, CA	#600250
Lysing enzymes	Sigma Chemical Co., St. Louis, MO	#L-1412

All enzymes were used following the manufacturer's instructions, in the appropriate reaction buffers.

2.1.3 Molecular weight markers

DNA:

100 bp DNA ladder	New England Biolabs, Beverly, MA	#323-1.
1 kb DNA ladder	New England Biolabs, Beverly, MA	#323-2.

Protein:

Precision Plus Protein™ Standards (All Blue)	BIO-RAD, Hercules, CA	#161-0373

2.1.4 Radioactive isotopes and nucleotides

- [α-^{32}P] dATP (3000 Ci/mmol) Radiochemical Centre, Amersham, UK. #AA0004.

- Ultrapure dNTP Set Amersham Pharmacia Biotech Inc., Piscataway, NJ #27-2035-02.

2.1.5 Bacterial strain

The *Escherichia coli* strain used for cloning and DNA manipulations was DH5α (F^- *mcrA* Δ*lacU*169 (ϕ80*d lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (Hanahan, 1983).

2.1.6 *Aspergillus nidulans* strains

The genotypes and origin of *A. nidulans* strains used in this study are presented in Table 2.1.

Table 2.1 Strains of *A. nidulans* used in this study.

Strain	Genotype	Transformed plasmid	Origin
Wild type (yellow)	*yA1; riboB2*		Shroff *et al*., 1997
Wild type (green)	*biA1; niiA4*		Pateman *et al.*, 1967
1. study on CreA interaction with CreB, and the proteomics approach.			
creB1937	*yA1 pabaA1; creB1937; riboB2*		Lockington and Kelly, 2001
TA1	*yA1 pabaA1 creAΔ4; riboB2*	pPL3; pGPDMycCreA	Lockington RA, unpublished
T3	*yA1 pabaA1; creB1937; riboB2*	pPL3; pGPDCreBFLAG	Lockington and Kelly, 2001
T3.5	*yA1 pabaA1; creB1937; riboB2*	pPL3; pGPDMycCreA; pGPDCreBFLAG	Lockington RA, unpublished
TA6	*yA1 pabaA1 creAΔ4; riboB2*	ppL3; pGPDCreAHAGFP	Lockington RA, unpublished
TA7	*yA1 pabaA1 creAΔ4; riboB2*	ppL3; pGPDCreAHAGFP	Lockington RA, unpublished
T2	*yA1 pabaA1 creAΔ99; creB1937; riboB2*	pPL3; pGPDCreBFLAG	Lockington RA, unpublished
T3.6	*yA1 pabaA1 creAΔ99; creB1937; riboB2*	pPL3; pGPDCreBFLAG	Lockington RA, unpublished
T4.15	*yA1 pabaA1 creAΔ99; creB1937; riboB2*	pPL3; pGPDCreBFLAG	Lockington RA, unpublished
T7	*yA1 pabaA1; creB1937 creC956; riboB2*	pPL3; pGPDCreBFLAG; pGPDCreCHA	Lockington and Kelly, 2002
TA3H2	*yA1 pabaA1 creAΔ4; riboB2*	pPL3; pGPDCreA2HAHis	Lockington RA, unpublished
TA3H2/T3.6	*yA1 pabaA1 creAΔ4; riboB2* | *yA1 pabaA1 creAΔ99; creB1937; riboB2*	pPL3; pGPDCreA2HAHis; pGPDCreBFLAG	diploid from TA3H2 and T3.6 strains (This work)
2. Study on PrnB interaction with CreB			
pabaA1; prnB6	*pabaA1; prnB6*		Supplied by Arst HN.
T1 (T1A, T1B)	*pabaA1; prnB6*	pPrnBHA; pPaba	Ferragamo D. and Kelly JM, unpublished
T4 (T4A, T4B)	*pabaA1; prnB6*	pPrnBHA; pPaba	Ferragamo D. and Kelly JM, unpublished
Tpaba	*pabaA1; prnB6*	pPaba	Ferragamo D. and Kelly JM, unpublished
TP1 (TP1-1, TP1-2, TP1-3)	*pabaA1; prnB6*	pGPDPrnBHA; pPaba	This work
TP2 (TP2-1, TP2-2)	*pabaA1; prnB6*	pGPDPrnBHA; pPaba	This work
TP3 (TP3-1, TP3-1)	*pabaA1; prnB6*	pGPDPrnBHA; pPaba	This work
TP6 (TP6-1, TP6-2)	*pabaA1; prnB6*	pGPDPrnBHA; pPaba	This work

Table 2.1 Strains of *A. nidulans* used in this study (cont.).

Strain	Genotype	Transformed plasmid	Origin
3. Study on QutD experiments			
qutD312	*biA1; pyroA4; riboB2 qutD312*		Ferragamo D. and Kelly JM, unpublished
TQ1	*biA1; pyroA4; riboB2 qutD312*	pPL3; pGEMTQutDHA	This work
TQ5	*biA1; pyroA4; riboB2 qutD312*	pPL3; pGEMTQutDHA	This work
CreBFLAG;QutDHA	*yA1 pabaA; creAΔ99; creB1937; riboB2* | *biA1 pyroA4; riboB2 qutD312*	pCreBFLAG; pGEMTQutDHA; pPL3; pPaba	diploid from T2 and TQ1strains (this work)
TQBD5	*yA1 creB1937*; *pyroA4; riboB2 qutD312*	pGEMTQutDHA; pPL3	This work
TQBD6	*biA1 creB1937; pyroA4; riboB2 qutD312*	pGEMTQutDHA; pPL3	This work

2.1.7 Plasmids and Oligonucleotides

Vectors and plasmids used in this study are presented in Table 2.2. Custom oligonucleotides used in this study were purchased from Geneworks (http://geneworks.com.au; Adelaide, Australia) and are presented in Table 2.3.

Table 2.2 Plasmids and vectors used in this study.

Plasmid name	Description	Source/Origin
pPaba	*A. nidulans pabaA*$^{+}$ gene in pGEM®-T easy vector, used as a selectable marker for co-transformation in *A. nidulans*	Supplied by Hynes, MJ.
pPL3	*A. nidulans riboB*$^{+}$ gene in pUC19 vector, used as a selectable marker for co-transformation in *A. nidulans*	Oakely *et al*. (1987)
pGPDMycCreA	*MyccreA* gene expressed from the *A. nidulans gpdA* promoter in pCB1004 vector.	Lockington RA., unpublished
pGPDCreAHA	*creAHA* gene expressed from the *A. nidulans gpdA* promoter in pCB1004 vector.	Lockington RA., unpublished
pGPDCreA2HAHis	*creA2HAHis* gene expressed from the *A. nidulans gpdA* promoter in pBluescrip II SK vector.	Lockington RA., unpublished
pGPDCreBFLAG	*creBFLAG* gene expressed from the *A. nidulans gpdA* promoter in pCB1004 vector.	Lockington RA., unpublished
pPRNB-HA	*prnBHA* gene from PCR expressed from the native promoter in pBluescript II SK vector.	Ferragamo D. and Kelly JM., unpublished
pGDPMycPrnB	*MycprnB* gene express from the *A. nidulans gpdA* promoter in pBluescript II SK vector.	Ferragamo D. and Kelly JM., unpublished
pGPDCreAHis	*creAHis* gene express from the *A. nidulans gpdA* promoter. Used as a vector to create pGPDPrnBHA and pGPDMycQutD.	Lockington, RA., unpublished
pGPDPrnBHA	*prnBHA* gene express from *A. nidulans gpdA* promoter in pBluescript II SK vector.	This work
pGPDMycQutD	*MycqutD* gene express from the *A. nidulans gpdA* promoter pBluescript II SK vector.	Ferragamo D. and Kelly JM., unpublished
pGEMTQutD	*qutD* gene fron PCR expressed from the native promoter in pGEM®-T easy vector. This plasmid contains 2 *Xho*I-sites and was used to create pGEMTQutDHA.	This work
pGEMTQutDHA	*qutDHA* gene expressed from the native promoter in pGEM®-T easy vector.	This work
pGEM®-T easy	Vector for Cloning of PCR products	Promega

Table 2.3 Oligonucleotide primers used in this study.

Primer name	Primer sequences (5' to 3')	Use
NKQHAF	TGT AAC TGA CGA CCC GAC AA(20)	PCR *qutD* region/ sequencing.
NKQXhoHAR	TTA CTC TCG AGC CTC CTC GAC ATA CTC CTT (30)	PCR *qutD* region/ sequencing.
NKPHAR	AGC TGG ATC CTA CGC GTA ATC(21)	PCR *prnBHA* region.
NKPHAF	GGC ACC GAA TTC AAA ATG AGT(21)	PCR *prnBHA* region.
OligoHA1 [RAL]	T CGA TAT CCG TAT GAT GTC CCC GAC TAT GCG TT (33)	HA annealing oligonucleotides
OligoHA2 [RAL]	TCG AAA CGC ATA GTC GGG GAC ATC ATA CGG ATA (33)	HA annealing oligonucleotides

2.1.8 Kits and miscellaneous materials

2.1.8.1 Commercial Kits used in this study were as follows.

Bio-Rad Protein Assay Kit	Bio-Rad Laboratories, Hercules, CA.	#500-0006
DNeasy® Plant Mini Kit	Qiagen, Valencia, CA.	#69104
FLAG® Tagged Protein Immunoprecipitation Kit	Sigma, Saint Louis, MI.	#FLAGIPT-1
HisTrap™ HP Kit	GE Healthcare Bioscience AB, Uppsala.	#307417
QIAquick® Gel extraction Kit	Qiagen, Valencia, CA.	#28704
QIAquick® PCR Purification Kit	Qiagen, Valencia, CA.	#28104
RNeasy® Plant Mini Kit	Qiagen, Valencia, CA.	#72904
SilverQuest® Silver staining Kit	Invitrogen, Carlsbad, CA.	#LC6070
UbiQapture™-Q Kit	BIOMOL®, Exeter, UK.	#UW8995
Wizard® *Plus* SV Minipreps DNA purification systems Kit	Promega, Madison, WI.	#A1460

2.1.8.2 Miscellaneous materials used in this study were as follows.

Anti-HA Affinity Matrix	Roche Diagnostics GmbH, Germany	#39446622
Complete, Mini, EDTA-free Proteinase inhibitor cocktail tablet	Roche Diagnostics GmbH, Germany	#11836170001
His buffer for His-Trap™ HP Kit	GE Healthcare Bioscience AB.	#11003400
Hybond-N+ nylon membrane	Amersham, UK.	#RPN303B
Hybond-P+ PVDF membrane	Amersham, UK.	#RPN303F
LongLife Gel sample buffer	Life Therapeutics, NSW, Australia.	#BG-165

LongLife Gel Transfer buffer	Life Therapeutics, NSW, Australia.	#BG-168
Lumi-Light Western Blotting substrates	Roche Diagnostics GmbH, Germany	#2015200
Pre-cast Polyacrylamide gel (10%)	Life Therapeutics, NSW, Australia	# NH31-010
Minisart sterilized filter (0.2 μm)	Sartorius, Hannover, Germany	#16534060231
Sand (purified by acid)	BDH Laboratory Supplied, England	#K27041992002
TRIS SDS HEPES LongLife gel running buffer	Life Therapeutics, NSW, Australia	#BG-161
X-ray film	Photo Film Co., Ltd., Tokyo, Japan	#03E220

2.2 Media, Solutions and Buffers

2.2.1 Media

Aspergillus complete and minimal media were as described by Cove (Cove, 1966). Carbon sources were added to carbon free minimal medium at a final concentration of 1.0% (w/v) unless mentioned otherwise. Nitrogen sources were added to 1% glucose minimal medium at a final concentration of 10mM unless noted otherwise.

Standard bacterial media (L-broth, Luria Bertani Agar) were prepared as described in Sambrook *et al*., (1983). Ampicillin or chloramphenicol was added, to a final concentration of 50 μg/ml or 15 μg/ml, when required. Bacteria were grown at 37^{o}C overnight, shaken at 120 rpm.

2.2.1.1 *Aspergillus* Nitrogen-free Medium (ANM); 2% (v/v) salt solution, 1% (w/v) D-glucose, 1% or 2.2% (w/v) Bacteriological agar, pH 6.5.

2.2.1.2 Carbon-free Broth (C-free broth); 2% (v/v) salt solution, pH 6.5.

2.2.1.3 Carbon-free Medium (C-free Medium); 2% (v/v) salt solution, 1% or 2.2% (w/v) Bacteriological agar, pH 6.5 with 1M NaOH.

2.2.1.4 Complete Medium; 1% (w/v) D-glucose. 0.2% (w/v) Bacteriological peptone, 0.15% (w/v) casein hydrolysate, 0.1% (w/v) yeast extract, 0.1% (v/v) vitamin solution, 0.2% (v/v) salt solution, 25 μg/ml riboflavin, 1% or 2.2% (w/v) Oxoid class 3 agar, pH 6.5.

2.2.1.5 1% L Agar; 25 g Luria Broth Base, 10 g Bacteriological Agar, adjusted total volume to 1L.

2.2.1.6 Protoplast regeneration medium; 1M sucrose in ANM agar. Nitrogen sources and amino acid supplements are added to a final concentration of 10 mM.

2.2.2 Solutions and Buffers

All solutions were prepared using MilliQ reverse osmosis water and, where appropriate, were autoclaved. Solutions not able to be autoclaved were sterilized by filtration through a 0.2 μm sterilized membrane filter (Sartorius). Solutions and buffers routinely used in this study were as follows.

2.2.2.1 Salt solution; 26 g KCL, 26 g $MgSO_4$, 76 g KH_2PO_4, 50 ml *Aspergillus* trace element solution, 2 ml $CHCl_3$, made up to 1000 ml with MilliQ water.

2.2.2.2 Vitamin solution; 40 mg para-amino benzoic acid, 50 mg thiamine, 1mg biotin, 400 mg inositol, 100 mg nicotinic acid, 200 mg calcium D-pathothenate, 100 mg riboflavin, 50 mg pyridoxine, 2 ml $CHCl_3$, made up the total volume to 1000 ml with MilliQ water.

2.2.2.3 Trace elements solution; 40 mg Sodium borate, 400 mg copper sulphate, 1 g ferric orthophosphate, 800 mg sodium molybdate, 8 g zinc sulphate, 600 mg manganese sulphate, made up the total volume to 1000 ml with MilliQ water.

2.2.2.4 Protoplast wash solution; 1.2 M sorbitol, 10 mM Tris-HCl pH 7.5.

2.2.2.5 Supplement solutions;

Supplements	Stock	Working stock	Final concentration
Biotin	10 mg/100 ml	0.1 mg/100 ml	0.001 mg/100 ml
Para amino benzoic acid	50 mg/100 ml	5 mg/100 ml	0.05 mg/100 ml
Pyridoxin	50 mg/100 ml	5 mg/ml	0.05 mg/100 ml
Riboflavin	25 mg/100 ml	-	0.25 mg/100 ml

2.2.2.6 Blotto; 10% skim milk powder, 2 mg/ml sodium azide.

2.2.2.7 10X DNA loading dye; 0.25% bromophenol blue, 0.25% xylene cyanol FF, 25% Ficoll 400.

2.2.2.8 10X MOPS buffer; 200 mM MOPS, 50 mM CH_3COONa (anhydrous), 10 mM EDTA, adjusted to pH 7.0.

2.2.2.9 10X Oligolabelling buffer; 0.5 M Tris-HCl pH 6.9, 0.1 M $MgSO_4$, 1 mM DTT, and 0.6 mM each of dCTP, dGTP, and dTTP.

2.2.2.10 Osmotic medium; 1.2 mg $MgSO_4$ made up in 0.01 M Na_2HPO_4/NaH_2PO_4 (pH7.0), pH5.8 with 0.2 M Na_2HPO_4, adjusted total volume to 1000 ml with MilliQ water.

2.2.2.11 1X PBS; 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, pH 7.4 with HCl, adjusted total volume to 1000 ml with MilliQ water.

2.2.2.12 1X PBS-T; 1X PBS 1000 ml, 1 ml tween 20 (Sigma, P1379).

2.2.2.13 Prehybridisation (Formamide) solution; 40% (v/v) formamide, 4X SSPE, 1%(v/v) SDS, 5% (v/v) Blotto, and 100 μg sonicated salmon sperm DNA.

2.2.2.14 Prehybridisation (Phosphate) solution; 10 mM sodium orthophosphate, 5X SSC, 2% (v/v) Blotto, 0.4% (v/v) SDS, and 100 μg sonicated salmon sperm DNA.

2.2.2.15 RNA loading buffer (MOPS); 50% (v/v) formamide, 37% (v/v) formaldehyde, 1X MOPS buffer, 1X DNA loading dye, and 10 mg/ml ethidium bromide.

2.2.2.16 RNA loading buffer (phosphate); 50% (v/v) formamide, 12% (v/v) formaldehyde, 10 mM sodium orthophosphate, 1X DNA loading dye, and 10 mg/ml ethidium bromide.

2.2.2.17 1X SSC; 0.15 M NaCl, 15 M $Na_3C_6H_5O_7.2H_2O$, pH 7.2.

2.2.2.18 1X STC buffer 1.2 M Sorbitol, 10 mM Tris-HCL pH 7.5, and 10 mM $CaCl_2$.

2.2.2.18 Stop buffer; 10 mM Tris base, 1 mM EDTA, 2% (w/v) SDS.

2.2.2.19 1X TAE; 40 mM Tris base, 20 mM CH_3COONa, 2 mM EDTA, pH 7.8.

2.2.2.20 1X TE; 10 mM Tris-HCl pH 8.0, 1.0 mM EDTA.

2.2.2.21 Trapping buffer; 0.6 M sorbitol, 10 mM Tris-HCl pH 7.0.

2.2.2.22 Tris-glycine SDS-PAGE (10%, resolving gel); 30% Acrylamide 3.3ml, 10% SDS 0.1 ml, 1M Tris pH 8.8 3.75 ml, 10% $(NH_4)_2S_2O_8$ 0.1 ml, TEMED 0.004 ml, adjusted volume to 10ml with MilliQ water.

2.2.2.23 Tris-glycine SDS-PAGE (5%, stacking gels); 30%(v/v) Acrylamide 0.5 ml, 1 M Tris pH 6.8, 0.38 ml, 10% (v/v) SDS 0.03 ml, 10% (v/v) $(NH_4)_2S_2O_8$ 0.03 ml, TEMED 0.003 ml, adjusted volume to 3 ml with MilliQ water.

2.3 Methods

Standard molecular techniques were performed as outlined in Sambrook *et al.* (1989). Growth testing and genetic manipulations were carried out using techniques described by Cove (1966) and Clutterbuck (Clutterbuck, 1993). Transformation experiments were performed using a modification to the method of Tilburn *et al.* (1983), typically by adding 100 mg Lysing Enzymes (Sigma Chemical) to digest the fungal cell wall. Transformants from co-transformation experiments were selected using the *riboB*$^+$ selectable marker plasmid pPL3 (Oakley *et al.*, 1987) on media lacking riboflavin, or using the *pabaA*$^+$ selectable marker plasmid pPaba (Supplied by Hynes, MJ.) on media lacking para-amino benzoic acid.

2.3.1 Plasmid amplification

E. coli strain DH5-α was made competent by the $CaCl_2$ method (Sambrook *et al.*, 1989) for transformation. 1-5 ng of plasmid DNA was added to 100 µl of competent cells, which were incubated on ice for at least 10 mins, heat shocked at 42 ^oC for 50 secs, and rapidly transferred to ice for 2 mins, when 500 µl of L-broth was added. After incubation for 30 mins at 37 ^oC, *E. coli* cultures were plated directly onto L-agar with ampicillin antibiotic. Plates were incubated at 37 ^oC overnight when the colonies were screened for the presence of recombinant plasmids. The plasmid DNA was isolated

from *E. coli* using the Wizard® *Plus* SV Miniprep DNA purification system Kit (Qiagen), according the manufacturer's instructions.

2.3.2 Conditions for producing *A. nidulans* mycelia in liquid culture

Conidia were produced by inoculating spores to 2.2% complete medium and incubation for 2-3 days at 37 ^{o}C. The conidia were scraped, vortexed in 0.01% sterile tween 20, and added to 800 ml flasks of supplemented liquid media for protoplast preparation, and 200 ml flasks of supplemented liquid media for DNA or RNA preparation and protein extraction. The cultures were incubated overnight at 37 ^{o}C, shaken at 120-150 rpm.

2.3.3 Preparation of *A. nidulans* protoplasts

Mycelium from an 800 ml overnight culture of the desired strain was harvested through sterile muslin and washed with cold 0.6 M $MgSO_4$ and pressed dry with sterile paper toweling. The weight of the semi-dried mycelium was recorded. The mycelium was resuspended in osmotic medium (5 ml per gram of mycelium) in a 150 ml conical flask and Lysing Enzyme (Sigma) was added to a concentration of 100 mg per gram of mycelia. The flask was left on ice for 5 mins., when 250 µl/g of a 12 mg/ml BSA solution was added. The flask was incubated, shaking, at 37 ^{o}C until the surface of solution become milky, due to released protoplasts, after approximately 2 hrs. The contents of the flask were transferred to a 30 ml corex tube, overlayed with an equal volume of trapping buffer, and centrifuged at 3700 rpm for 10 minutes in a pre-cooled swing-out centrifuge (Beckmen TJ-6). The band of protoplasts which formed at the interface of the solutions was recovered using a sterile bent-tip pipette and transferred to a 15 ml corex tube, and an equal volume of protoplast wash was added. The protoplasts were centrifuged at 3700 rpm for 10 minutes in a pre-cooled swing-out centrifuge (Beckmen TJ-6), and the pellet was resuspended in 100 µl of 1X STC and kept on ice.

2.3.4 Transformation of *A. nidulans* protoplasts (Tilburn *et al.*, 1983).

For co-transformation, 5 µg of each plasmid (target and marker plasmids) was added to an aliquot of protoplasts (50-100 µl) in a 1.5 ml eppendorf tube. An aliquot of protoplasts to which no DNA had been added was used as a control. 25 µl of 60% PEG (polyethylene glycol)/$CaCl_2$ was added and mixed by inversion before incubation on ice for 20 mins. 1 ml of 60% PEG/$CaCl_2$ was added and roll mixed, and the tube was

left at room temperature for 5 mins., before plating onto the protoplast medium. Regeneration plates of control protoplasts were made by diluting to 10^{-2}, 10^{-3}, and 10^{-4} in both distilled water (MilliQ water) and 1X STC before plating onto the regeneration medium. The regenerated transformants were isolated and streaked on the appropriate solid media until a purified strain was obtained.

2.3.5 Meiotic cross in *A. nidulans*

The two *A. nidulans* strains to be crossed were inoculated 5mm apart on 1% complete medium and incubated for 2 days at 37 ^{o}C. Small pieces of the agar were taken from the intersection zone where mycelia of the 2 strains had grown together and placed onto selective media to allow heterokaryon formation. After 2 days, the plates were taped and incubated for a further 7 days for cleistothecium formation. The cleistothecia were cleaned by rolling on a 3% sterile agar plate, and broken in 200 µl sterile water in an eppendorf tube. A 10 µl aliquot from each cleistothecia were placed on complete medium to test whether the cleistothecia was formed by a cross or self. 100 µl of each crossed sample was vortexed in 1 ml sterile water and then plated out onto complete medium. Master plates were produced, and replica plated onto a variety of screening media to establish their genotype.

2.3.6 Diploid formation in *A. nidulans*

The two haploid strains were inoculated 5 mm apart on 1% complete medium and incubated for 2 days at 37 ^{o}C. Mycelia were transferred from the intersecting zone and placed onto selective media to allow heterokaryon formation. Strong regions of heterokaryon growth were transferred to fresh selective plates until a diploid strain formed.

2.3.7 Nucleic acids (DNA and RNA) isolation

A. nidulans mycelia for genomic DNA isolation was grown overnight in appropriate medium. Growth conditions were as in section 2.3.2. DNA was isolated using the DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was isolated from *A. nidulans* mycelia grown in specified conditions, using the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

2.3.8 Polymerase Chain Reaction (PCR)

The design of primers and the calculation of optimum annealing temperatures were performed using the OLIGO 4.01 program (National Biosciences Inc.). In general, PCR reactions were 100 ng of double-strained DNA and 100 ng of each specific oligonucleotide primer in a final volume of 50 µl (Innis and Gelfand, 1990). DNA was typically amplified in a programmable Mastercycle Gradient Engine (Eppendorf) using an initial denaturation at 95 ^{o}C for 2 mins., followed by 25-35 cycles of denaturation at 95 ^{o}C for 1 min., a 30 sec. annealing step, and extension at 72 ^{o}C for 1 min. The annealing temperature was modified to suit each specific template/primers set and ranged between 50 ^{o}C and 65 ^{o}C. The extension time also varied depending on the size of the fragment to be amplified, allowing a 1 kb/min. synthesis rate. When necessary, the PCR products were purified with the QIAquick® PCR Purification Kit (Qiagen) following the manufacturer's instructions.

2.3.9 DNA sequencing

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) prior to direct sequencing. The plasmid DNA was prepared using the SV Wizard^{+} SV Mini Kit. For some experiments, DNA fragments were cloned in the pBluescrip SK(+) (Stratagene, CA.) or pGEM-T easy (Promega), and the plasmid DNA required no further purification before sequencing. Sequencing reactions were performed using a Big Dye Terminator Kit (Applied Biosystems-Perkin Elmer) as outlined in the manufacturer's instructions. The reaction mixture contained 8µl template DNA, 1µl oligomer, and 6µl Big Dye sequencing mix. The PCR reaction was performed for 25 cycles at 95 ^{o}C for 30 secs, 50 ^{o}C for 20 secs, and 60 ^{o}C for 3 mins. The products were resolved on an ABI Prism Model 377 automated sequencing machine at the Institute of Medical and Veterinary Science (IMVS) sequencing facility, Adelaide, SA.

2.3.10 DNA restriction endonuclease digestion

DNA was incubated with the appropriate restriction enzyme (s) and buffer, with MilliQ water making up the required volume, according to the manufacturer's instructions (Boehringer-Mannheim, and Promega).

2.3.11 DNA Gel purification

DNA fragments for cloning were recovered and purified from agarose gel using the QIAquick® Gel extraction Kit (Qiagen), following the manufacturer's instructions.

2.3.12 Radioactive labelling of probes

Generally, DNA probes were radioactively labeled using the random oilgonucleotide primer method described by Hodgson and Fisk with [α-^{32}P]dATP (Hodgson and Fisk, 1987). Radiolabelling by PCR was also performed, when indicated in the experiments, using the PCR product as the template in the standard PCR protocol, except that the dNTPs were replaced by a mixture of dCTP, dGTP, dTTP, and [α-^{32}P]ATP, and with a typical reaction cycle at 10 cycles. Column purification (Bio-Gel P-60 Gel) was used to determine the efficiency of the labeling reaction.

2.3.13 DNA gel electrophoresis and Southern transfer

Southern blotting was performed using the alkali transfer method described in Sambrook *et al*. (1989), based on the technique devised by Southern (Southern, 1975). DNA was separated by 1% agarose gel electrophoresis in 1X TAE buffer, depurinated by treatment with 0.25 M HCl for 10 minutes and transferred to Hybond N+ nylon membrane (Amersham) overnight in 0.4 M NaOH. The filters were prehybridised with 10 ml prehybridisation (phosphate) solution for 2 hrs., rotated at 15 rpm at 65 ^{o}C. The denatured labeled DNA probe was added directly to the prehybridisation mix and incubated for a further 16 to 24 hours. Filters were washed at room temperature with 2X SSC, 0.1% SDS for 20 mins., 0.5X SSC, 0.1% SDS for 20 mins., and 0.1% SSC, 0.1% SDS for another 20 mins., and then exposed to X-ray film or to phosphor-imager plate. Long exposure, in a case of low sensitivity, was acchieved by keeping the cassette at -80 ^{0}C for 1 week.

2.3.14 RNA gel electrophoresis and Northern transfer

Two methods of RNA gel electrophoresis were used in this research. Using the "MOPS" method, 1 μg – 5 μg of total RNA was electrophoresed through 1.2% agarose-0.6M formaldehyde gel in 1X MOPS running buffer. RNA samples were added to RNA loading buffer (MOPS) and heated to 68 ^{o}C for 15 mins. prior to loading. Alternatively, using the "phosphate" method, 1 μg – 5 μg of total RNA was electrophoresed in 1.5% agarose- 0.6 M formaldehyde-10mM sodium orthophosphate gels in 10 mM sodium orthophosphate running buffer. RNA samples were added to RNA loading buffer (phosphate) and heated to 68 ^{o}C for 15 mins prior loading to the 1.2% agarose - 0.6 M formaldehyde gel.

RNA was transferred to Hybond N+ nylon membrane (Amersham) by alkaline transfer in 0.04 N NaOH for 3-4 hrs. Denatured radioactively labeled probe was added directly to the prehybridisation mix and incubated overnight. Filters were washed with 2X SSC, 0.1% SDS for 20 mins., and then 0.5X SSC, 0.1% SDS for 20 mins., before autoradiography. An additional 20 mins washing with 0.1X SSC, 0.1% SDS was required if the radioactive signal was indicative of a high non-specific background. The hybridizations were performed at 65 ^{o}C.

2.3.15 Protein analysis

2.3.15.1 Total protein extracts from *A. nidulans*

Mycelia were harvested from 200 ml cultures grown overnight at 37 ^{o}C (Growth conditions as in section 2.3.2). The mycelia were pressed dry between sheets of paper towel and weighed to determine the amount of buffer to add to the ground mycelium in non-denaturing lysis buffer supplied by the manufacturer (GE Healthcare Bioscience AB or Roche Diagnostics GmbH). Complete proteinase cocktail inhibitor (Roche) was added to the buffer at the recommended concentration from manufacture's instructions. The mycelia were ground in pre-cooled mortar and pestles, with added sand (BDH Laboratory Supplied). The ground mixtures were transferred to a microcentrifuge tube and spun at 12000 rpm for 2 mins. The supernatant was carefully removed to a new pre-cooled microcentrifuge tube. The protein sample was diluted in an appropriate buffer (PBS or extraction buffer) to the required concentration before use.

2.3.15.2 Immunoprecipitaion and Western analysis.

Protein purification and Co-immunopreciptations (Co-IP) were performed according to the following protocols;

- CreBFLAG was precipitated from the total protein extract using anti-FLAG monoclonal antibodies conjugated with agarose following the manufacturer's instructions (Sigma).

- CreA2HAHis was purified from the total protein extract using His-Trap™ HP column (GE Healthcare) and anti-HA affinity matrix (Roche) following the manufacturer's instructions.

- QutDHA was purified from the protein extract using anti-HA (high affinity) matrix following the manufacturer's instructions (Roche).

The fractions were separated by SDS- PAGE gel electrophoresis, and transferred to a polyvinyl diflouride (PVDF) membrane. Western blots were performed according to the following protocols;

- Protein transfer by the Hybond-P+ PVDF membrane following the manufacturer's instruction manual for immunodetections (Amersham).

- Anti-FLAG was detected using the anti-FLAG::M2® Monoclonal Anitibody-Peroxidase Conjugate following the manufacturer's instructions (Sigma).

- Anti-HA was detected using the anti-HA-Peroxidase High Affinity (3F10) Rat Monoclonal Antibody following the manufacturer's instructions (Roche).

- Anti-Myc was detected using the anti-[C-myc]-peroxidase (3E10) monoclonal antibody following the manufacturer's instructions (Roche).

- Western blotting detection was performed using the Lumi-Light Western Blotting substrate following the manufacturer's instructions (Roche Diagnostics).

2.3.15.3 Ubiquitinylated protein enrichment and Immunoprecipitation.

Total protein extract was obtained from fungal mycelia by grinding in a pre-cooled mortar in HA-lysis buffer (Roche Diagnostics GmbH) with added protease inhibitor. The ubiquitinylated proteins were immunoprecipitated with the UbiQapture™-Q Kit (MolBiol), and the immunoprecipitated proteins were detected by the ubiquitin-conjugate specific primary antibody and the HRP-Donkey anti-mouse secondary antibody, according to the manufacturer's instructions (Rockland Immunochemical).

2.4 Bioinformatic tools

2.4.1 *Aspergillus nidulans* online database

The *A. nidulans* online database at the Broad Institute was used to extract DNA sequences: http://www.broad.mit.edu/annotation/fungi/ aspergillus/index.html.

2.4.2 DNA sequence analysis

DNA sequence data obtained from automated sequencing reactions were visualized using the Chromas V2.23 (Technelysium) program. Sequence analyses were performed using programs accessed via the Australian National Genomic Information Service (ANGIS;http://www1.angis.org.au/pbin/WebANGIS/wrapper.pl) facility, Biomanager (http://biomanager.angis.org.au), and NEBcutter Version 2 (http://tools.neb.com/NEBcutter2/index.php). Database searches were performed via the National Center for

Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). The Primer3 program was sourced from http://biotoold.umassmed.edu/bioapps/primer3 www.cgi.

2.4.3 Protein analysis

Proteomics analysis was performed at the Adelaide Proteomics Centre, SA. Purified fractions and protein extracts from fungal mycelia were separated by SDS-PAGE electrophoresis. The gel pieces were destained using 15 mM potassium ferricyanide and 50 mM sodium thiosulphate, alkylated with iodoacetamide and digested with trypsin. Protein samples were dired and reconstituted in 1% formic acid before chromatohraphy using Agilant Protein ID liquid chromatography-ESI mass spectrometry (MS&MS/MS) (Bruker Daltonik GMbH). MS and MS/MS spectra were subjected to peak detection using DataAnalysis (Version 2.4, Bruker Daltonik GmbH), and submitted to Mascot database-searching engine (Matrix Science: http://www.matrixscience.com) with the following specification;

Taxonomy:	fungi
Database:	MSDB20060831
Enzyme:	Trypsin
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)
Mass tol MS:	0.3 Da
MS/MS tol:	0.6 Da
Peptide charge:	1+, 2+ and 3+
Missed cleavages:	1

CHAPTER 3: STUDY OF CREA, A CANDIDATE PROTEIN TARGET FOR THE CREB DEUBIQUITINATION PROTEIN

3.1 Introduction

CreA is a DNA-binding protein of the Cys_2-His_2 class, and it is key regulatory protein involved in CCR (Dowzer and Kelly, 1989, 1991; Hynes and Kelly, 1977). The *creB* gene encodes a functional deubiquitinating enzyme (Lockington and Kelly, 2001). The *creC* gene encodes a regulatory protein composed of a proline-rich region, a nuclear localization motif and WD40 motif repeats (Todd *et al.*, 2000). CreB and CreC are present in a complex *in vivo* as determined by co-immunoprecipitation (Lockington and Kelly, 2002), and it had been proposed that the CreB/CreC deubiquitination complex is involved in regulating the stability of ubiquitinated protein targets, such as CreA (reviewed in Kelly, 2004). Mutation in *creB* or *creC* show loss of CCR, and these mutants also grow poorly on some substrates such as quinate and proline (Lockington and Kelly, 2001). Suppressors of *creB* and *creC* mutant phenotypes revealed genes involved in ubiquitination in *A. nidulans*, including *creD*, indicating that CreB and CreC form part of a regulatory ubiquitination/deubiquitination network (Boase and Kelly, 2004).

The targets of the CreB deubiquitination enzyme have not been identified. This chapter reports experiments to test whether CreA is a deubiquitinatied protein, a target for the CreB deubiquitinating enzyme complex. Co-immunoprecipitation using specific antibodies against either epitope tagged CreA or CreB were used in this approach. This approach had previously been used to identify targets of other deubiquitination enzymes, such as the Fat facets/Liquid facets in eye development of *Drosophila* (Cadavid *et al.*, 2000) and Dao4 in yeast (Amerik and Hochstrasser, 2004; Richter *et al.*, 2007).

3.2 Epitope tagged version of CreA and CreB constructs.

Since there are no antibodies to CreA or CreB, plasmids expressing epitope tagged versions of CreA and CreB proteins were constructed in the laboratory by R.A. Lockington (unpublished data; Lockington and Kelly, 2002). The *creA* gene was ligated with sequences encoding Myc at the amino (N)-terminus to create pGPDMycCreA, or ligated with sequences encoding HA near the carboxy (C)-terminus

and sequences encoding GFP at the C-terminal of CreA to create pGPDCreAHAGFP. *creB* was tagged with sequences encoding FLAG near to the C-terminus to create pGPDCreBFLAG. The pGPDCreA2HAHis is containing the *creA* gene ligated with 2 sequences encoding HA in the non-esential region of *creA*, and the sequence encoding Histidine at the C-terminus (Figure 3.1). MycCreA, CreAHAGFP, CreA2HAHis and CreBFLAG were expressed from the *A. nidulans gpdA* promoter. *A. nidulans* strains containing each of these plasmids were already constructed and available in our laboratory. The epitope tagged proteins were in all cases capable of wild-type levels of complementation of mutant strains and thus were fully functional.

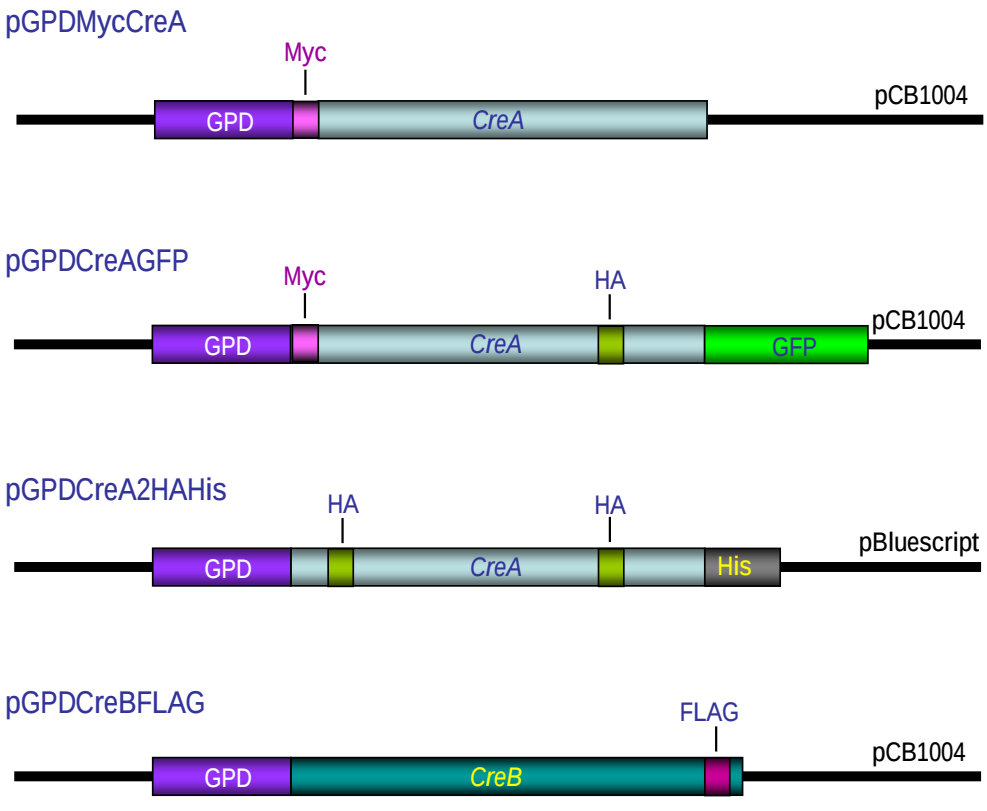


Figure 3.1 Diagram showing epitope tagged versions of *creA* and *creB* constructs. These plasmids express from the *A. nidulans gpdA* promoter.

3.2.1 Co-immunoprecipitation experiment using a strain containing pMycCreA and pCreBFLAG.

In order to determine whether CreA and CreB form a complex *in vivo*, strains containing only MycCreA [TA1; *creA*Δ4::pGPDMycCreA], CreBFLAG [T3; *creB1937*::pGPDCreBFLAG], and a strain containing both MycCreA and CreBFLAG [T3.5; pGPDMycCreA, pGPDCreBFLAG] were grown in medium containing glucose as the sole carbon source, at 37 ^{o}C for 18 hrs and shaking at 120 rpm. The mycelia were harvested and protein extracted by the method described in Materials and Methods. CreBFLAG was precipitated from the total protein extracts using anti-FLAG monoclonal antibody conjugated to agarose (anti-FLAG::M2-agarose affinity gel, Sigma). In each case, the total protein extract, supernatant and immunoprecipitation fractions were separated with SDS-PAGE, and transferred to PVDF membrane. Western analysis was performed using anti-Myc monoclonal antibody conjugated with peroxidase to detect CreA, and the filters were further probed with anti-FLAG monoclonal antibody conjugated with peroxidase to detect CreB.

Figure 3.2A shows that the antibody to detect the Myc-epitope tagged CreA did not detect any protein at the appropriate size (~47 kDa) from the TA1 control strain (containing MycCreA). The anti-Myc antibody showed cross reaction with *A. nidulans* proteins at a lower molecular weight, but these were not MycCreA as they were present in the T3 (containing CreBFLAG) strain as well as the TA1 strain. The filter was stripped and probed with the anti-FLAG antibody to detect CreBFLAG, and a band of appropriate size (~86 kDa) was present in the immunoprecipitated fractions of T3 and T3.5 (Figure 3.2B). The anti-FLAG antibody also showed cross reaction with others lower molecular weight proteins which were precipitated with anti-FLAG::M2-agarose affinity gel. CreBFLAG was detected more strongly in the immunoprecipitate of T3.5 than T3 because of the difference in protein concentration in the extracts.

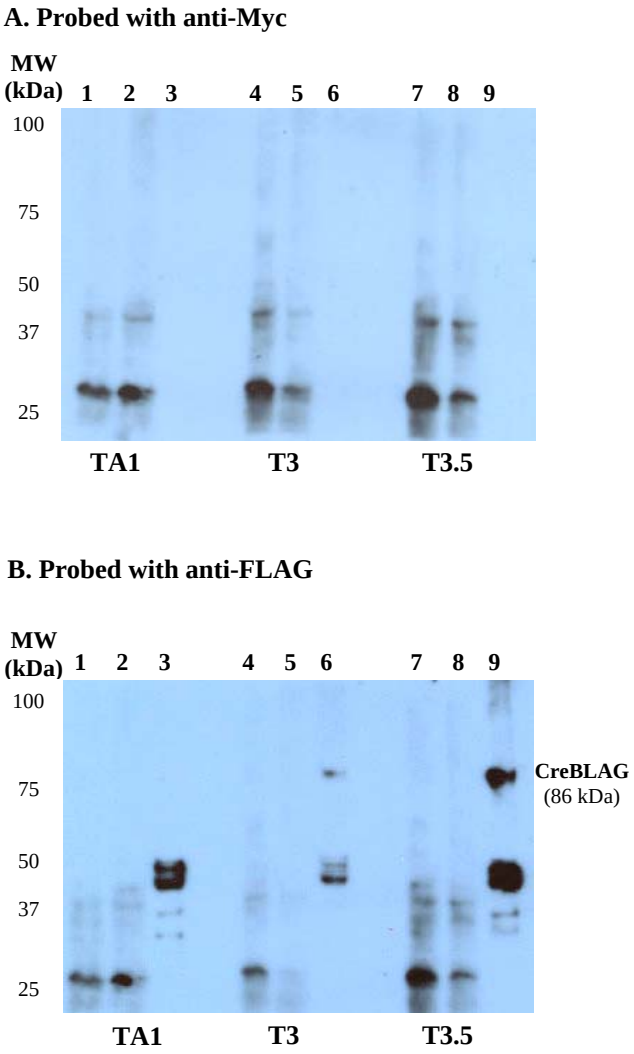


Figure 3.2 Immunoprecipitation using anti-FLAG antibody. Strains TA1 (MycCreA), T3 (CreBFLAG) and T3.5 (MycCreA; CreBFLAG) were grown in medium containing glucose as the sole carbon source. Mycelium was harvested and protein extracted. Proteins were purified using anti-FLAG::M2-agarose. A) The Western probed with anti-Myc monoclonal antibody to detect Myc tagged CreA. B) The Western probed with anti-FLAG::peroxidase-linked monoclonal antibody to detect the FLAG tagged CreB. Tracks 1, 4 and 7 contain approximately equal samples of protein lysate; tracks 2, 5 and 8 contain supernatant from the IP; tracks 3, 6 and 9 contain the immunoprecipitate.

The Myc tagged version of CreA was not detected using an antibody directed against the Myc epitope tag in strain TA1. Thus this epitope tagged version of CreA could not be used to detect whether CreA had been co-precipitated with CreB in the strain containing both MycCreA and CreBFLAG. Lockington R.A. (unpublished data) subsequently made constructs with a GFP (Green Fluorescent Protein) tag at the N-terminal end of CreA followed by an HA tag, and found that the N-terminal GFPHA tag was cleaved from the fusion protein and could be detected by Western analysis with antibodies directed against the HA epitope tag. Thus, N-terminal tags are removed from CreA and cannot be used to detect CreA. Therefore, a new construct was made in which a heamaglutinin (HA) epitope tag was inserted into a non essential region of the CreA peptide (Table 2.2 in Materials and Methods, and Figure 3.1). The *creAHA* plasmid was introduced into a *creA* mutant strain and complemented the mutant phenotype. Diploid strains which contained both the CreA and CreB epitope tagged proteins were produced in order to determine the interaction of these proteins.

3.2.2 Construction the CreAHA and CreBFLAG containing strain.

Two strains were available that contained an integrated copy of pGPDCreAHAGFP (TA6 and TA7), and three strains were available that contained an integrated copy of pCreBFLAG in a *creA* deletion background (T2, T3.6 and T4.15). Six diploids were created (TA6/T2; TA6/T3.6; TA6/T4.15; TA7/T2; TA7/T3.6; TA7/T4.15). These diploids were grown in glucose medium, and proteins were extracted to determine whether CreAHAGFP and CreBFLAG could be detected by Western analysis using anti-HA and anti-FLAG antibodies, respectively. The diploid between T2 [*creAΔ99;creB1937*::pGPDCreBFlAG] and TA7 [*creAΔ4*::pGPDCreAHAGFP] showed expression of CreBFLAG and CreAHA that was readily detected in Western analysis.

3.2.3 CreAHA and CreBFLAG were precipitated with anti-FLAG antibody.

Strains containing tagged constructs of both CreAHA and CreBFLAG expressed from the *gpdA* promoter, and CreAHA or CreBFLAG alone, were used in co-immunoprecipitation experiments with anti-FLAG monoclonal antibody to test whether CreA and CreB are present in a complex *in vivo*. The strains were grown overnight in medium containing glucose as a sole carbon source and ammonium tartrate as a nitrogen source.

The fungal mycelia were harvested and protein extracted as described in Materials and Methods. CreBFLAG was precipitated from the total protein extracts using the anti-FLAG monoclonal antibody conjugated with agarose (FLAG tagged Protein Precipitation Kit; Sigma). Samples of the total protein extract, supernatant and immunoprecipitate fractions were separated with SDS-PAGE, and transferred to PVDF membrane. The membrane was incubated with an anti-HA monoclonal antibody conjugated to peroxidase (Roche), and detection of CreAHA was made using Lumi Light Western Blotting reagents (Roche). The filter was further probed using anti-FLAG monoclonal antibody conjugated with peroxidase for CreBFLAG detection (Figure 3.3).

Antibodies against the FLAG epitope detected CreBFLAG at about 86 kDa in the immunoprecipitation of the T2 strain and the diploid of T2/TA7 as expected, but not in the TA7 strain (Figure 3.3A). This showed that the antibody was specific for CreBFLAG. CreBFLAG could not be detected in the total cell lysate of T2 and diploid strains presumably because the level of CreBFLAG was too low in concentration in these fractions when compared to the purified fraction in the immunoprecipitate. Antibodies against the HA epitope detected CreAHAGFP at about 70 kDa in the cell lysis extract of TA7 and the diploid of TA7/T2, but not in the T2 strain as expected (Figure 3.3B), indicating that this antibody was specific to the HA epitope. Additionally, the 48 kDa fragment was detected in the immunoprecipitate of both TA7 and the diploid strains. Possibly the GFP tag was cleaved from the fusion protein, leaving the CreAHA fragment. This would have a molecular weight of about 48 kDa, consistent with what is seen in the Western. Anti-HA antibodies detected CreAHAGFP in the diploid after immunoprecipitation with anti-FLAG antibody, but CreAHAGFP was also detected in the immunoprecipitate fraction in the TA7 strain, which did not contain a FLAG tagged protein. This suggested that CreAHAGFP was binding nonspecifically to the anti-FLAG agarose.

Thus, a single purification step using the anti-FLAG::M2 agarose was not sufficient to remove the CreAHA in a strain that does not contain a FLAG-tagged protein, and a more complex procedure was required.

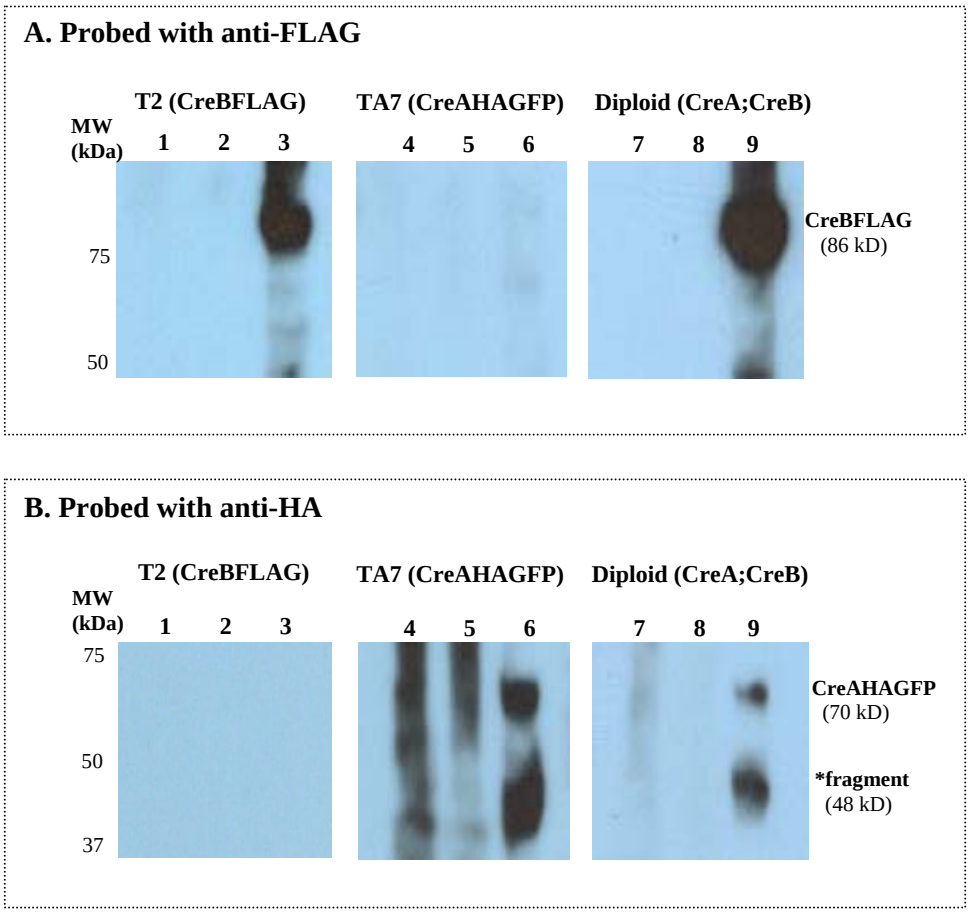


Figure 3.3 Co-immunoprecipitation of CreAHA and CreBFLAG. Strains T2 (CreBFLAG), TA7 (CreAHAGFP) and diploid (CreAHAGFP; CreBFLAG) were grown in medium containing glucose as the sole carbon source. Mycelium was harvested and protein extracted. Proteins were purified using anti-FLAG::M2 agarose. Western transfers probed with anti-HA high affinity monoclonal antibody detecting HA tagged CreA protein are shown in panel B. The same filter was stripped and probed with anti-FLAG monoclonal antibody detecting FLAG tagged CreB, shown in panel A. Tracks 1, 4 and 7 contain samples of protein lysate; tracks 2, 5 and 8 contain supernatant from the immunoprecipitates; tracks 3, 6 and 9 contain the immunoprecipitate. (* fragment of CreAHAGFP at about 48 kDa).

3.2.4 Construction of the CreA2HAHis and CreBFLAG containing strain.

The co-immunoprecipitaiton experiment using CreAHAGFP indicated that CreAHAGFP was not completely removed in the control TA7 strain when the protein extract was immunoprecipitated with anti FLAG::M2 agarose. This indicated that a one step purification was not sufficient, and thus a strain was constructed to allow a double purification of CreA. A strain containing a CreA construct with a C-terminal Histidine tag as well as the HA epitope tag was available. This allowed a double purification of CreA using a His-Trap HP column (GE Healthcare) for the first purification step, and a anti-HA affinity matrix (Roche) for the second purification of step. A diploid was created between TA3H2 [*creAΔ4*::pGPDCreA2HAHis] and T3.6 [*creAΔ99;creB1937*:: CreBFLAG) haploid strains. TA3H2, T3.6 and the diploid (TA3H2/T3.6; pGPDCreA2HAHis, pGPDCreBFLAG) strains were grown in the appropriate liquid medium and the proteins were extracted and analysed.

3.2.5 CreA2HAHis and CreBFLAG were precipitated with a His-Trap column and anti-HA matrix.

Strains containing overexpressing tagged constructs of both CreA2HAHis and CreBFLAG, and CreA2HAHis or CreBFLAG alone, were used in co-immunoprecipitation experiments to test whether there is an interaction between CreA and CreB *in vivo*. The purified diploid, TA3H2/T3.6, was grown overnight and processed as described previously. The fungal mycelia were harvested and protein was extracted as described in Materials and Methods. CreA2HAHis was precipitated from the total protein extracts using a His-Trap column. The eluate from the His-Trap column was subjected to a further anti-HA matrix purification step. Samples of the total protein extract (L), Flow through fraction of the His-Trap column (FT), Washed fraction of the His-Trap column (WF), eluted fraction of the His-Trap column (EF_{His}), supernatant of anti-HA matrix (S_{HA}), washed fraction of anti-HA matrix (WF_{HA}) and immunoprecipitate fractions of anti-HA matrix (EF_{HA}) were separated with SDS-PAGE, and transferred to PVDF membrane. The membrane was incubated with an anti-FLAG monoclonal antibody conjugated to peroxidase (Roche) to detect CreBFLAG, and detection was made using Lumi Light Western Blotting (Roche). The filter was further incubated with anti-HA high affinity monoclonal antibody conjugated with peroxidase for CreAHA detection.

Figure 3.4 shows the results of the experiment to determine whether CreB remained after a two-step purification of CreA. The T3.6 control strain showed that when CreA was purified using a His-Trap column, some CreBFLAG was also present, but this was not present after further purification using anti-HA matrix (Figure 3.4A). The TA3H2 control strain showed that the anti-FLAG antibody did not detect any signal when no FLAG tagged protein was present in the cell (Figure 3.4B). In the diploid strain constructed from TA3H2 and T3.6, CreBFLAG was present in the precipitate after two rounds of precipitation (Figure 3.4C), although some CreB was lost in the second purification step (as seen in the S_{HA} fraction). The same filter was also probed with antibodies directed against the HA epitope tag (Figure 3.5). The T3.6 control strain showed that the anti-HA antibody was specific, and did not detect any proteins in this strain (Figure 3.5A; the bands seen are from the previous detection using anti-FLAG). The TA3H2 control strain showed that the purification of CreA2HAHis was very efficient, with high levels of detection of a protein of expected size (~50 kDa; Figure 3.5B). In the diploid strain there was a high level of CreA2HAHis in the precipitate after two rounds of precipitation using a His-Trap column and anti-HA matrix (Figure 3.5C). These experiments show that CreB is able to be co-immunoprecipitated with CreA, and thus, at least some CreB and CreA exist in a complex *in vivo* in *A. nidulans*. It was not determined whether the immunoprecipitate of CreA2HAHis using an anti-HA matrix was unbiquitinated-CreA because the specific antibody to the ubiquitinated protein was not available at that time.

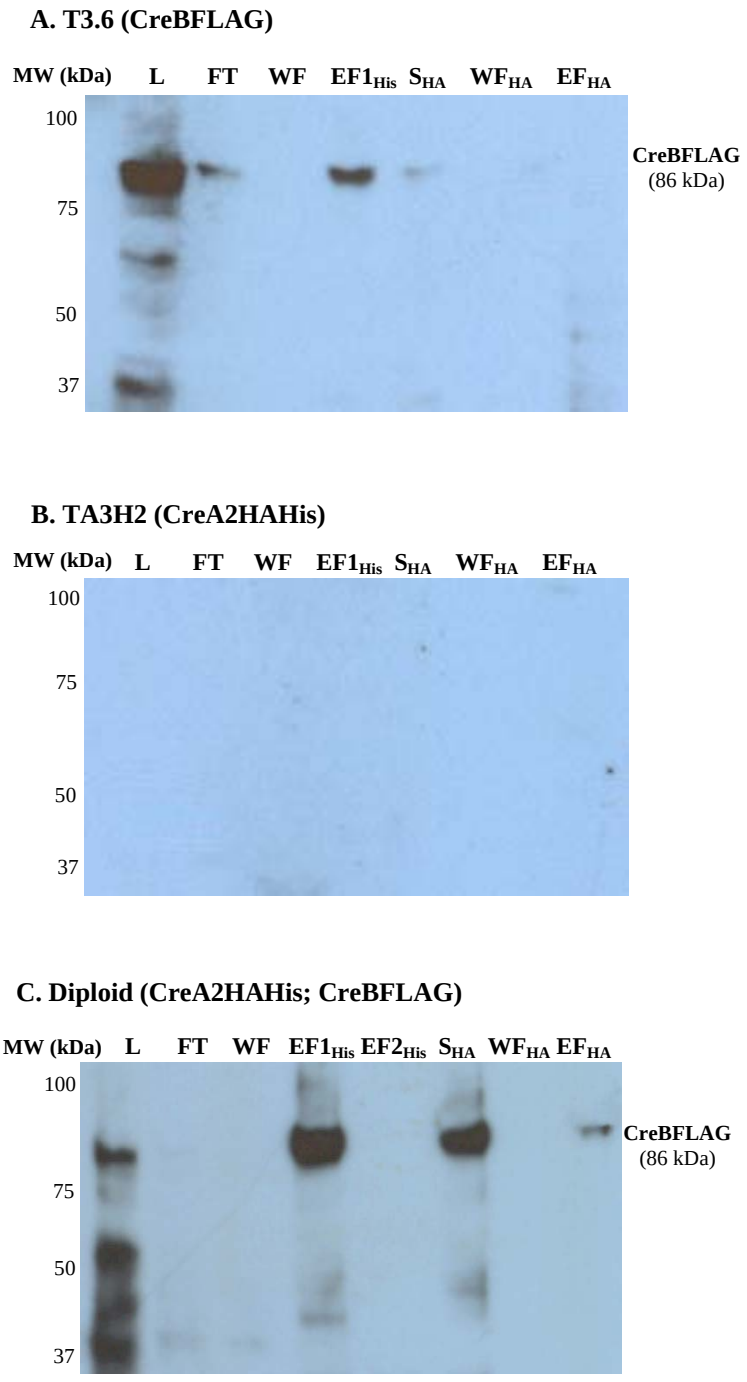


Figure 3.4 Immunoprecipitation of CreAHA probed with anti-FLAG antibody. In each cast: L, cell lysate; FT, flow though fraction; WF, wash fraction; $EF1_{His}$ and $EF2_{His}$, eluted fractions from His-Trap column; S_{HA}, supernatant of HA-matrix; WF_{HA}, wash fraction of HA-matrix; EF_{HA}, eluted fraction of HA-matrix.

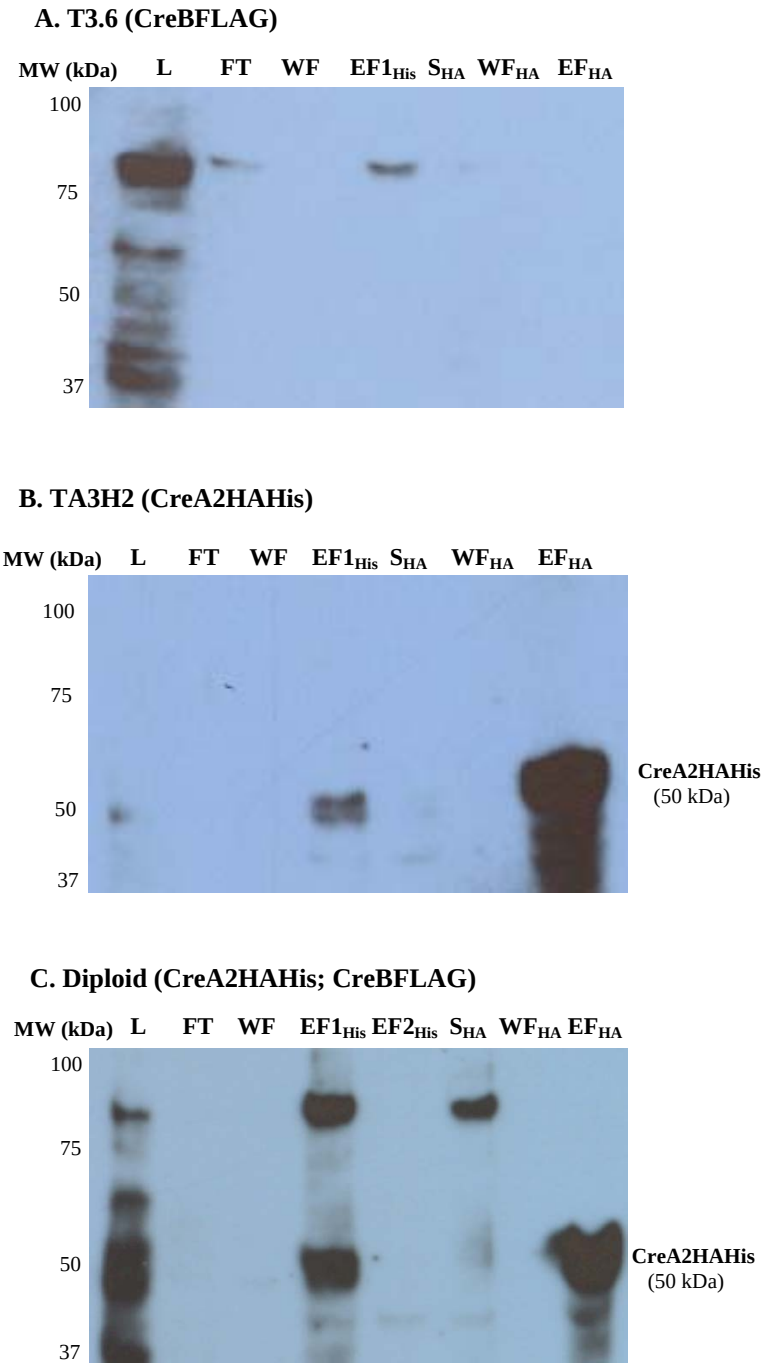


Figure 3.5 Immunoprecipitation of CreAHA when probed with anti-HA antibody, after probing with anti-FLAG antibody (in Figure 3.4). In each cast: L, cell lysate; FT, flow though fraction; WF, wash fraction; $EF1_{His}$ and $EF2_{His}$, eluted fractions from His-Trap column; S_{HA}, supernatant of HA-matrix; WF_{HA}, wash fraction of HA-matrix; EF_{HA}, eluted fraction of HA-matrix.

3.3 CreA is a ubiquitinated protein.

CreB is a deubiquitinating enzyme, and the co-immunoprecipitation results from the previous experiments to detect CreA and CreB show that some CreA was bound to CreB in the cell. Thus, it is possible that CreA might be a ubiquitinated protein in the cell and a target of the CreB deubiquitinating enzyme *in vivo*. In order to determine whether CreA is ubiquitinated *in vivo,* a protein extract from the tagged CreAHAHis (TA3H2) strain was separated using a UbiQapture-Q matrix (BIOMOL) to purify the ubiquitinated proteins in the cell. The cultures of TA3H2 and wild-type strains were grown, shaking at 120 rpm. for 18 hrs at 37 ^{o}C, in medium containing glucose as a sole carbon source. Fungal mycelia were recovered and proteins extracted as described in Materials and Methods. Ubiquitinated proteins were separated from the total protein extracted using the UbiQapture-Q kit (BIOMOL). Three fraction samples (total extract, unbound fraction and immunoprecipitate) of either TA3H2 or wild-type; *biA1niiA4* (Pateman *et al.*, 1967) were separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. Figure 3.6 shows the results of a Western blot where the three fractions were probed with anti-HA antibody. The Western results indicated that CreAHA was precipitated using UbiQapture-Q matrix.

Anti-HA antibody detected a very strong band in the total protein lysate and unbound fractions (track 4 and 5) of the TA3H2 strain, and a weaker band in the immonoprecipitate of TA3H2 (Figure 3.6A). As there was no signal in the wild-type tracks, this indicated that this was CreA2HAHis. Thus some CreA protein in the cell was ubiquitinated and trapped with the matrix of the UbiQapture-Q kit. In the immunoprecipitaited fraction there are clearly two bands detected with the anti-HA antibody. This may be due to populations of CreA with different numbers of ubiquitin moieties. Thus a proportion of CreA2HAHis was precipitated from the protein extract when using the UbiQapture-Q matrix. The membrane was also probed with an Ubiquitin-conjugate specific antibody. Ubiquitin was detected mainly in the total protein extracts ranging from high molecular weight to low molecular weight, and small amounts were found in the unbound and immunoprecipitate fractions (Figure 3.6B). This result shows that only a very small proportion of ubiquitinated proteins, when compared to the total protein in the lysate, were precipitated using the UbiQapture-Q matrix. Thus a proportion of CreA is ubiquitinated in the cell.

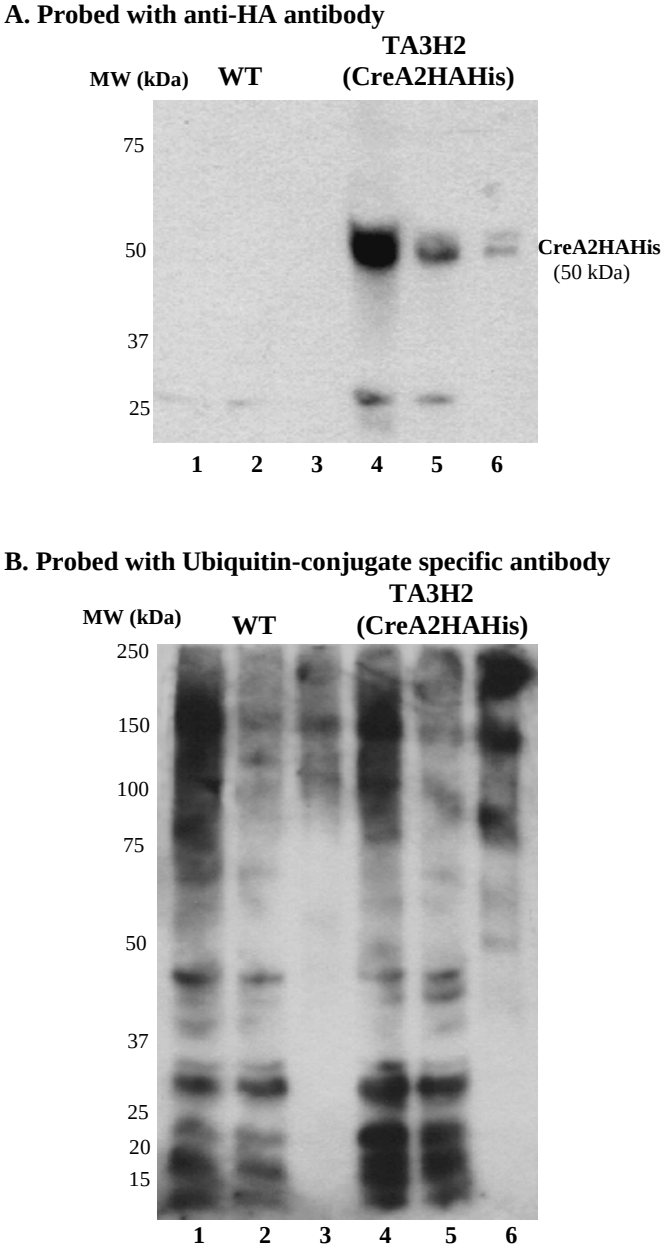


Figure 3.6 CreA is a ubiquitinated protein. Mycelium was harvested and protein extracted. Proteins were purified using an UbiQapture-Q matrix. The results of the Western where the precipitated ubiquitinated-proteins were probed with anti-HA monoclonal antibody detecting the HA tagged CreA (A). The same filter was probed with Ubiquitin-conjugate specific antibody to detected ubiquitinated proteins (B). Track 1-3; wild-type, track 4-6; TA3H2. Tracks 1 and 4 contain samples of protein lysate; tracks 2 and 5 contain unbound fractions from the immunoprecipitation; and tracks 3 and 6 contain the immunoprecipitates.

3.4 Summary

To determine whether CreA, a DNA binding protein, interacts with the CreB deubiquitination protein *in vivo*, epitope tagged versions of CreA and CreB were constructed and introduced into *A. nidulans*. Co-immunoprecipitations (Co-IP) with specific antibodies to either CreA or CreB epitope tags was used to test for a specific interaction between CreA and CreB.

The Western results of Co-IP experiments, using anti-FLAG, of the strain containing the Myc-tagged CreA and FLAG-tagged CreB show that the anti-FLAG monoclonal antibody can detect CreB in immunoprecipitated fractions of both the CreB containing and the diploid strain, but not in the control strain. However, anti-Myc antibody did not detect any MycCreA in any fractions from the strain containing MycCreA or in the diploid. This suggests that MycCreA may not be expressed well in these strains, or CreA may be processed at the N-terminus. Therefore, a new construct designed to incorporate the HA tag in an inessential region toward the C-terminal end of CreA was made and introduced into *A. nidulans*.

A diploid strain containing CreAHAGFP and CreBFLAG was made. Western results of the Co-IP experiments with anti-FLAG antibody showed that full length CreAHAGFP was present in the precipitate of the diploid, and CreBFLAG was also present in the precipitate of the T2 and diploid strains. As expected, the anti-HA and anti-FLAG antibodies did not detect any particular protein in the T2 and TA7 strains, respectively. However, anti-HA detects a protein in the immunoprecipitate of TA7, when precipitated with antiFLAG::M2 agarose, at a similar size to that in the precipitate of the diploid. Therefore, a single purification step with anti-FLAG was insufficient to remove all CreAHA in a strain not containing CreBFLAG.

When using both an anti-HA matrix and a His-Trap column in a double purification of CreA2HAHis, CreBFLAG was also detected in the diploid, but not in the haploid strains. The concentration of the CreA protein in the total extract of TA3H2 and the diploid strains was increased after purifying with the His-Trap column and anti-HA matrix. However, the proportion of CreBFLAG was decreased when purified by this method. The lower detection of CreBFLAG after purification with anti-HA matrix may indicate the molecular ratio of CreA and CreB in the deubiquitinating complex.

Furthermore, an experiment was undertaken to provide evidence to show that CreA is an ubiquitinated protein *in vivo*. When a UbiQapture-Q matrix was used to purify ubiquitinated proteins from a strain containing CreA2HAHis, CreA2HAHis was also purified, indicating that CreA was a ubiquitinated protein. These results, in summary, indicated that CreA was ubiquitinated in *A. nidulans*, and clearly bound with CreB deubiquitinating protein when CreA was purified with His-Trap column and anti-HA matrix, and CreB was precipitated with anti-FLAG::M2 agarose, and detected using the specific antibody to CreA (anti-HA) or to CreB (anti-FLAG). However, this result represents the growth of these strains in repressing condition, where CreA is expected to be active, and thus highly ubiquitinated. Further experiments in which strains are grown in derepressing conditions, such in ethanol, will need to be undertaken to determine the amount of ubiquitinated proteins, ans to confirm the ubiquitination of CreA and other proteins.

CHAPTER 4: STUDY OF PROLINE PERMEASE A CANDIDATE PROTEIN TARGET FOR THE CREB DEUBIQUITINATION PROTEIN

4.1 Introduction

Genetic analysis in *A. nidulans* had shown that the carbon regulation involves ubiquitination and deubiquitination pathways ((Lockington and Kelly, 2001, 2002). However, deubiquitination is difficult to study *in vivo*, and there are relatively few cases where the target protein, ubiquitination complex, and deubiquitination complex, are known. The identification of CreB and CreC as part of a deubiquitination complex allows an opportunity to identify the targets of the CreB deubiquitination complex in *A. nidulans* (Lockington and Kelly, 2002).

Because mutations in *creB* and *creC* grow poorly on medium containing proline and quinate as sole carbon sources, it has been suggested that the CreB/CreC deubiquitinating complex plays a role in the turnover of some permeases, such as those required for proline and quinate uptake, in both the presence and absence of carbon catabolite repression (Lockington and Kelly, 2002). In addition, there is evidence that the uptake of proline into mycelia was decreased in these mutants (Arst, 1981; Hynes and Kelly, 1977; Kelly and Hynes, 1977).

In *A. nidulans*, the *prnB* gene encodes a highly specific transporter for L-proline (Sophianopoulou and Diallinas, 1995; Tazebay *et al.*, 1994; Tazebay *et al.*, 1995). The expression of the *prn* gene cluster is induced by proline, which is transported into the fungal cell by the proline permease (PrnB) (Tazebay *et al.*, 1997). The *prnB* gene shows complex regulation, being under both nitrogen catabolite repression and carbon catabolite repression in addition to induction, reflecting the fact that it can provide both a nitrogen and carbon source for the cell (Sophianopoulou and Diallinas, 1995). Growth testing of *creB* and *creC* mutants indicated that CreB may affect the amount of the proline permease (Hynes and Kelly, 1977) thus, PrnB is a likely candidate for interaction with the CreB/CreC deubiquitinating complex, and the CreB/CreC complex may act to stabilize this permease via the deubiquitination pathway.

Whether the CreB deubiquitination enzyme directly targets the PrnB protein has not previously been tested. To test whether PrnB is a target for the CreB deubiquitinating

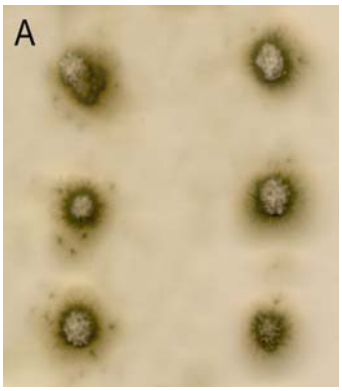
enzyme complex, DNA constructs to allow the expression of epitope-tagged versions of PrnB were produced and transformed into a *prnB* mutant strain, and Southern blotting was used to monitor these integrations. Western analysis was used to detect PrnB expression using specific antibodies against the epitope tagged PrnB. The results of these experiments are presented and discussed in this chapter.

4.2 Expression of the pGPDMycPrnB and pPRNB-HA

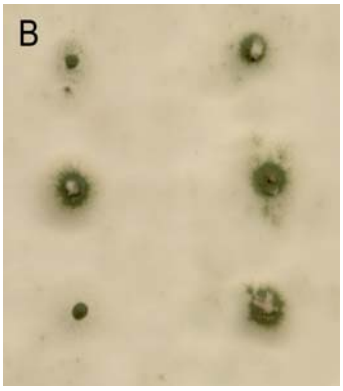
Transformants containing pGPDMycPrnB, which was designed to express PrnB with an amino-terminal Myc-epitope tag from the *gpdA* (glyceraldehydes-3-phosphate dehydrogenase) promoter, had been previously tested and none showed complementation of the phenotype due to the *prnB6* mutation on medium containing proline (Ferragamo and Kelly, unpublished data). This may be due to the epitope tag at the N-terminus of the protein interfering with function by preventing correct insertion of the protein into the membrane.

A second construct was made, pPRNB-HA, which was designed to express PrnB with a carboxy-terminal HA-epitope tag from the native *prnB* promoter. This construct was co-transformed with a plasmid pPaba, which contains the *A. nidulans pabaA*$^{+}$ gene, into a *pabaA1; prnB6* strain, and para-aminobenzoic acid (PABA) independent transformants were available, but had not been tested for the *prnB*6 complementation (Ferragamo and Kelly, unpublished data). Two PABA independent transformants, T1 and T4, were purified and two single colonies were selected from each purification, T1A and T1B, and T4A and T4B. These four strains were inoculated on proline medium together with wild-type and a control transformant, Tpaba, which was transformed only with the pPaba plasmid (Figure 4.1). T1B, T4A and T4B transformants showed complementation of the *prnB6* mutation on medium containing glucose and proline.

Glucose + NH_4



Glucose + proline



Key to strains

T1A	**T1B**
T4A	**T4B**
Tpaba	**WT**

Figure 4.1 pPRNB-HA can complement the *prnB6* mutation. Strains as indicated (T1A, T1B, T4A, T4B, Tpaba and wild-type) were inoculated on media as indicated. All media contain biotin, and plates were incubated at 37 ^{o}C for 2 days.

4.3 Southern analysis of pPRNB-HA transformants

Although some of the transformants using pPRNB-HA could complement the *prnB6* mutation, Southern analysis was required to determine whether the functional copy of *prnB* contained an HA-epitope, as a homologous single-crossover event could produce a transformant with a wild-type copy of *prnB*, as could a double crossover event (Figure 4.2). A Southern blot was used to determine whether a HA-tagged copy of *prnB* was present in T1B, T4A and T4B transformants which exhibited wild-type growth on glucose and proline medium. A 15 kb DNA sequence of *A. nidulans*, including the *prnB* gene, was identified in the *A. nidulans* database and an *in silico* digestion analysis was performed using the NEBcutter website. The *Eco*RI restriction enzyme was selected as it does not cut within the region containing *prnB* used as a probe (Figure 4.3). The detection was made using a ^{32}P labelled DNA probe specific to the *prnB* gene using PCR and the same primers as were used for construction of pPRNB-HA (Table 2.3 in Materials and Methods). The result of the Southern hybridization is shown in Figure 4.4A and 4.4B. The Southern confirmed the presence of the native *prnB* fragment at 6.2 kb in the genomic DNA of both the complementing transformants and wild-type strains. Additional, fragments of about 5 kb were also present in T1B, T4A and T4B transformants. T1A did not show a band in addition to the 6.2 kb band, consistent with the fact that it did not show complementation on the plate test (in Figure 4.1). The results of the Southern confirmed that T1B, T4A and T4B contained a heterologous integrated copy of *prnBHA*, and thus the functional copy of PrnB is predicted to contain an HA tag. Western analysis was used to determine the expression of the PrnBHA in these transformants in the medium containing glucose and proline, but no expression was detected using the anti-HA antibody (Figure 4.4C), even though this construct was designed to include the native upstream promoter of *prnB* to ensure expression, and phenotypic complementation was seen. Western analysis was done in parallel with the positive control strain (T7) which contains both CreBFLAG and CreCHA

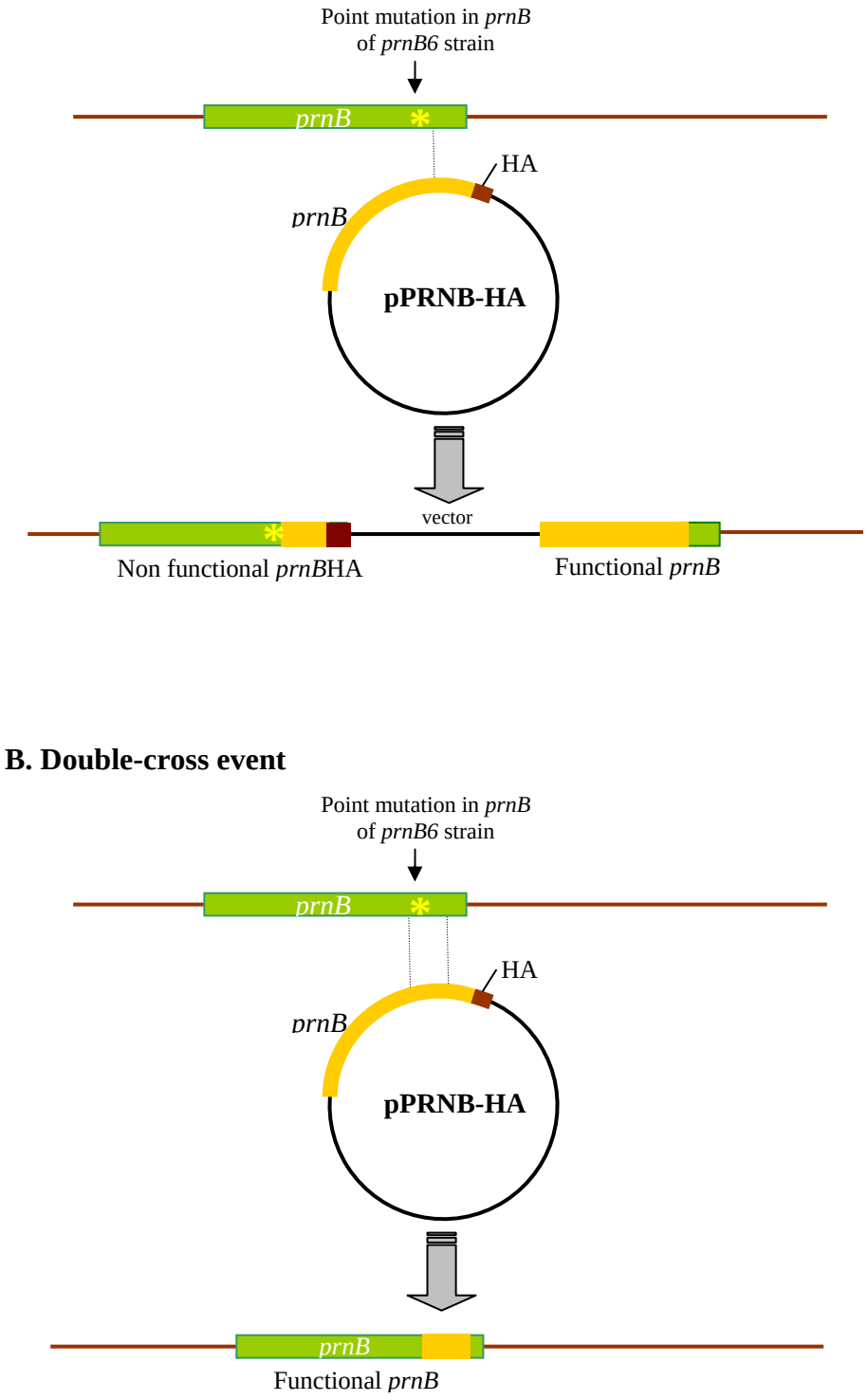
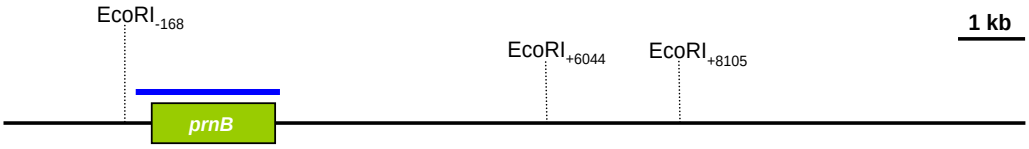


Figure 4.2 Homologous integration events producing PrnB without a HA-tag. A): single crossover and B): double crossover.

A. *Eco*RI sites in the *prnB* region



B. Restriction map of pPrnB-HA

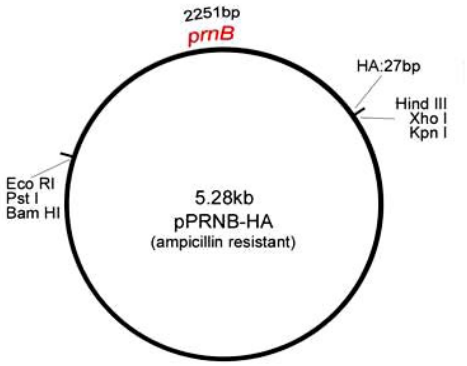
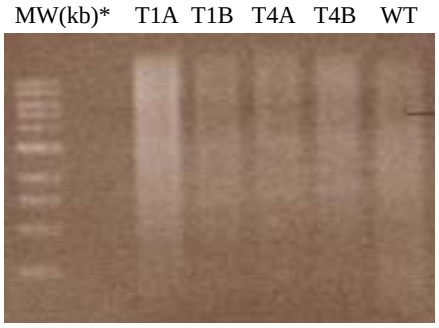


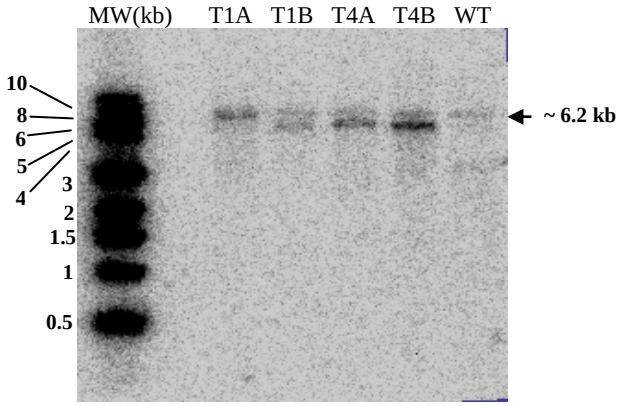
Figure 4.3 *Eco*RI sites in native *prnB* and in pPRNB-HA. *Eco*RI digestion produces a 6.2 kb fragment containing the native *prnB* (A). There is a *Eco*RI restriction sequence upstream of *prnB* in pPrnB-HA (B). The blue bar in (A) represents the probe specific to the *prnB* gene. Number in A indicats the position of restriction site of *Eco*RI compared to the start codon of the *prnB* gene.

A. Ethidium bromide stained gel



* molecular weight maker are indicated in B.

B. Southern analysis of transformants



C. Western analysis

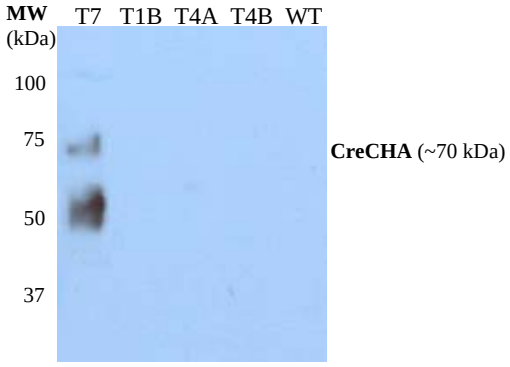


Figure 4.4 Southern and Western analysis of pPRNB-HA transformants. *Eco*RI digested genomic DNA from T1A, T1B, T4A, T4B and wild-type (WT) were separated by 0.8% agarose gel electrophoresis (A) and transferred to membrane. The blot was hybridized with the *prnB* specific probe (B). A 1kb DNA ladder was use as a reference for the Southern. Protein extracts of T7, T1B, T4A, T4B and WT were separated on SDS-PAGE, and Western blot was probed with anti-HA antibody (C).

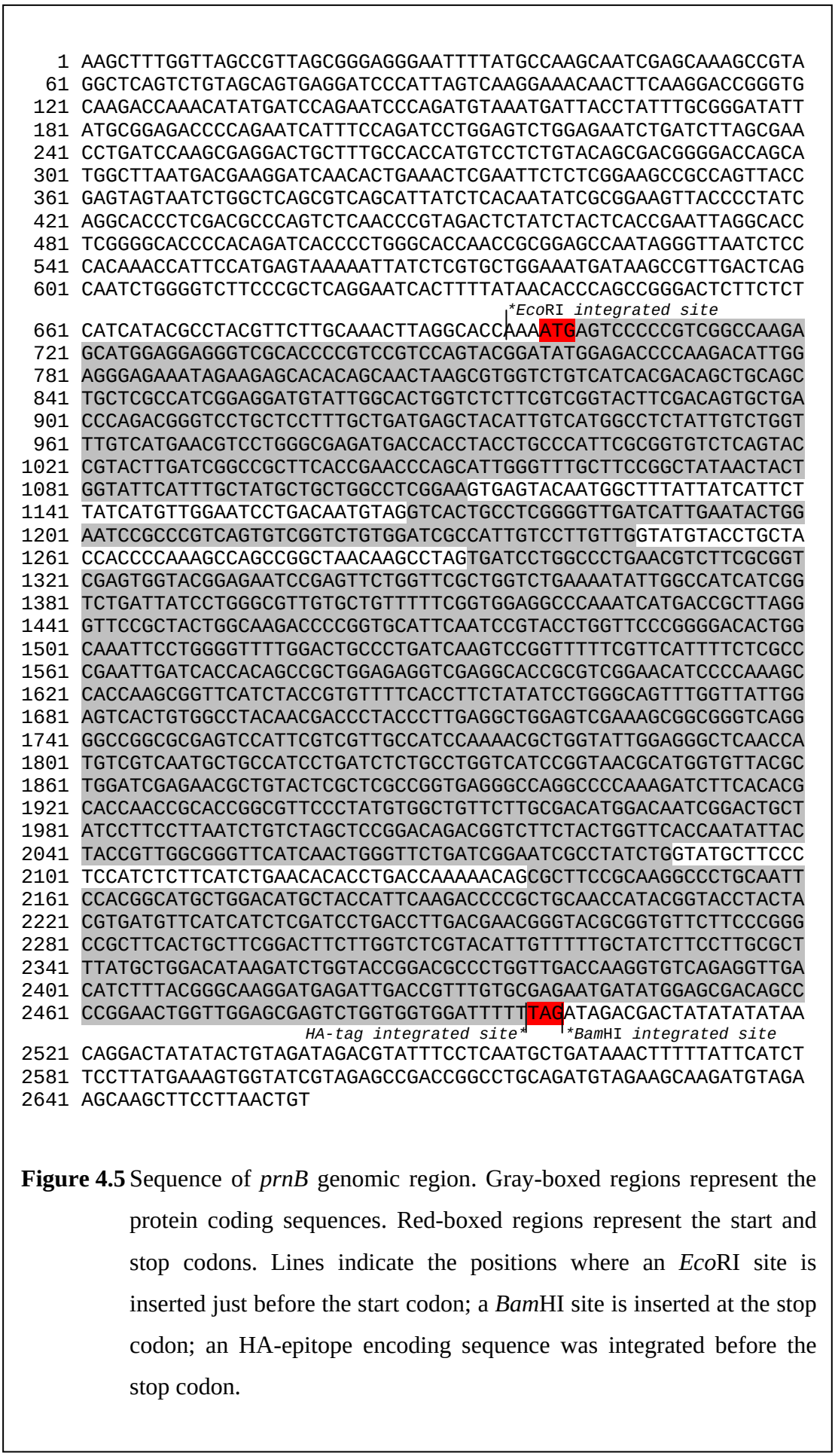
4.4 pGPDPrnBHA construction

4.4.1 PCR amplification and re-design of epitope-tagged PrnBHA

Since the constructs which contained an N-terminal epitope tagged PrnB (MycPrnB) did not complement the *prnB* mutant strain, and the HA-tag at the C-terminal end expressed under the control of the native promoter of the *prnB* gene (PrnBHA) was not able to be detected using Western analysis, a new HA epitope-tagged version of PrnB was designed to express at a high level from the *A. nidulans gpdA* promoter. The forward primer (NKPHAF; Table 2.3 in Materials and Methods) was designed to include the *Eco*RI restriction endonuclease recognition sequence just before the start codon of the *prnB* gene. The reverse primer (NKPHAR; Table 2.3 in Materials and Methods) was designed to include the *Bam*HI restriction endonuclease recognition sequence adjacent to the HA sequence at the 3'-end of the gene (Figure 4.5). PCR was used to generate the *prnBHA* fragment using the pPRNB-HA plasmid (Figure 4.6A) as a template. The PCR products were digested with both *Eco*RI and *Bam*HI restriction enzymes, and the digested DNA was separated on a 0.8% agarose gel. Fragments of the expected size, 1.9 kb, were purified from the gel by using the QIAquick® Gel extraction Kit.

4.4.2 Ligation of *prnBHA* into a pGPD-containing vector and plasmid amplification

The pGPD-containing vector was derived from the pGPDCreAHis (Figure 4.6B) plasmid by digesting this plasmid with both *Eco*RI and *Bam*HI restriction enzymes to remove the CreAHis fragment and produce the plasmid-fragment containing *gpdA* promoter. The purified and digested 1.9 kb of the *prnBHA* fragment, from the PCR of pPRNB-HA plasmid, was ligated together with the vector fragment resulting in the recombinant plasmid, pGPDPrnBHA, as outlined in Figure 4.6C. pGPDPrnBHA was amplified by transformation of competent *E. coli* DH5α. Selected *E. coli* colonies on the chloramphenical-antibiotic-containing agar were grown overnight in L-Broth at 37 ^{o}C followed by plasmid extraction using the Miniprep® DNA Purification Kit.

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   1 AAGCTTTGGTTAGCCGTTAGCGGGAGGGAATTTTATGCCAAGCAATCGAGCAAAGCCGTA
  61 GGCTCAGTCTGTAGCAGTGAGGATCCCATTAGTCAAGGAAACAACTTCAAGGACCGGGTG
 121 CAAGACCAAACATATGATCCAGAATCCCAGATGTAAATGATTACCTATTTGCGGGATATT
 181 ATGCGGAGACCCCAGAATCATTTCCAGATCCTGGAGTCTGGAGAATCTGATCTTAGCGAA
 241 CCTGATCCAAGCGAGGACTGCTTTGCCACCATGTCCTCTGTACAGCGACGGGGACCAGCA
 301 TGGCTTAATGACGAAGGATCAACACTGAAACTCGAATTCTCTCGGAAGCCGCCAGTTACC
 361 GAGTAGTAATCTGGCTCAGCGTCAGCATTATCTCACAATATCGCGGAAGTTACCCCTATC
 421 AGGCACCCTCGACGCCCAGTCTCAACCCGTAGACTCTATCTACTCACCGAATTAGGCACC
 481 TCGGGGCACCCCACAGATCACCCCTGGGCACCAACCGCGGAGCCAATAGGGTTAATCTCC
 541 CACAAACCATTCCATGAGTAAAAATTATCTCGTGCTGGAAATGATAAGCCGTTGACTCAG
 601 CAATCTGGGGTCTTCCCGCTCAGGAATCACTTTTATAACACCCAGCCGGGACTCTTCTCT
                                                  *EcoRI integrated site
 661 CATCATACGCCTACGTTCTTGCAAACTTAGGCACCAAAATGAGTCCCCCGTCGGCCAAGA
 721 GCATGGAGGAGGGTCGCACCCCGTCCGTCCAGTACGGATATGGAGACCCCAAGACATTGG
 781 AGGGAGAAATAGAAGAGCACACAGCAACTAAGCGTGGTCTGTCATCACGACAGCTGCAGC
 841 TGCTCGCCATCGGAGGATGTATTGGCACTGGTCTCTTCGTCGGTACTTCGACAGTGCTGA
 901 CCCAGACGGGTCCTGCTCCTTTGCTGATGAGCTACATTGTCATGGCCTCTATTGTCTGGT
 961 TTGTCATGAACGTCCTGGGCGAGATGACCACCTACCTGCCCATTCGCGGTGTCTCAGTAC
1021 CGTACTTGATCGGCCGCTTCACCGAACCCAGCATTGGGTTTGCTTCCGGCTATAACTACT
1081 GGTATTCATTTGCTATGCTGCTGGCCTCGGAAGTGAGTACAATGGCTTTATTATCATTCT
1141 TATCATGTTGGAATCCTGACAATGTAGGTCACTGCCTCGGGGTTGATCATTGAATACTGG
1201 AATCCGCCCGTCAGTGTCGGTCTGTGGATCGCCATTGTCCTTGTTGGTATGTACCTGCTA
1261 CCACCCCAAAGCCAGCCGGCTAACAAGCCTAGTGATCCTGGCCCTGAACGTCTTCGCGGT
1321 CGAGTGGTACGGAGAATCCGAGTTCTGGTTCGCTGGTCTGAAAATATTGGCCATCATCGG
1381 TCTGATTATCCTGGGCGTTGTGCTGTTTTTCGGTGGAGGCCCAAATCATGACCGCTTAGG
1441 GTTCCGCTACTGGCAAGACCCCGGTGCATTCAATCCGTACCTGGTTCCCGGGGACACTGG
1501 CAAATTCCTGGGGTTTTGGACTGCCCTGATCAAGTCCGGTTTTTCGTTCATTTTCTCGCC
1561 CGAATTGATCACCACAGCCGCTGGAGAGGTCGAGGCACCGCGTCGGAACATCCCCAAAGC
1621 CACCAAGCGGTTCATCTACCGTGTTTTCACCTTCTATATCCTGGGCAGTTTGGTTATTGG
1681 AGTCACTGTGGCCTACAACGACCCTACCCTTGAGGCTGGAGTCGAAAGCGGCGGGTCAGG
1741 GGCCGGCGCGAGTCCATTCGTCGTTGCCATCCAAAACGCTGGTATTGGAGGGCTCAACCA
1801 TGTCGTCAATGCTGCCATCCTGATCTCTGCCTGGTCATCCGGTAACGCATGGTGTTACGC
1861 TGGATCGAGAACGCTGTACTCGCTCGCCGGTGAGGGCCAGGCCCCAAAGATCTTCACACG
1921 CACCAACCGCACCGGCGTTCCCTATGTGGCTGTTCTTGCGACATGGACAATCGGACTGCT
1981 ATCCTTCCTTAATCTGTCTAGCTCCGGACAGACGGTCTTCTACTGGTTCACCAATATTAC
2041 TACCGTTGGCGGGTTCATCAACTGGGTTCTGATCGGAATCGCCTATCTGGTATGCTTCCC
2101 TCCATCTCTTCATCTGAACACACCTGACCAAAAACAGCGCTTCCGCAAGGCCCTGCAATT
2161 CCACGGCATGCTGGACATGCTACCATTCAAGACCCCGCTGCAACCATACGGTACCTACTA
2221 CGTGATGTTCATCATCTCGATCCTGACCTTGACGAACGGGTACGCGGTGTTCTTCCCGGG
2281 CCGCTTCACTGCTTCGGACTTCTTGGTCTCGTACATTGTTTTTGCTATCTTCCTTGCGCT
2341 TTATGCTGGACATAAGATCTGGTACCGGACGCCCTGGTTGACCAAGGTGTCAGAGGTTGA
2401 CATCTTTACGGGCAAGGATGAGATTGACCGTTTGTGCGAGAATGATATGGAGCGACAGCC
2461 CCGGAACTGGTTGGAGCGAGTCTGGTGGTGGATTTTTTAGATAGACGACTATATATATAA
                          HA-tag integrated site*    *BamHI integrated site
2521 CAGGACTATATACTGTAGATAGACGTATTTCCTCAATGCTGATAAACTTTTTATTCATCT
2581 TCCTTATGAAAGTGGTATCGTAGAGCCGACCGGCCTGCAGATGTAGAAGCAAGATGTAGA
2641 AGCAAGCTTCCTTAACTGT
```

Figure 4.5 Sequence of *prnB* genomic region. Gray-boxed regions represent the protein coding sequences. Red-boxed regions represent the start and stop codons. Lines indicate the positions where an *Eco*RI site is inserted just before the start codon; a *Bam*HI site is inserted at the stop codon; an HA-epitope encoding sequence was integrated before the stop codon.

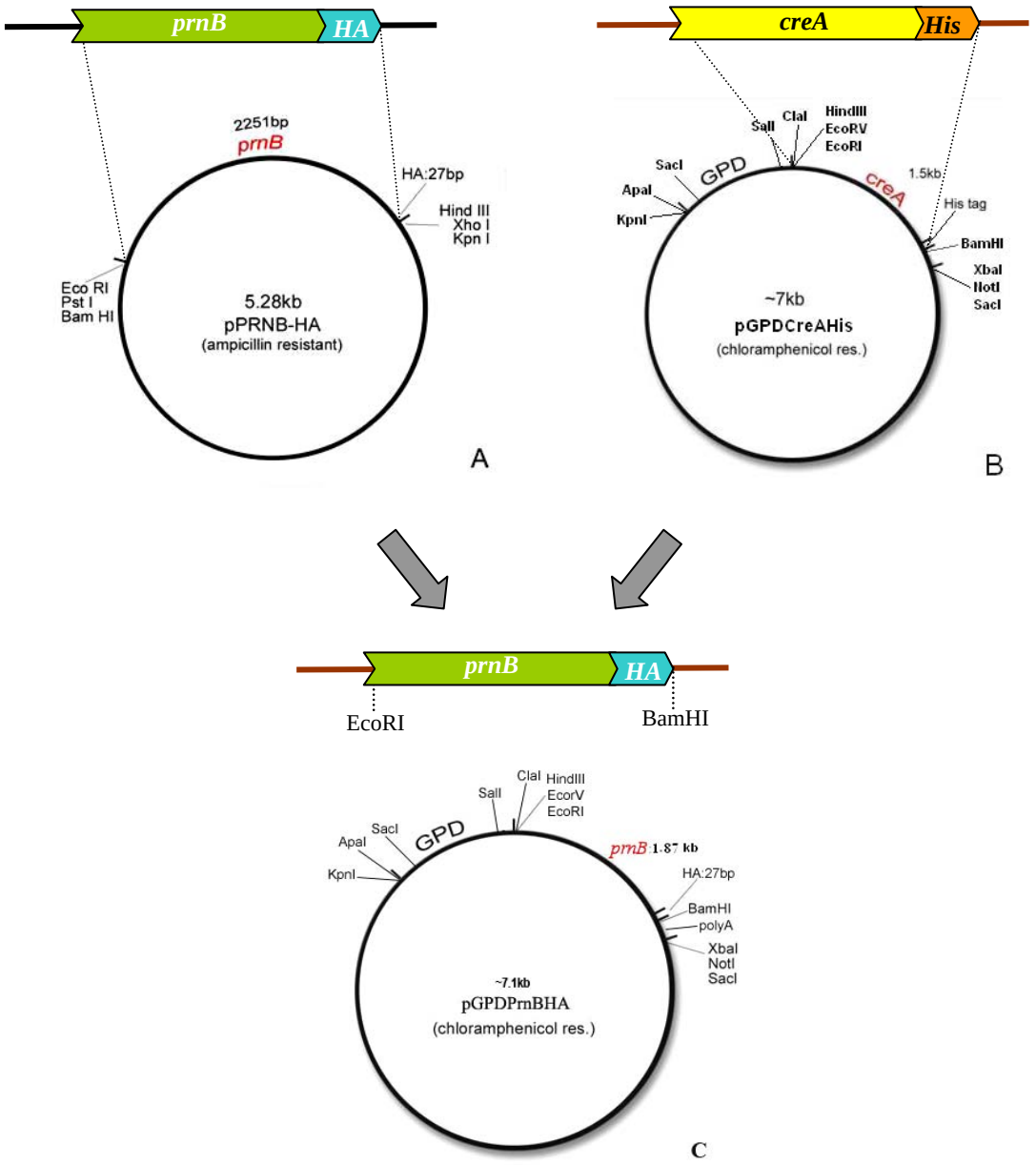


Figure 4.6 Construction of pGPDPrnBHA. The *prnBHA* fragment (1.87 kb) was amplified from pPRNB-HA (A) by using the specific primers designed to include *Eco*RI and *Bam*HI restriction sit at the 5' and 3' end, respectively. pGPDCreAHis (B) was digested with *Eco*RI/*Bam*HI to remove the CreAHis fragment before religation with the *prnBHA* fragment from pPRNB-HA to create the pGPDPrnBHA plasmid (C), which *prnBHA* gene is expressed from the *gpdA* promoter. Experimental details are in section 4.4.1-2, and diagrams are not in scale.

In order to confirm that the selected plasmids contained the correct sized insert of 1.87 kb, the purified plasmids from transformed *E. coli* colonies were double digested with *Eco*RI and *Bam*HI, and resolved on a 0.8% agarose gel. The result in Figure 4.7 shows that the recombinant plasmids contain the *prnBHA* fragment at 1.87 kb (lane 3 and 4) and the precursor fragment at ~6.0 kb. The precursor plasmid (pGPDCreAHis) contained the 1.5 kb fragment of CreAHis (lane 2) and the same 6.0 kb fragment as in the recombinant products (lane 3 and 4).

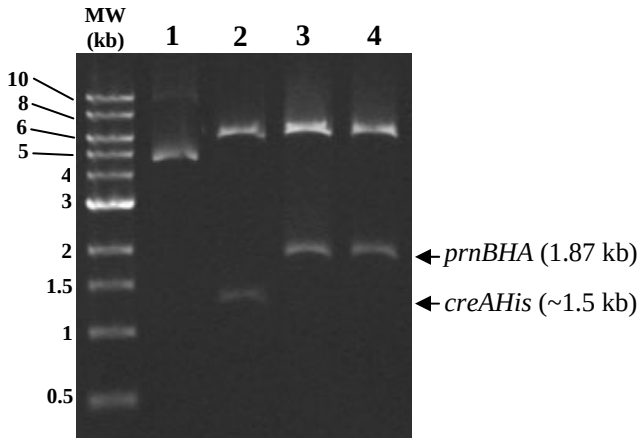


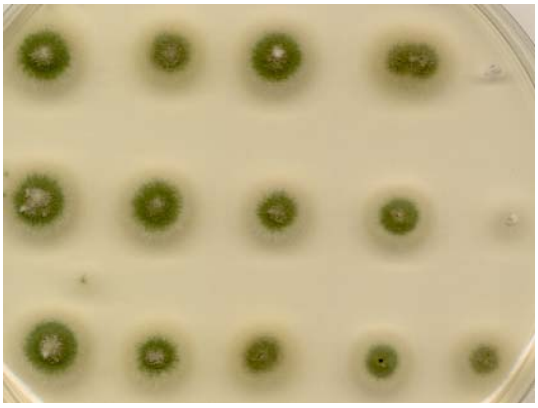
Figure 4.7 Confirmation of pGPDPrnBHA. Double-digestion of pGPDCreAHis and pGPDPrnBHA was performed with *Eco*RI and *Bam*HI restriction enzyme for 4 hrs at 37 ^{O}C. Lane 1, pGPDCreAHis; Lane 2, pGPDCreAHis digested with *Eco*RI/*Bam*HI; Lane 3, pGPDPrnBHA transformant I digested with *Eco*RI/*Bam*HI; Lane 4, pGPDPrnBHA transformant II digested with *Eco*RI/*Bam*HI.

4.5 Transformation and Expression of pGPDPrnBHA in *A. nidulans*

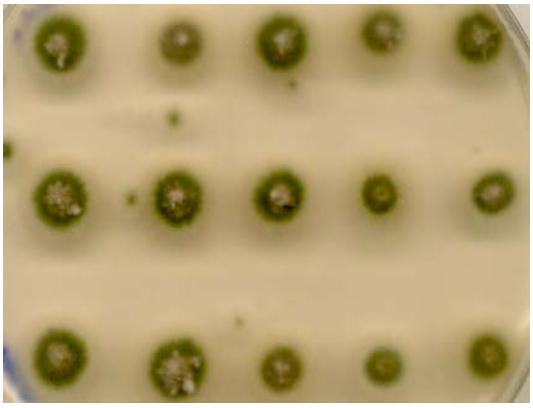
In order to confirm that the constructs encoded a functional copy of proline permease (*prnB*) that was able to complement the mutation, the pGPDPrnBHA plasmids were introduced into the *pabaA1; prnB6* mutant strain, which requires PABA for growth, by co-transformation with the pPaba plasmid containing the *pabaA*$^{+}$ gene as described previously, and selecting for PABA independent colonies. These transformants were analysed further to determine if they had also been co-transformed with the plasmid containing the *prnBHA* construct.

Six PABA independent colonies were selected for further analysis, and these transformants were further purified and phenotypically tested (Figure 4.8). Transformant TP1 isolates (TP1-1, TP1-2 and TP1-3) showed full complementation of the phenotype due to the *prnB6* mutation on proline medium. Transformant TP2, TP3 and TP6 isolates (TP2-1, TP2-2, TP3-1, TP3-2, TP6-1 and TP6-2) showed partial complementation of the phenotype due to the *prnB6* on proline medium, whereas transformant TP8 and TP9 isolates (TP8-1, TP8-2, TP9-1 and TP9-2) showed no complementation.

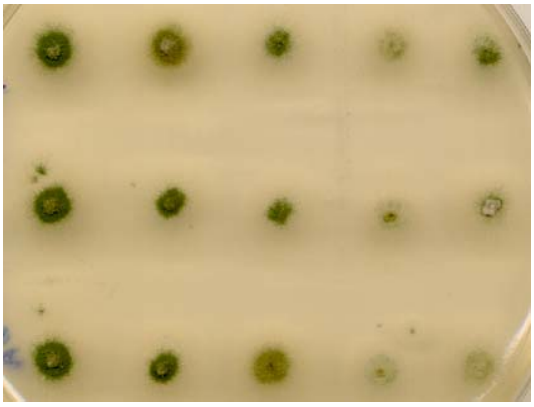
A. Glucose + NH_4



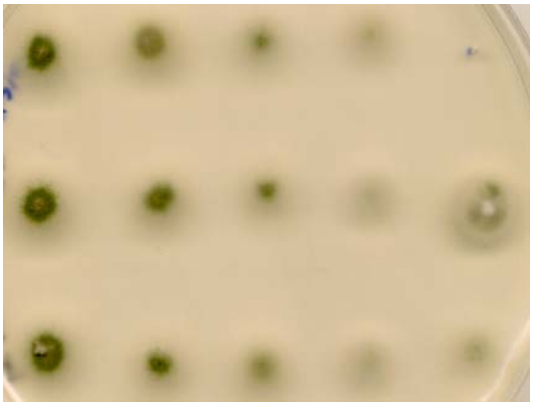
B. Glucose + NH_4 + biotin + paba



C. Glucose + proline + paba



D. Glucose + proline + biotin



E. Key to placement of strains

TP1-1	**TP3-1**	**TP6-2**	**TP9-1**	***pabaA1 prnB6***
TP1-2	**TP2-2**	**TP6-1**	**TP8-2**	**WT**
TP1-3	**TP2-1**	**TP3-2**	**TP8-1**	**TP9-2**

Figure 4.8 Phenotype analysis of pGPDPrnBHA transformants. Glucose was added at 1%, Nitrogen sources were added at 10 mM. Plates were incubated at 37^{O}C for 2 days. WT is *biA1;niiA4*.

4.6 Southern analysis of pGPDPrnBHA transformants

A Southern blot was used to confirm that an ectopically integrated copy of the *prnB* was present in the complementing transformants. Genomic DNA of complementing transformants was extracted and digested with *Eco*RI and *Bam*HI restriction enzymes, and separated by agarose gel electrophoresis before transfer to nylon membrane (Figure 4.9A). The detection was made using a ^{32}P labelled probe specific to the *prnB* gene made using PCR and the same primers as were used for construction of pGPDPrnBHA. The transformant T1B containing pPRNB-HA DNA was used as a control.

The result of the Southern hybridization is shown in Figure 4.9B and details showing the *Eco*RI and *Bam*HI endonuclease restriction sites are in Figure 4.9C and 4.9D. The Southern confirmed the present of the native *prnB* fragment at 6.2 kb in the genomic DNA of both pGPDPrnBHA complementing transformants (TP1, TP2 and TP3 strains) and the T1B strain. Southern analysis of isolate of TP1 and TP3 showed a 1.9 kb *Eco*RI/*Bam*HI fragment as expected if the *prnBHA* region was integrated ectopically. The TP2 transformant did not contain a 1.9 kb *Eco*RI/*Bam*HI fragment, but there was an additional fragment at about 5 kb of the DNA of TP2, and thus the integration event may not have reproduced the complete *prnBHA* region.

The results of the Southern confirmed that the TP1 and TP3 transformants contained a heterologous integrated copy of *prnBHA*, and thus the functional copy of *prnB* is predicted to contain an HA epitope tag. Western analysis was used to determine the expression of PrnBHA in these transformants. However, the Southern does not definitively show that the *gpd* promoter is still adjacent to the *prnBHA* gene, and this could affect the level of expression in the transformant. However, a number of independent transformants were tested.

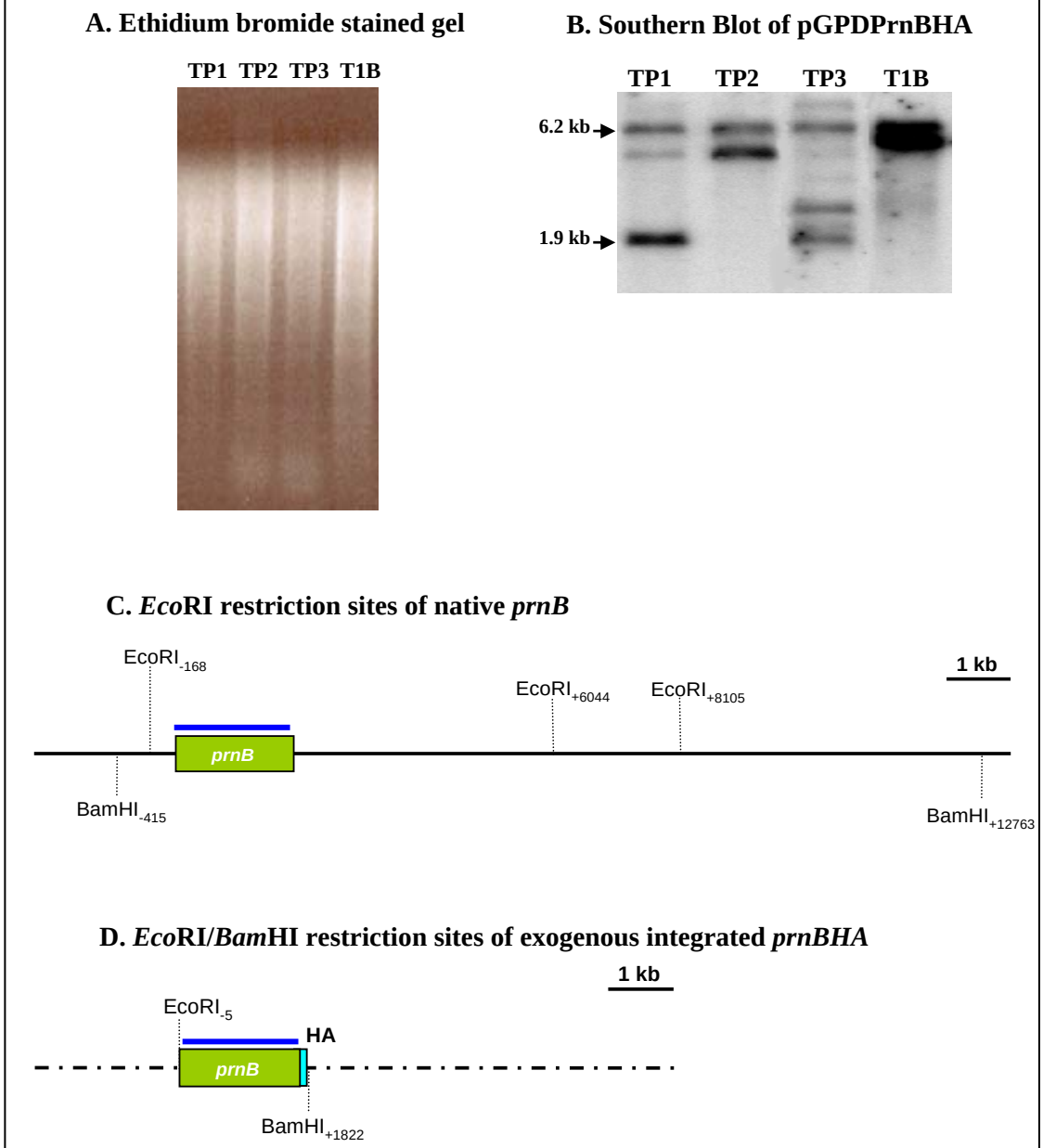


Figure 4.9 Southern hybridization of pGPDPrnBHA transformants. *Eco*RI/*Bam*HI digested genomic DNA from 3 transformants and a T1B strains were separated on 0.8% agarose gel electrophoresis (A). The blot was hybridized with the *prnB* specific probe (B); *In silico* analysis of native *prnB* region showed a 6.2 kb of *Eco*RI digestion fragment (C), and a complemented *prnBHA* gene shows a 1.87 kb of *Eco*RI/*Bam*HI digestion fragment. (lane 1: TP1; lane 2: TP2; lane 3: TP3; and lane 4: T1B, containing pPRNB-HA). Blue bars in C and D represent the probe specific to *prnB*.

4.7 Western analysis of pGPDPrnBHA transformants

Isolates of transformants 1, 2 and 3 (TP1, TP2 and TP3) from pGPDPrnBHA were selected and analysed for expression of the *prnBHA* fusion protein by Western analysis. To test for the presence of pGPDPrnBHA, the transformants were grown overnight in glucose medium containing proline as a sole nitrogen source at 37 ^{o}C and shaking at 120 rpm. The mycelia were harvested and proteins extracted as in Material and Methods. Total protein extracts were separated by SDS-PAGE before transfer to PVDF membrane. Western blots were performed to detect the PrnBHA protein by using high affinity anti-HA monoclonal antibody conjugated with peroxidase. The detections were done in parallel with the positive-control of HA expression strain (T7, containing CreBFLAG and CreCHA; Lockington and Kelly, 2002).

The result of the Western analysis is shown in Figure 4.10. CreCHA was detected in the positive control strain, T7, at the expected size (track 7). However, anti-HA did not detect any PrnBHA protein from the pGPDPrnBHA containing transformants. Anti-HA interacted with a low molecular weight protein fragment in all of transformants, and also in the CreCHA containing strain (T7). This suggested that the antigen-antibody interaction and the detection worked well. Because transcription was driven from the *gpdA* promoter, it is unlikely that the level of gene transcription is a problem. More likely, the proline permease (PrnB) might be processed or modified before travelling to, and functioning at the cell membrane. This processing may result in cleavage of the C-terminus of PrnB where the HA epitope tag was positioned and loss of the HA epitope tag from the protein.

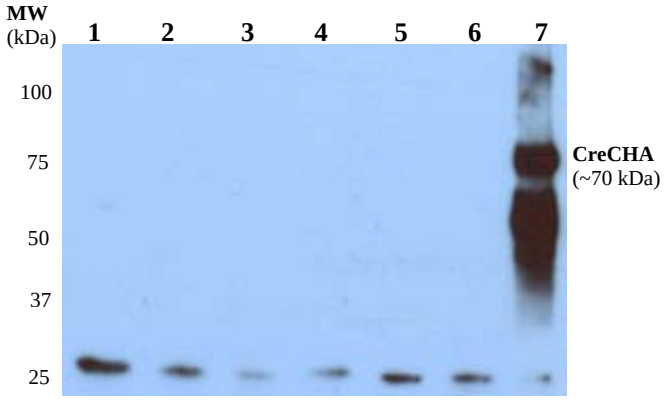


Figure 4.10 Western analysis of pGPDPrnBHA transformants and the T7 strain. An anti-HA immunodetection exposed for 15 min. Cultures were grown on the proline assay medium for 16 hrs at 37 ^{o}C before harvest. (lane1: TP1-1; lane2: TP1-2; lane3: TP2-1; lane4: TP2-2; lane5: TP3-1; lane6: TP3-2; lane7: T7 containing CreBFLAG and CreCHA)

4.8 Summary

To determine whether the proline permeases (PrnB) is a direct-target substrate of the CreB/CreC deubiquitinating complex, epitope tagged versions of PrnB were constructed and introduced into the *prnB6* mutant strain. Transformants were checked for *prnB* expression both by phenotype testing on specific media, Southern analysis to analyse the intergration sites, and Western analysis using specific antibodies directed against the HA-epitope and Myc-epitope tags.

Initially, *A. nidulans* mutant strains, containing a C-terminal tagged PrnB-HA, or an N-terminal tagged MycPrnB, had already been made and were available in the laboratory but not analysed. Phenotype testing results showed that a C-terminal HA-tagged PrnB could complement a mutant strain, but none of the colonies of pGPDMycPrnB transformants resembled wild-type growth on proline medium, and thus constructs with an N-terminal tag could not complement the *prnB6* mutation.

Four pPRNB-HA transformants were selected for further analysis by Southern blot to determine where they had inserted. Specifically, because the *prnB6* mutation is a point mutation, an integration event could have occurred that reconstituted a wild-type *prnB* gene with no epitope tag. Thus, a Southern was used to check that the construct had integrated other than in the *prnB* genomic region. The expected 6.2 kb of the native *prnB* gene and a copy of *prnBHA* was detected in these complemented PrnBHA transformants. This confirmed the correct insertion of a functional copy of the *prnBHA* genes in the genome of transformants. However, no HA epitope tag was detected by Western blot although a high affinity anti-HA antibody was used in this experiment. It may be that the expression of *prnB* from its own promoter is not sufficient for detection by the Western analysis. Therefore, a new construct to expresses the HA tagged PrnB protein from a highly active promoter was constructed. Phenotype testing showed that transformants obtained with this construct showed complementation of the *prnB* mutation and showed wild-type growth on proline medium. Southern analysis showed the *prnBHA* fragment (~1.9 kb) was present in the genome of the fully complementing transformants in addition to the native *prnB* fragment at ~6.2 kb, and so the incoming DNA had not integrated at the *prnB* locus, thus the HA epitope tag was expected to be present on the functional copy of the PrnB protein. The pGPDPrnBHA containing transformants were tested to determine whether *prnBHA* could be detected in a Western analysis. The Western analysis did not show any evidence of PrnBHA. A control strain containing CreCHA (T7) was used as a positive control and the HA tagged CreC protein was easily detected, showing that the Western procedure had been correctly carried out.

Thus, the N- and C-terminal epitope tagged versions of PrnB could not be detected using Western analysis and the epitope tagged version of PrnB could not be used to determine whether PrnB was a substrate of CreB in the strain containing both PrnBHA and CreBFLAG using co-immunoprecipitation. Therefore, a study of the interaction of CreB deubiquitinating enzyme with another permease, quinate permease (QutD), was initiated and is the focus of the next chapter.

CHAPTER 5: STUDY OF QUINATE PERMEASE, QUTD, CANDIDATE PROTEIN TARGET FOR CREB DEUBIQUITINATION PROTEIN

5.1 Introduction

Carbon catabolite repression acts to prevent the expression of a range of enzymes that are required for the utilization of alternative carbon sources in the presence of glucose or other strong carbon sources. Three genes had been involved in this system in *A. nidulans*, *creA*, *creB* and *creC*. Mutation in *creB* and *creC* show derepressed expression of some genes that are normally subject to CCR, and in addition they show reduced utilization of some sole carbon sources such as quinate or proline. The *creB* gene encodes a deubiquitinating enzyme, and *creC* gene encodes a WD40 repeat containing protein. *creB* and *creC* form part of a deubiquitination complex (Lockington and Kelly, 2001, 2002; Todd *et al.*, 2000).

As *creB* and *creC* mutations have phenotypic effects in derepressing condition as well as in repressing conditions, it is likely that CreB and CreC play distinct roles in carbon catabolite repressing and derepressing conditions. *A. nidulans* can use quinate as a carbon source (Wheeler *et al.*, 1996), and the genes required are present in a cluster. The induction of genes in the *qut* cluster is initiated by the uptake of quinate across the plasma membrane to the fungal mycelia by the quinic acid permease (QutD) encoded by the *qutD* gene (Hawkins *et al.*, 1988), and this induction is greatly decreased in the presence of glucose due to the effect of CCR rather than substrate exclusion (Whittington *et al.*, 1987). In the absence of glucose *creB* mutant strains are unable to utilize quinate as a carbon source, suggesting some role for the *creB* gene in quinate utilization. Ubiquitination is known to directly regulate transporter-proteins in other organisms. For example, in *S. cerevisiae*, the uptake of amino acids is regulated by the general amino acid permease, Gap1p, (Helliwell *et al.*, 2001). Both the stability and functionality of Gap1p are regulated by the nitrogen conditions, where the addition of the preferential nitrogen source (NH_4^+) to cells growing in a poor nitrogen source induces rapid internalization and ubiquitination of the Gap1p permease followed by the degradation of these complexes in the yeast vacuole (Springael and Andre, 1998; Springael *et al.*, 1999a).

Although it is known that *creB* and *creC* mutations lead to drastically reduced utilization of quinate, whether the quinate permease (QutD) is ubiquitinated, and whether the ubiquitinated protein is a direct target of the CreB deubiquitination enzyme has not been demonstrated. Epitope-tagged versions of the QutD permease were constructed and expressed in the *qutD312* mutant strain. Results of the co-immunoprecipitation of CreB and QutD, and expression of QutD in the wild-type and *creB* mutant background are presented in this chapter.

5.2 Transformation of pGPDMycQutD into the *qutD*312 mutant strain.

An N-terminal tagged pGPDMycQutD plasmid was already constructed and available in the laboratory (Ferragamo D. and Kelly JM., unplublished data). This plasmid expressed MycQutDHA from the highly active *A. nidulans gpdA* promoter. Several clones of pGPDMycQutD were tested for expression by co-transformation with pPL3 plasmid (Oakely *et al.* 1987) into the *qutD* mutant strain (*biA1; pyroA4; riboB2 qutD312)* and riboflavin-independent colonies were selected. Although some of these transformants could grow on the selection medium lacking riboflavin, none of them showed complementation of *qutD312* on quinate medium.

This result indicated that an N-terminal tag on QutD interfered with its function in *A. nidulans*, even though this construct was designed to express *qutD* from the highly active promoter of *A. nidulans gpdA*. The results from this experiment were similar to those of pGPDMycPrnB where the N-terminal tagged version of PrnB permease also failed to complement the *prnB* mutant strain. The failure of the N-terminal tagged permease to complement the mutant strain, either *prnB6* or *qutD312*, may be due to the epitope-tag interfering with these permease proteins localization, folding or function.

5.3 pGEMTQutDHA construction

5.3.1 Redesign of epitope tag and PCR amplification of *qutDHA*

Since the constructs which contained an N-terminal epitope tagged QutD (pGPDMycQutD) did not complement the *qutD* mutant strain, a new HA epitope-tagged version of *qutD* was designed in which a HA tag was added to the C-terminus of the protein. This included 682 base pairs upstream of the *qutD* start codon containing the promoter region of the gene. The *qutD* gene of 1705 base pairs was isolated by

PCR. The forward primer (NKQHAF; Table 2.3 in Materials and Methods) was designed to prime from a sequence 681-700 base pairs upstream of the *qutD* gene to ensure that the PCR product included the promoter region of the *qutD* gene. The reverse primer (NKQXhoHAR; Table 2.3 in Materials and Methods) was designed to insert a *Xho*I recognition sequence prior to the stop codon of the *qutD* gene. Details of the *qutD* sequence and the regions specific to the PCR primers are shown in Figure 5.1.

Gradient PCR was used to optimize the annealing reaction temperatures of those two primers and *A. nidulans* genomic DNA (H17A12RAS(3)) as a template. The optimum annealing condition was found to be 60 ^{o}C (Figure 5.2). The cleaned 2.3 kb PCR product was cloned using the pGEM®-T easy vector.

5.3.2 Directional cloning of the *qutD* into the pGEM®-T easy vector and plasmid amplification

The *qutD* fragment was ligated into the pGEM®-T easy vector in order to create the pGEMTQutD plasmid. The plasmid was then amplified in the *E. coli* competent cells which were selected for growth on an L-agar containing the ampicillin antibiotic (Section 2.3.1 in Materials and Methods). pGEMTQutD contains two *Xho*I restriction sites, one at 153 base pairs upstream of *qutD,* and the designed *Xho*I restriction site prior to the stop codon of *qutD* (Figure 5.3). To romove the *Xho*I site 153 base pairs upstream of *qutD*, a partial digestion of pGEMTQutD with *Xho*I was separated and purified from an agarose gel, before end-filling and religation of the filled fragment. The re-ligated plasmids were completely digested with *Xho*I restriction endonuclease, and separated using an agarose gel. The singly digested fragment was removed and purified before religation and transformation into *E. coli* competent cells. To identify which of the two *Xho*I sites had been destroyed, the resulting plasmids were completely digested with *Xho*I and *Sph*I restriction endonucleases and separated using an agarose gel.

Figure 5.4 shows that plasmid T2# contains a *Xho*I site just before the stop codon of the *qutD* gene, resulting in fragments of 3.0 kb and 2.3 kb. When pGEMTQutD contains a *Xho*I site upstream of *qutD,* the digestion products are 0.5 kb and 4.8 (T2#). The correct pGEMTQutD plasmid was then used for ligation with an annealed HA oligonucleotide (Figure 5.5).

```
   1  GGCATTGTAACTGACGACCCGACAAATTTGGGATTGGAGCGGACGGAGGAGAGGAAGGTG
  61  GATATTTCCGGTGATCTCGTGAACGATGTACCAGTGGCTGTAGACAGAGGGATCTGAGTC
 121  CAGTTCAGGCCAAGCGCGTTGTAGACTGTTTGATGAAGAGGAGGAGAGAGCGAGTTGCGC
 181  AGTGGGTGACCGTAAAGGTAGGCGACACCGTCGCGGTCGGTGGGAATGGTGATTGGCTCC
 241  ATGTTTGTTATGATATAATGATTTTTCCCTTTGCGGGAAATGGGGAAGTACTTGGAAGTG
 301  ACGGAGAGGAGAGGGAAAAGCTGGGGTGTTATAATTTATACGGGGAGCGTTTAGCCGGCT
 361  GCGACCCCCGGAAGAACACTTTCGCCCCCTAGTGGCAGCGGGTCATGCAGAGTCAAAACC
 421  TGCACTCGCTTAACCGGGATCGAAACCCAGCACCCCGTGGGGATTTTTACAGATTGTCGC
 481  TCGTTTAAACTGCTTTAGCTCAGGGTGGTAAATGCTTTCCCCACAATCTCTGCTCGAGTC
 541  ATGCCCAGTGAAATCCTTTCATCCCCGGGTTTCCTTTACCCAGGCCGCCCTCCCCCCGGC
 601  CGCATATAAGGTTCCCTTGAGTCTCCGCTATTTTCCTCCCCTTCTCCATCACCACGTCCT
 661  ATTCTTCTACCTTTAATCAGGGCAAAATGTCCATCCTCGCCTTGGTCGAAGACCGCCCAA
 721  CTCCCCGCGAGGTTTACAACTGGCGCGTCTACCTCCTGGCCGCAGTCGCCTCCTTCACAT
 781  CATGCATGATCGGCTACGACAGCGCCTTCATCGGCACAACGCTGTCTCTGCAATCCTTTC
 841  AAAATGAATTTAATTGGGAATCGCTCAATACGGATCTCATCAGCGCGAATATCGTCTCGC
 901  TATATCAAGCAGGTGCCTTCTTCGGCGCTCTTTTCGCGTACCCCATCGGCCACTTCTGGG
 961  GCCGCCGTTGGGGTCTTATGTTCTCTGCTCTTATTTTCTTCCTTGGTGCCGGCATGATGC
1021  TCGGTGCGAATGGGGATCGAGGGCTAGGGCTGATCTATGGTGGCCGAGTGCTTGCGGGCA
1081  TTGGCGTCGGCGCCGGGTCGAACATCTGTCCTATCTACATCTCAGAAATGGCACCTCCCG
1141  CTATTCGGGGTCGCCTTGTTGGGGTTTATGAGCTGGGTTGGCAGATTGGTGGCGTCGTTG
1201  GGTTCTGGATCAATGTACTTCCCCTTATTTTCTCTGGTTTTCCAGGTTTGCTAACAAGAA
1261  TAGTACGGCGTGGACGAAACCCTTGCCCCAAGCCACAAACAATGGATCATCCCCTTTGCC
1321  GTGCAATTGATTCCTGCTGGCCTGCTCATTATCGGCGCCCTCTTGATCCGCGAATCTCCT
1381  CGTTGGCTCTTCCTCCGCGGTAACCGTGAAAAGGGCATTGAGACCCTGGCCTGGATCCGC
1441  AACCTGCCAGCCGACCATATCTATATGGTCGAGGAAATCAACATGATCGAGCAGTCGCTC
1501  GAACAACAGCGTGTCAAAATTGGGCTTGGTTTCTGGAAACCCTTTAAAGCAGCCTGGACG
1561  AACAAGCGTATTCTCTACCGCCTCTTTTTGGGGTCTATGTTGTTCCTCTGGCAGAACGGG
1621  TCAGGCATCAATGCGATCAACTATTACAGCCCGCGCGTCTTCAAGAGTATTGGCGTATCG
1681  GGTGGCAATACCTCACTCTTGACAACGGGCATTTTCGGTGTCGTCAAGGCGGTCATTACC
1741  TTTGTTTGGCTGTTGTATTTGATCGATCATTTTGGACGACGGAACTTGCTGCTTGTTGGT
1801  GCGGCGGGCGGTTCAGTCTGCCTTTGGATTGTGGGCGGATACATCAAGATCGCGAAGCCA
1861  GAGAACAACCCCGAGGGAACGCAGCTTGATAGCGGTGGCATTGCGGCTATATTCTTCTTC
1921  TACCTGTGGACGGCCTTCTATACACCATCTTGGAACGGCACGCCTTGGGTCATCAACTCG
1981  GTAAGTCCTCCTTCGTGGCCTTCCAAAGAAAGCACATATTAACAGTGTCCGCAGGAAATG
2041  TTCGATCCCACCGTCCGCTCCCTTGCACAAGCCTGTGCTGCCGCCTCAAACTGGCTCTGG
2101  AACTTTCTGATCTCTCGCTTTACGCCCCAGATGTTCACGTCCATGGGATACGGAGTCTAC
2161  TTCTTCTTTGCATCGTTGATGATCCTCTCGATCGTGTTTGTTTTCTTCCTCATTCCCGAG
2221  ACGAAGGGCGTGCCGCTGGAGAGCATGGAGACTCTATTTGACAAGAAACCGGTTTGGCAC
2281  GCCCATTCGCAGCTAATCAGGGAGCTGAGGGAGAATGAGGAGGCGTTCCGGGCGGATATG
2341  GGTGCGAGTGGGAAGGGAGGTGTGACGAAGGAGTATGTCGAGGAGGCTTAAGTAAGCTTG
2401  GTTTCAAGTGATGTATATATGTTATGAGGATATAATATGAACCGAAAGACGATGTTTCTT
2461  GTGAATATTTACGTGATAGTTGTCTGTCTAATATGGTACACCACTAGAACAACTACATAC
2521  GGTCACTACTTACAGCCCTAGTCATTCCCTCCCTGATTGCCTACCATTTATACACTTTGA
```

Figure 5.1 Sequence of *qutD* genomic region. Gray-boxed regions represent three protein coding regions. Red-boxed regions represent the start and stop codons. Yellow-boxed regions represent sequences used to design forward (NKQHAF) and reverse (NKQXhoHAR) primers for PCR (Table 2.3 in Materials and Methods). NKQXhoHAR was designed to incorporate the *Xho*I restriction sequence before the stop codon of the gene.

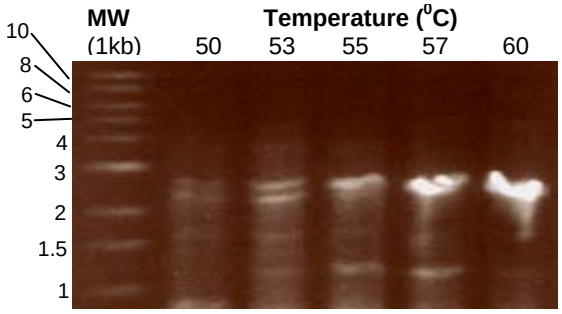


Figure 5.2 Amplification of the *qutD* region from *A. nidulans* genomic DNA using NKQHAF and NKQXhoHAR primers. The gradient PCR temperature ranged from 50 ^{0}C to 60 ^{0}C.

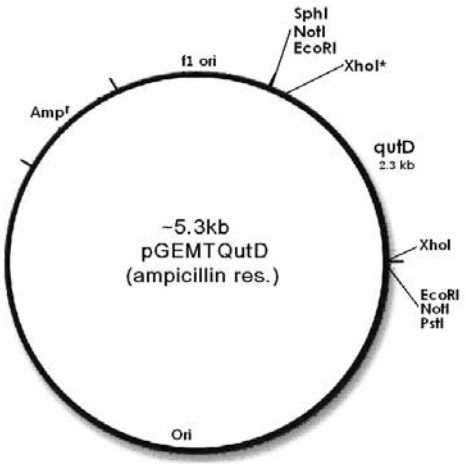


Figure 5.3 Diagram of the pGEMTQutD plasmids. pGEMTQutD contains a 2.3 kb fragment of *qutD* and two *Xho*I sites at -153 upstream (*) and at the 3' end of *qutD*.

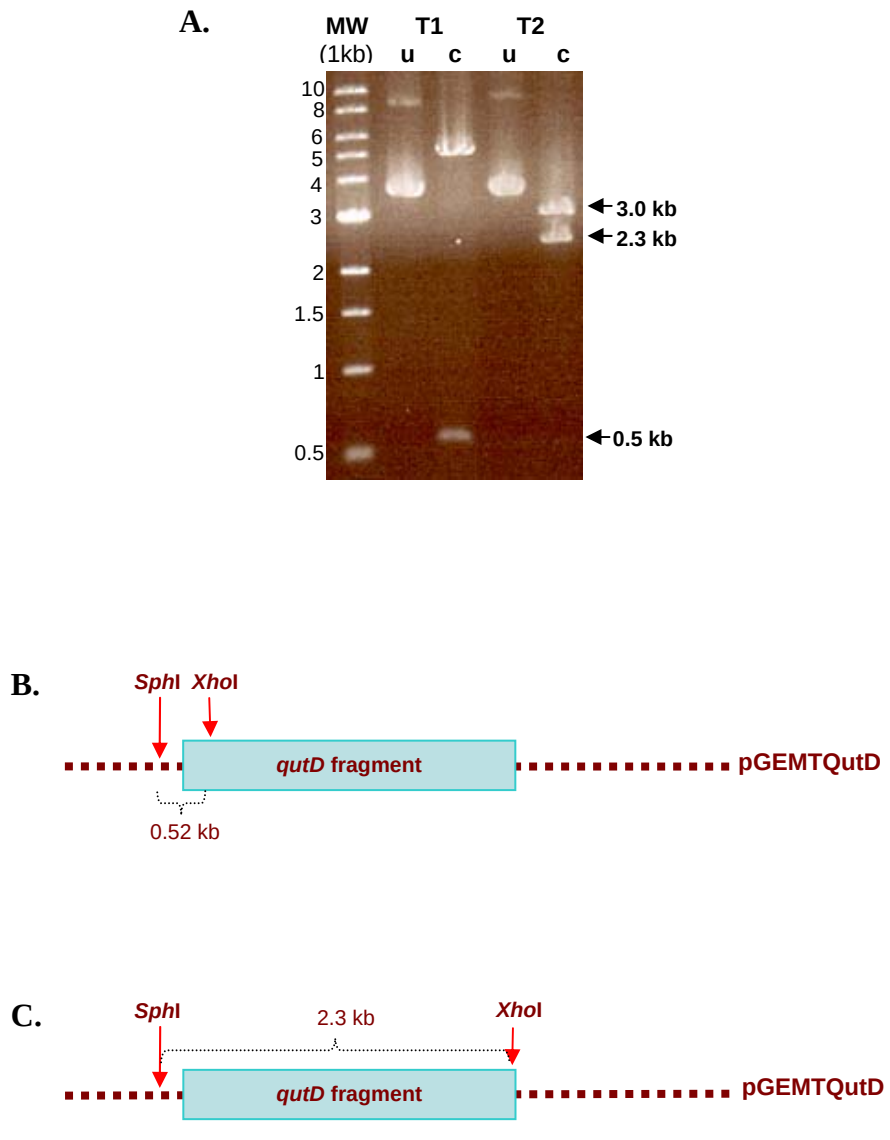


Figure 5.4 pGEMTQutD digestion with *Xho*I and *Sph*I. A: digestion products (C) after digested with *Xho*I and *Sph*I compared to non-digested plasmid (U). B. diagram showing digested fragments when a *Xho*I site is present at 153 base pairs upstream of the *qutD* gene. C: diagram showing digested fragments when a *Xho*I site is at the 3' end of the *qutD* gene. T1# and T2# are the different *E. coli* colonies. Diagrams in B and C are not to scale.

A. HA *Xho*I annealing oligonucleotides:

```
5′ T CGA TAT CCG TAT GAT GTC CCC GAC TAT GCG TT        3′ OligoHA1 [RAL]
3′      ATA GGC ATA CTA CAG GGG CTG ATA CGC AAA GCT   5′ OligoHA2 [RAL]
    arg  Y   P   Y   D   V   P   D   Y   A  phe arg
```

B. *qutD* 3’ coding region:

```
5′ … GGT GCG AGT GGG AAG GGA GGT GTG ACG AAG GAG TAT GTC GAG GAG GCT TAA GTAAGCTT 3′
3′ … CCA CGC TCA CCC TTA CCT CCA CAC TGC TTC CTC ATA CAG CTC CTC CGA ATT CATTCGAA 5′
      G   A   S   G   K   G   G   V   T   K   E   Y   V   E   E   A   *
```

C. PCR of *qutD* 3’ region with inserted *Xho*I sequence:

```
5′ AAG GAG TAT GTC GAG GAG GCT CGA GAG TAA 3′
3′ TTC CTC ATA CAG CTC CTC CGA GCT CTC ATT 5′
    K   E   Y   V   E   E   A  arg asp  *
```

D. Ligation of the *Xho*I digested *qutD* fragment with HA annealing oligonucleotides:

```
5′ AAG GAG TAT GTC GAG GAG GCT CGA TAT CCG TAT GAT GTC CCC GAC TAT GCG TTT CGA GAG TAA 3′
3′ TTC CTC ATA CAG CTC CTC CGA GCT ATA GGC ATA CTA CAG GGG CTG ATA CGC AAA GCT CTC ATT 5′
    K   E   Y   V   E   E   A  arg  Y   P   Y   D   V   P   D   Y   A  phe arg asp  *
```

Figure 5.5 Scheme for insertion HA tag into pGEMTQutD. The *Xho*I site is underlined. The *Xho*I sequence is in blue and the HA tag is in orange.

The purified pGEMTQutD plasmid was digested with *Xho*I restriction enzyme followed by ligation with annealed oligonucleotides coding for the haemagglutinin (HA) tag to create pGEMTQutDHA (Figure 5.6). The annealed HA-oligonucleotides contained ends complementary with those generated by the *Xho*I restriction endonuclease, but which fail to regenerate a *Xho*I site. This allowed the selection of plasmids incorporating the HA epitope tag by digesting plasmids with *Xho*I restriction enzyme, with those containing a correct insertion lacking a *Xho*I site (as indicated in Figure 5.5). The correct insertion of the HA epitope tag in pGEMTQutDHA was confirmed by DNA sequencing and the result is shown in Figure 5.7.

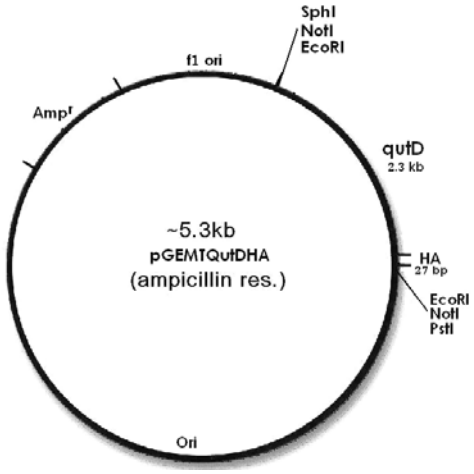


Figure 5.6 Diagram of the pGEMTQutDHA plasmid. The pGEMTQutDHA plasmid contains a HA epitope tag at the 3' end of *qutD*, and does not contains any *Xho*I restriction site.

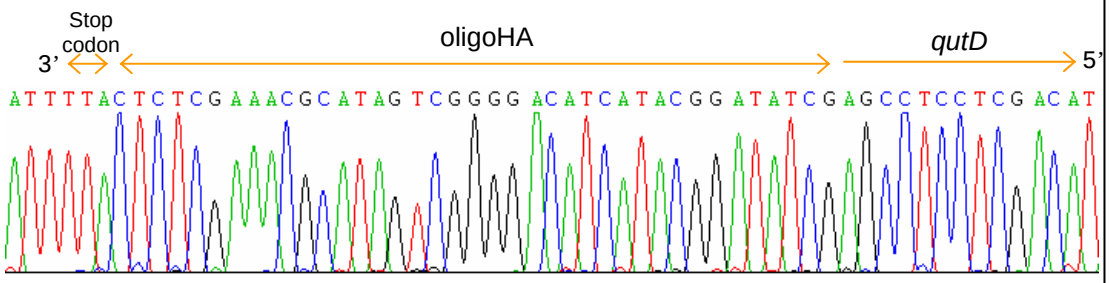


Figure 5.7 Sequencing result of pGEMTQutDHA plasmids showed the correct insertion of HA epitope tag before the stop codon of the *qutD* gene.

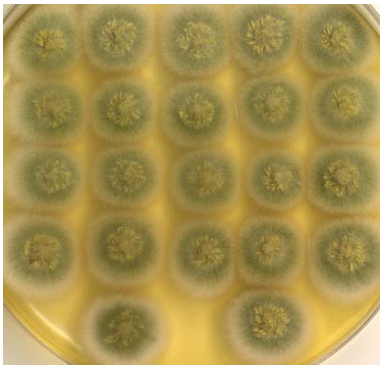
5.4 Transformation of pGEMTQutDHA into the *qutD* mutant strain

The pGEMTQutDHA plasmid was co-transformed with the pPL3 plasmid, as a selectable marker, into a *qutD* mutant strain of genotype *biA1; pyroA4; riboB2 qutD312*. Riboflavin independent transformants were selected, and these were screened for complementation of the *qutD312* mutant phynotype using medium containing 50 mM quinate as the sole added carbon source (Figure 5.8). Of six transformants which grew on selection medium that were tested, one fully complemented (TQ1; isolate 1-1, 1-2, 1-3 and 1-4) the *qutD312* strain, one showed partial complementation (TQ5; isolate 5-3), and four did not show complementation (TQ2, TQ3 and TQ4), and the growth of the fully complementing transformant was similar to the wild-type (*biA1; niiA4*).

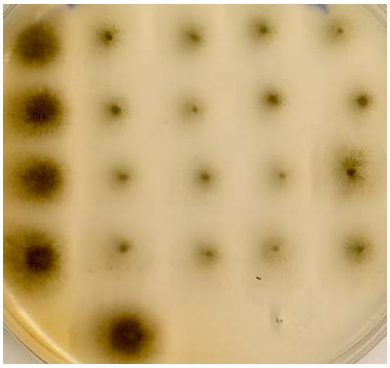
5.5 Southern Analysis of the *qutDHA* transformants

Fully complemented transformants TQ1-1, TQ1-2 and TQ1-3, and partial complemented transformant TQ5-3, were grown in medium containing 50mM quinate as a sole carbon source for 18 hr at 37 ^{0}C, shaking at 120 rpm. Genomic DNA was extracted from the parent strain (*qutD312*), TQ1 and TQ5, digested with *Eco*RI, and transferred to nylon membrane. The membrane was probed with a specific *qutD* probe. As expected, the 6.1 kb native *qutD* gene from the *Eco*RI digestion was detected in all strains, and a additional sequence at 2.3 kb representing *qutDHA* was present in TQ1 and TQ5 transformants (Figure 5.9). Thus, this Southern result showed that the functional copy of the *qutDHA* gene was heterologous integrated into the genome of TQ1 and TQ5 transformants. However, a number of copies of *qutDHA* in these transformants was not precisely quantified, but it can be approximated as 2 copies of *qutDHA* from the experimental results.

A. Complete medium



B. Quinate medium

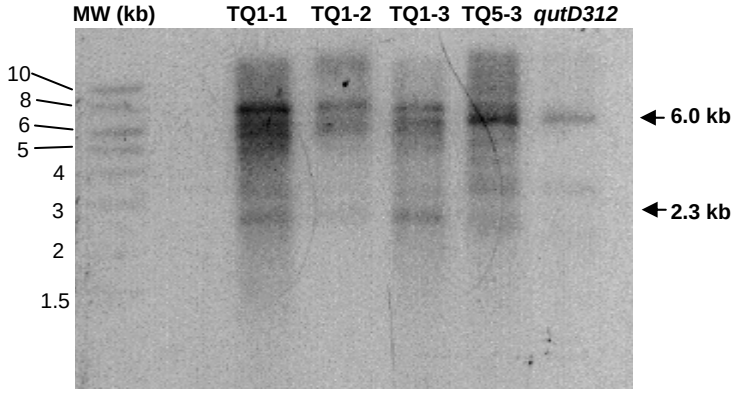


C. Key to strains

TQ1-1	TQ2-1	TQ3-1	TQ4-1	TQ5-1
TQ1-2	TQ2-2	TQ3-2	TQ4-2	TQ5-2
TQ1-3	TQ2-3	TQ3-3	TQ4-3	TQ5-3
TQ1-4	TQ6-1	TQ6-2	TQ6-3	TQ5-4
	WT		*qutD312*	

Figure 5.8 Complementation of *qutD312* by pGEMTQutDHA. TQ1, TQ2, TQ3, TQ4, TQ5 and TQ6 are independent-riboflavin independent transformants grown at 37 ^{0}C for 2 days on medium containing quinate as the sole carbon source. Blue in the Key to strains represents full complementation, red color represents partial complementation, and blak color represents non complementation.

A. Southern analysis of *qutDHA* transformants



B. *Eco*RI restriction sites of *qutD*

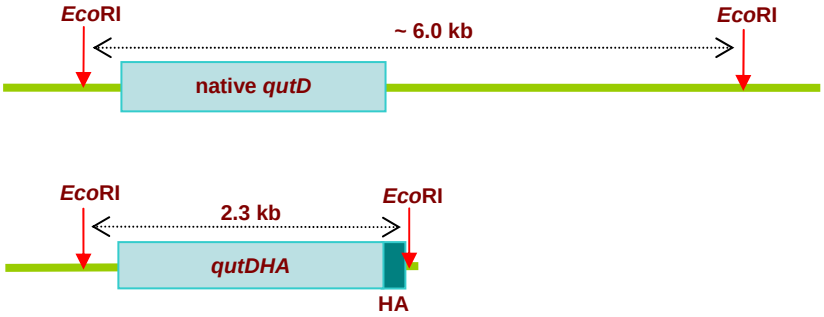


Figure 5.9 Integration of *qutDHA* in the *qutD*312 strain. A) Genomic DNA of TQ1, TQ5 and *qutD312* was extracted and digested with *Eco*RI, transferred to nylon bembrane, and probed with a *qutD* specific probe. B) Diagram showing the expected size of the native *qutD* and heterologous integrated *qutDHA* in *A. nidulans* when digested with *Eco*RI restriction endonuclease.

5.6 Expression of pGEMTQutDHA in a *qutD* mutant strain

Expression of pGEMTQutDHA in the *qutD* mutant strain was analysed by Western analysis. Strains containing QutDHA, TQ1-1, TQ1-2, TQ1-3, and TQ5, were grown overnight in medium containing 50 mM quinate as sole added carbon source at 37 ^{o}C, shaking at 120 rpm. Fungal mycelia were harvested and protein extracts were analysed by acrylamide gel electrophoresis followed by Western blotting. The membranes were probed with peroxidase conjugated with anti-HA monoclonal antibody to identify the HA tagged quinate permease. The result is shown in Figure 5.10A. High levels of a lower molecular weight (40kDa) N-terminally processed form of QutDHA were detected, and lower levels of the predicted full length QutDHA peptide (60kDa) were detected in an undiluted protein extract. There was more QutDHA in TQ1 transformants compared with TQ5 transformed strains, consistent with the observation that TQ1 complements fully whereas TQ5 is only partially complemented for growth on quinate medium.

Expression of *qutDHA* was also compared in repressing and derepressing conditions by growing TQ1-1 in medium containing either glucose (repressing condition), glucose and quinate (induced-repressing condition), or quinate (induced-derepressing condition) as a carbon source(s). Western analysis with anti-HA is shown in Figure 5.10B. The QutDHA protein was detected only in the induced-derepressing condition in medium containing quinate as the sole carbon source, but not in the repressing condition either in medium containing only glucose or glucose plus quinate. Thus, the expression of QutDHA protein is strongly repressed by glucose.

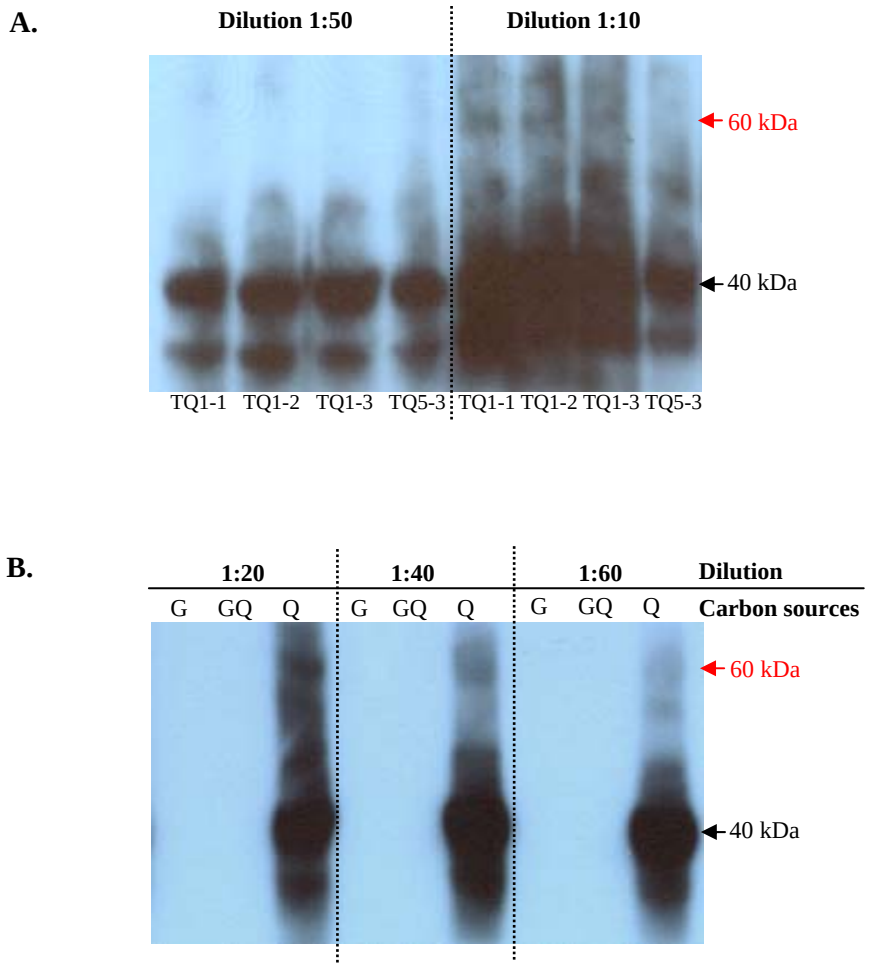


Figure 5.10 Westerns analysis to determine the expression of QutDHA in a *qutD312* strain. A) Western blot of total protein extracted from strains TQ1 (TQ1-1, TQ1-2, TQ1-3) and TQ5. QutDHA was detected at 40 kDa (black arrow), and at 60 kDa (red arrow). B) Western blot of total protein extract of TQ1-1 grown in medium containing glucose (G), glucose and quinate (GQ), and quinate (Q) and a sole carbon source(s). Culture conditions for both A and B were at 37 ^{o}C for 18 hr. Protein extracts were separated by SDS-PAGE and the Western was probed with anti-HA antibody.

5.7 Co-Immunoprecipitation of CreBFLAG and QutDHA

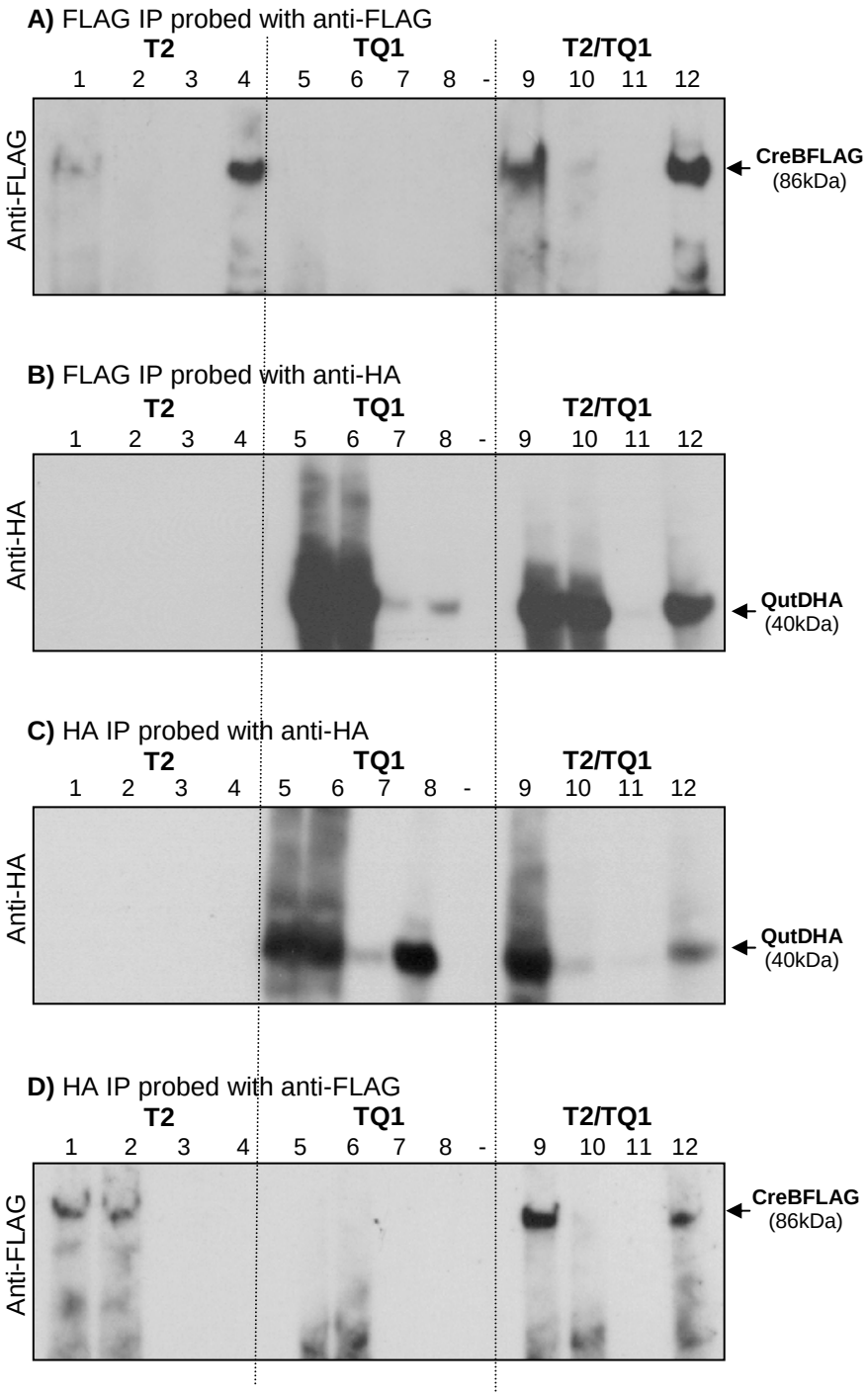
In order to test whether CreB and QutD form a complex *in vivo*, tagged CreB and QutD proteins were produced in the same strain by making a diploid strain from two haploid strains, T2 [*creB1937*::CreBFLAG] strain and TQ1 [*qutD312*::QutDHA]. This diploid displayed a wild-type phenotype, and expressed both wild-type and tagged forms of both proteins. The diploid and both parent haploid strains were grown, shaken for 18 hrs at 37 ^{o}C, in medium containing quinate as a sole carbon source, and mycelia were recovered and proteins extracted as described in experimental procedures. CreBFLAG and QutDHA were precipitated from the total protein extracts using anti-FLAG monoclonal antibody conjugated to agarose (antiFLAG::M2 agarose), and anti-HA affinity matrix, respectively. In each case, four samples (total extract; supernatant of the precipitation; washing fraction; and the immunoprecipitate) were separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Figure 5.11A and 5.11B show the results of Western Blots where the anti-FLAG precipitated proteins were probed with anti-FLAG and anti-HA antibodies, respectively, and Figure 5.11C and 5.11D show the results of Western Blots where the anti-HA precipitated proteins were probed with anti-HA and anti-FLAG antibodies, respectively.

As shown in Figure 5.11A, CreBFLAG was precipitated using anti-FLAG from T2 (track 4), and from the diploid (track 12) strains, but not from TQ1 (track 8) as expected. CreBFLAG was also detected in cell lysate of T2 and the diploid (track 1 and 9). When this filter was probed with anti-HA (Figure 5.11B), QutDHA was detected strongly in the cell lysate (track 5) and supernatant (track 6) but only very weakly in the wash fraction (track 7) and the fraction that was precipitated using anti-FLAG (track 8), whereas in the diploid strain, QutDHA was detected strongly in the fraction that was precipitated using anti-FLAG (track 12). Thus a proportion of QutDHA was co-immunoprecipitated from the protein extracts when anti-FLAG::M2 agarose was used to precipitate CreBFLAG.

A reciprocal experiment was also undertaken in which anti-HA affinity matrix was used to precipitate QutDHA. As shown in Figure 5.11C, QutDHA was precipitated using anti-HA from TQ1 (Track 8), and from the diploid strain (track 12), but not from T2 (track 4) as expected. When this Western was probed with anti-FLAG (Figure 5.11D), in the haploid T2, CreBFLAG was detected strongly in the cell lysate (track1) and supernatant (track 2) but not in the wash fraction (track 3) and the fraction that was

precipitated using anti-HA affinity matrix (track 4). TQ1 as expected showed no CreBFLAG at all. In the diploid strain, CreBFLAG was detected strongly in the fraction that was precipitated using anti-HA affinity matrix (track 12). Thus a proportional of CreBFLAG was co-immunoprecipitated from the protein extracts when anti-HA affinity matrix was used to precipitate QutDHA.

Figure 5.11 Co-immunoprecipitation of CreBFLAG and QutDHA. Strains T2, TQ1 and a Diploid strain between T2 and TQ1 were grown in medium containing 1% quinate as the sole carbon source. The mycelium was harvested and protein extracted. Proteins were purified using Anti-FLAG::M2 agarose or anti-HA affinity matrix. Figure A and B show the results of a Western where the anti-FLAG precipitated proteins probed with anti-FLAG and anti-HA antibodies, respectively, and Figure C and D show the results of a Western where the anti-HA precipitated proteins were probed with anti-HA and anti-FLAG antibodies, respectively. In each case, Track 1-4 are T2, tracks 5-8 are TQ1, and track 9-12 are the diploid between T2 and TQ1. Tracks 1, 5 and 9 contain sample of proteins lysate (L); tracks 2, 6 and 10 contain supernatant (S); track 3, 7 and 11 contain the wash fraction (W); and track 4, 8 and 12 contain the immumoprecipitate (I). The membranes were first probed with anti-FLAG::peroxidase-linked monoclonal antibody detecting the FLAG-tagged CreB protein, and reprobed with anti-HA::peroxidase-linked monoclonal antibody (high affinity) detecting the HA-tagged protein.


A) FLAG IP probed with anti-FLAG
T2
TQ1
T2/TQ1
1 2 3 4 5 6 7 8 - 9 10 11 12
Anti-FLAG
CreBFLAG
(86kDa)
B) FLAG IP probed with anti-HA
T2
TQ1
T2/TQ1
1 2 3 4 5 6 7 8 - 9 10 11 12
Anti-HA
QutDHA
(40kDa)
C) HA IP probed with anti-HA
T2
TQ1
T2/TQ1
1 2 3 4 5 6 7 8 - 9 10 11 12
Anti-HA
QutDHA
(40kDa)
D) HA IP probed with anti-FLAG
T2
TQ1
T2/TQ1
1 2 3 4 5 6 7 8 - 9 10 11 12
Anti-FLAG
CreBFLAG
(86kDa)

5.8 Expression of QutDHA in a strain containing a *creB* null mutation

5.8.1 *Aspergillus* crosses between QutDHA containing strain with *creB1937* mutant strain.

A strain containing *qutDHA* was crossed with a strain containing *creB1937*. To identify a strain containing both *qutDHA* and the *creB1937* gene, the progeny were tested on complete medium, quinate medium, and on minimal medium containing allyl alcohol (Figure 5.12). Progeny from the cross, TQBD5 and TQBD6, grew better than the *qutD312* and *creB1937* mutant strains on quinate media. However, these two progeny could not grow on glucose medium containing an allyl alcohol, similar to the *creB1937* mutant strain. The growth testing indicated that the selected progeny contain pGPDQutDHA in a *creB* mutant background.

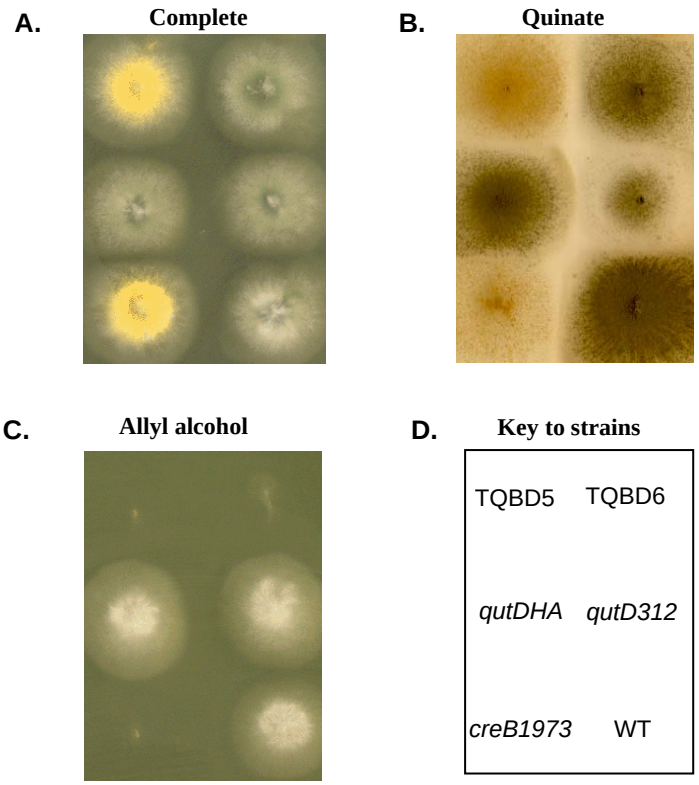


Figure 5.12 Growth testing of TQBD5 [*creB1937qutDHA*] and TQBD6 [*creB1937 qutDHA*]. A; media containing 1% glucose, B; 50mM quinate, and C; 1% D-glucose containing 12mM allyl alcohol, after culture at 37 ^{o}C for 2 days. The key to strain placement is show in D.

5.8.2 Southern analysis to confirm *qutDHA* in the progeny.

The presence of *creB1937* could be determined using specific growth tests, but Southern analysis was required to confirm that a copy of the *qutDHA* gene was also present in the potential *creB1937;qutDHA* containing strains (TQBD5 and TQBD6), although these strains grew better on quinate medium than the *creB* parent strain. Genomic DNA of TQ1, TQBD5, TQBD6, TQBD7 (negative control which could not grow on quinate medium), and *qutD312* mutant strains was extracted, digested with *Eco*RI restriction endonuclease, and separated in a 0.8% agarose gel before transferred to nylon membrane. A *qutD* specific probe detected a 2.3 kb *qutDHA* fragment in both potential *creBa937;qutDHA* strains, TQBD5 and TQBD6, and the parent strain (TQ1), but not in TQBD7 or *qutD312* mutant strains (Figure 5.13). The 6.1 kb of *Eco*RI digested fragment of the native *qutD* was detected in all strains. This result indicated that the heterogeneous integration of the *qutDHA* transgene in the *A. nidulans* genome seen in the TQ1 parent had been inherited by TQBD5 and TQBD6.

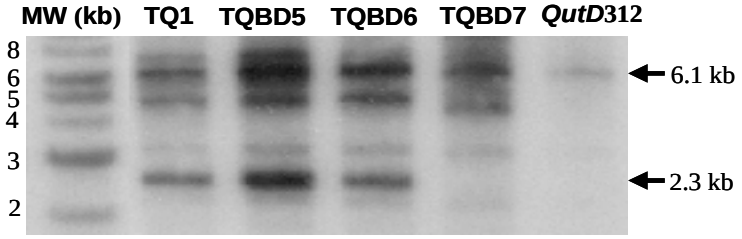


Figure 5.13 Southern analysis of *qutDHA* in a *creB1937* background. Genomic DNA was extracted from TQ1, TQBD5, TQBD6, TQBD7 and QutD312, digested with *Eco*RI, transferred to nylon membrane, and probed with a labeled *qutD* specific probe. The diagnostic 2.3 kb of *qutDHA* band and the native 6.1 kb DNA of *Eco*RI digested fragment are indicated by arrows.

5.8.3 Western and Northern analysis of *creB1937;qutDHA* strain.

Western blots were performed to analyse the amount of QutDHA protein in a *creB1937* mutant background compared to the wild-type (*creB*+) background. The strains were grown in medium containing quinate as the sole carbon source. Total protein extracts from TQ1, TQBD5 and TQBD6 were extracted from mycelia, separated on a SDS-PAGE gel, and transferred to the polyvinylidene difluoride (PVDF) membrane. QutDHA was detected using anti-HA monoclonal antibody conjugated with peroxidase as in the immunoprecipitation experiments and the result is shown in Figure 5.14A. Less QutDHA was detected in the strains lacking CreB than in the wild-type CreB containing parent strain, indicating that the level of the QutD protein is affected by the CreB protein in the cell. To rule out the possibility that the differences in the amount of QutDHA in a wild-type compared to CreB null strain were due to transcriptional differences, RNA was prepared from the strains after growth in the 1% glucose containing medium for 16 hrs and transfer to the quinic acid containing medium for a further 4 hrs, and the amount of *qutD* transcript determined by Northern transfer analysis (Figure 5.14B). Clearly, despite the lower level of the QutD protein seen by Western analysis, there is actually more messenger RNA for QutD in a *creB* mutant background. This may be the result of derepression of transcription due to the impaired carbon catabolite repression seen in *creB* mutant strain. This result clearly confirms that *creB* is required for stability of the QutD protein. The difference in level of expression of *qutDHA* between TQBD5 and TQBD6 strain was not further investigated.

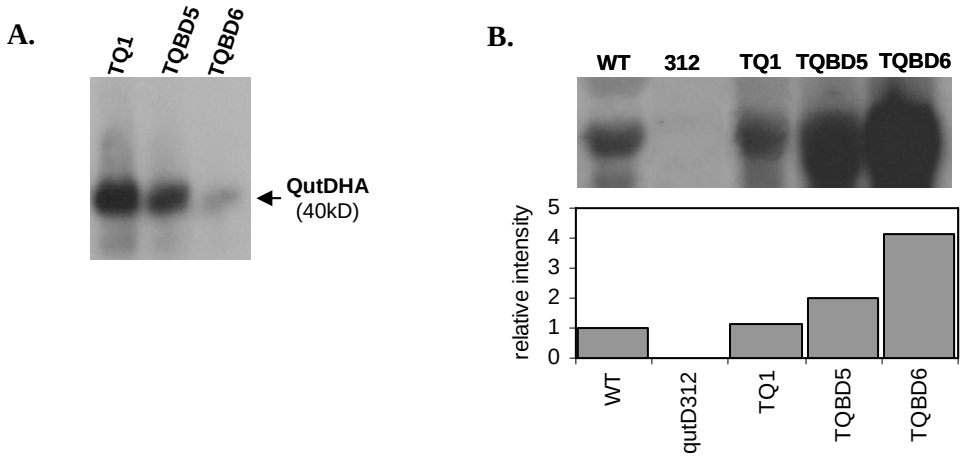


Figure 5.14 Expression of the *qutDHA* in the *creB*$^{+}$ and *creB*$^{-}$ background. A); Western analysis of QutDHA in total protein extract of TQ1, TQBD5 and TQBD6 strains and separated by SDS-PAGE. QutDHA was detected using anti-HA antibody. B); Northern analysis of *qutDHA*. TQ1, TQBD5, TQBD6, and *qutD312* were grown for 16 hours in medium containing 1% glucose and transferred for a further 4 hours to medium containing of 50mM quinate, before RNA was extracted from mycelia, separated by electrophoresis, and probed with a ^{32}P-labeled *qutD* specific probe. Intensity is shown relative to wild-type. Similar amounts of protein and RNA were used in all strains in the Western and Northern blots.

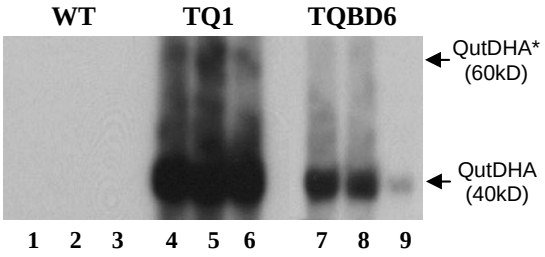
5.9 QutD is a ubiquitinated protein

In order to determine whether QutD is ubiquitinated and thus explaining the protective effect of CreB *in vivo*, the ubiquitination status of the tagged QutDHA protein, both in wild-type *creB* and *creB*-null backgrounds, was compared, along with a wild-type *A. nidulans* control strain (*biA1;niiA4*). Cultures were grown, shaking for 18 hrs at 37 ^{o}C, in medium containing quinate as a sole carbon source. Fungal mycelia were harvested and proteins extracted as described in the experimental procedures. Ubiquitinated proteins were separated from the total protein extracted using the UbiQapture-Q matrix kit (BIOMOL). Three fraction samples (total protein extract, unbound fraction and immunoprecipitate) of each strain were separated by SDS-PAGE, and transferred to

polyvinylidene difluoride (PVDF) membrane. Figure 5.15 shows the results of Western blot, probed with anti-HA antibody (Figure 5.15A) and ubiquitin-conjugate specific antibody (Figure 5.15B). The Western results showed that QutDHA was precipitated using UbiQapture-Q matrix. Anti-HA detection was very strong in the immunoprecipitated fraction of TQ1 strain (track 6 in Figure 5.15A) but not in the wild-type strain lacking HA tagged protein (track 3 in Figure 5.15A). Lower levels of QutDHA were present in the precipitated fraction of TQBD6 (track 9 in Figure 5.15A), which contains QutD protein in the *creB* mutant background. QutDHA was also detected weakly in the total protein extract and unbound fractions of TQBD6, and this result supports the results using this strain in the CreB/QutD co-immunoprecipitation experiment. QutDHA was also present in the unbound fraction of TQ1 and TQBD6 (track 5 and 8 in Figure 5.15A) because of the limiting capacity of the matrix used in the separation. The UbiQapture-Q matrix only precipitated a proportion of ubiquitinated protein, as there were significant amounts of ubiquitinated protein detected in the unbound fractions (track 2, 5, 8 in Figure 5.15B). Thus a proportion of QutDHA is ubiquitinated in the cell, but less ubiquitinated QutDHA is present in a *creB* mutant background, suggesting a role for CreB protein in stabilizing the ubiquitinated form of the quinate permease.

Thus, these results demonstrate that QutDHA is ubiquitinated, and the CreB deubiquitination enzyme is required to prevent degradation of QutDHA in the cell.

A. Probed with anti-HA antibody



B. Probed with Ubiquitin-conjugate specific antibody

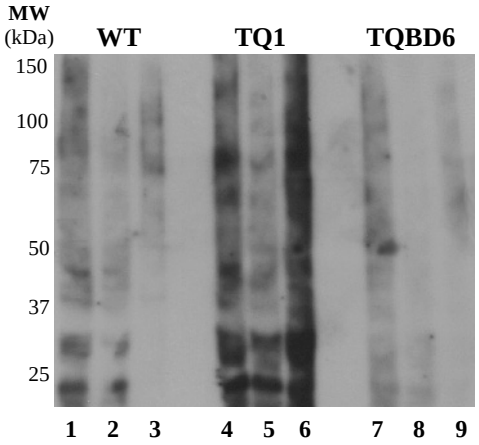


Figure 5.15 QutD is a ubiquitinated protein. Strains TQ1, TQBD6 and wild-type were grown overnight in medium containing 1% quinate as the sole carbon source. Figure A. shows the results of a Western where ubiquitinated proteins were purified using UbiQapture™-Q matrix, and the membrane was probed with anti-HA::peroxidase-linked monoclonal antibody detecting the HA-tagged protein. Figure B. shows the results of a Western of the same membrane when probed with the ubiquitin-conjugate specific primary antibody and the HRP-Donkey anti-mouse secondary antibody detecting the ubiquitinated protein. In each case, Track 1-3 are wild-type, tracks 4-6 are TQ1, and track 7-9 are the TQBD6. Tracks 1, 4 and 7 contain sample of proteins lysate (L); tracks 2, 5 and 8 contain unbound fractions (UB); and track 3, 6 and 9 contain the immumoprecipitate (I). (* full length of QutDA). Similar amounts of protein were used in all strains in this experiment. Pictures shown here represent the proportion of QutDHA in control (WT), in *creB*$^{+}$ background (TQ1) and in *creB*$^{-}$ background (TQBD6).

5.10 Summary

To determine whether the quinate permease (QutD) is a substrate of the CreB/CreC deubiquitination complex, epitope tagged versions of QutD were constructed and introduced into the *qutD312* mutant strain. Transformants were checked for *qutD* expression both by phenotype testing on specific media, Southern analysis to comfirm integration of the transgene, and Western analysis using specific antibodies directed against the HA epitope and Myc epitope tags.

An N-terminally tagged version of QutD was found not to be functional. Therefore, a new *qutD* construct was designed that expressed a C-terminally tagged version of *qutD* from its own promoter. This construct, pGEMTQutDHA, was transformed into the *qutD* mutant strain by co-transformation. One transformant fully complemented the *qutD* mutant strain, and another transformant partially complemented the *qutD* mutant on the quinate medium. These two transformants were selected for Southern analysis to determine whether they had *qutDHA* integrated heterogenously. Southern analysis showed that the functional copy of *qutDHA* was hetergenously integrated in the genome of those transformants, and that the native *qutD* fragment was intact in all strains. Thus, a copy of the *qutDHA* gene was present in the transformant genome. Western analysis was used to determine the expression of pGEMTQutDHA in the *qutD* mutant strain. The HA-tagged QutD could be easily detected on Western transfers probed with the anti-HA monoclonal antibody when QutD was induced by growth on quinate. QutDHA was detected by using an anti-HA antibody in both a fully and partial complementing transformant. However, the amount of QutHDA was lower in the partially complementing strain. QutDHA was detected only in mycelia grown in derepressing conditions, without glucose in medium, thus QutDHA is strongly regulated by carbon catabolite repression.

In order to determine whether QutD and CreB form a complex *in vivo*, a diploid containing QutDHA and CreBFLAG was made between a fully complementing transformant (TQ1) and a strain expressing a CreBFLAG construct (T2). Western results of the Co-IP experiments with anti-FLAG agarose showed that QutDHA was present in the precipitate of the diploid, and CreBFLAG was also present in the precipitate of T2 and the diploid strains confirming that the IP procedure worked satisfactorily. As expected, the anti-HA antibody and anti-FLAG antibodies did not detect any particular protein in T2 and TQ1 haploid strains, respectively. The reciprocal

experiment was also performed in which QutDHA was precipitated with anti-HA matrix. Anti-FLAG antibody detected CreBFLAG in the diploid and T2 strain, but not in the TQ1 strains. As well as anti-HA antibody detected QutDHA in the diploid and TQ1 strain, but not in the T2 strain. However CreBFLAG was only detected in the immunoprecipitate of the diploid strain. Thus, the CreB deubiquitinating enzyme is present in a complex with QutD *in vivo*.

To study the effect of CreB on the QutD protein, strains expressing QutDHA in a *creB* null mutant background were made by crossing. Copies of both the *creBFLAG* and the *qutDHA* genes were confirmed to be present in the progeny by phenotype testing, Southern and Western analysis. Western results showed less QutDHA was detected in a *creB* mutant background, indicating that the concentration of QutD is affected by the CreB protein in the cell. Northern analysis showed that the failure of *creB* mutant strains to grow on quinate medium was not due to a failure of transcriptional induction of *qutD*, as the amount of mRNA was higher in a *creB1937* mutant background compared to a *creB*$^{+}$ background.

Furthermore, an experiment was done to provide evidence to show that QutD is an ubiquitinated protein *in vivo*. Total proteins from the TQ1 and TQBD6 strains were precipitated with the UbiQapture-Q matrix, which interacted with ubiquitinated proteins, and separated with SDS-PAGE. When using the anti-HA monoclonal antibody, QutDHA was present in the immonuprecipitate, indicating that QutD was ubiquitinated in *A. nidulans*.

Thus, results present in this chapter showed that QutD was ubiquitinated *in vivo*, clearly bound with CreB deubiquitination protein when precipitated with the specific antibody to QutD (anti-HA) or to CreB (anti-FLAG), and the stability of QutD depended on the presence of CreB in the cell.

CHAPTER 6: IDENTIFICATION OF PROTEINS INTERACTING WITH CREA USING A PROTEOMIC APPROACH

6.1 Introduction

The DNA-binding protein, CreA, in *A. nidulans* is a member of the Cys2-His2 zinc finger class (Dowzer and Kelly, 1991) that has been shown to bind to a consensus sequence 5'-SYGGRG-3' in the regulatory region of genes subject to carbon catabolite repression to effect their repression (Kulmburg *et al.*, 1993). Experimental results on the interaction of CreA and CreB in Chapter 3 show that CreA is a ubiquitinated protein, and a probable substrate of the CreB/CreC deubiquitinating complex in *A. nidulans*. Althrough regions of CreA are similar to Mig1p in *S. cerevisiae* (Nehlin *et al.*, 1991), the repression mechanism of these two proteins are quite differenct. How CreA binding results in repression is not known, and other proteins that interact with CreA have not been identified. This chapter reports experiments aimed to identify proteins that interact with CreA. The approach used was immunoprecipitation of tagged CreA, followed by a proteomics approach to identify proteins that were co-immunoprecipitated.

6.2 Purification of CreA2HAHis and Western analysis

The wild-type strain, *biA1;niiA4*, and a strain containing CreA2HAHis, TA3H2 (Figure 3.1 in Chapter 3), were grown overnight in medium containing glucose as the sole carbon source, at 37 ^{o}C. In order to purify CreA2HAHis, total protein extracts were double purified, first with a His-Trap affinity column and then using a HA-matrix. SDS-PAGE was used to separate the protein fractions before transfer to a PVDF membrane. Western analysis using an anti-HA antibody was used to detect the CreA2HAHis protein in the total protein extract, purified fraction from the His-Trap column, and purified fraction from the HA-matrix of the CreA2HAHis containing and control strains. The result is shown in Figure 6.1. In the TA3H2 strain, CreA2HAHis was detected in the total protein extract, in the fraction from the His-Trap purification, and after HA-matrix purification. As expected, no signal was present in the control strain when using an anti-HA antibody. There were clearly two apparent molecular weight classes after His-Trap purification, possibly representing differently modified forms of the protein

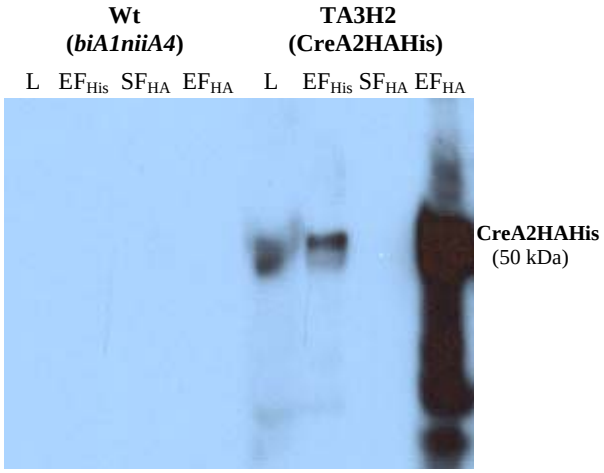


Figure 6.1 Two-step purification of CreA2HAHis. For each strain: L, cell lysate; EF_{His}, eluted fractions from His-Trap column; SF_{HA}, supernatant of HA-matrix; EF_{HA}, eluted fraction of HA-matrix. The membrane was probed with an anti-HA peroxidase conjugated antibody.

6.3 Silver staining of proteins

The fractions from this purification procedure were separated on a 10% polyacrylamide gel followed by further silver staining to identify the different proteins that might be present. Proteins were detected in the total protein lysate and all fractions of purification, both in TA3H2 and control strains. However, there were no obvious differences in the final purified fraction (EF_{HA}) between the control and TA3H2 strains (Figure 6.2), even though the detection of the HA epitope tag was very strong in the immunoprecipitate from the HA matrix in the TA3H2 strain. This is due to the far greater sensitivity of Western analysis compared to silver staining. However, there were many proteins present after two rounds of purification in both the experimental and control strains, and a 50 kDa band was present in the immunoprecipitate of both tracks indicating another protein was present in the same position as CreA2HAHis.

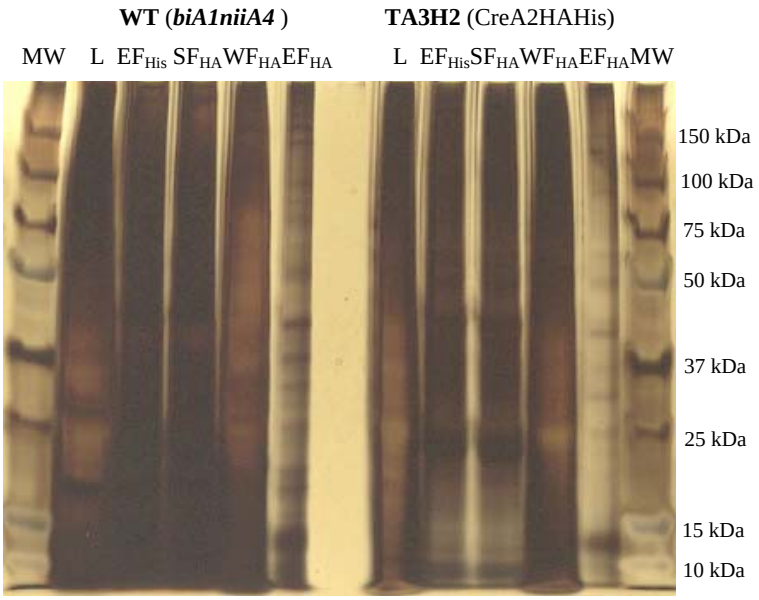


Figure 6.2 Silver staining of protein fractions of the control and TA3H2 strains when purified with a His-Trap column and anti-HA matrix. In each strain: L, cell lysate; EF_{His}, eluted fractions from His-Trap column; SF_{HA}, supernatant of HA-matrix; WF_{HA}, Wash fraction of HA-matrix; EF_{HA}, eluted fraction of HA-matrix.

A one step purification using only the HA-matrix was also used to purify CreA2HAHis from the total protein extract of the TA3H2 strain and the wild-type control, before separation on a SDS-PAGE and staining the gel with a silver staining kit (Invitrogen). Two separate cultures were used for protein extraction and purification. Silver staining of the gel showed 6 protein bands which were present in TA3H2 but not in the control strain. Three were present in the first batch, at about 75, 85 and 130 kDa, respectively (marked in Figure 6.3A). A further three were present in the second batch of total protein extract, at about 35, 50 and 75 kDa, respectively (marked in Figure 6.3B). These protein samples were extracted from the gel and further analyzed by the Adelaide Proteomics center.

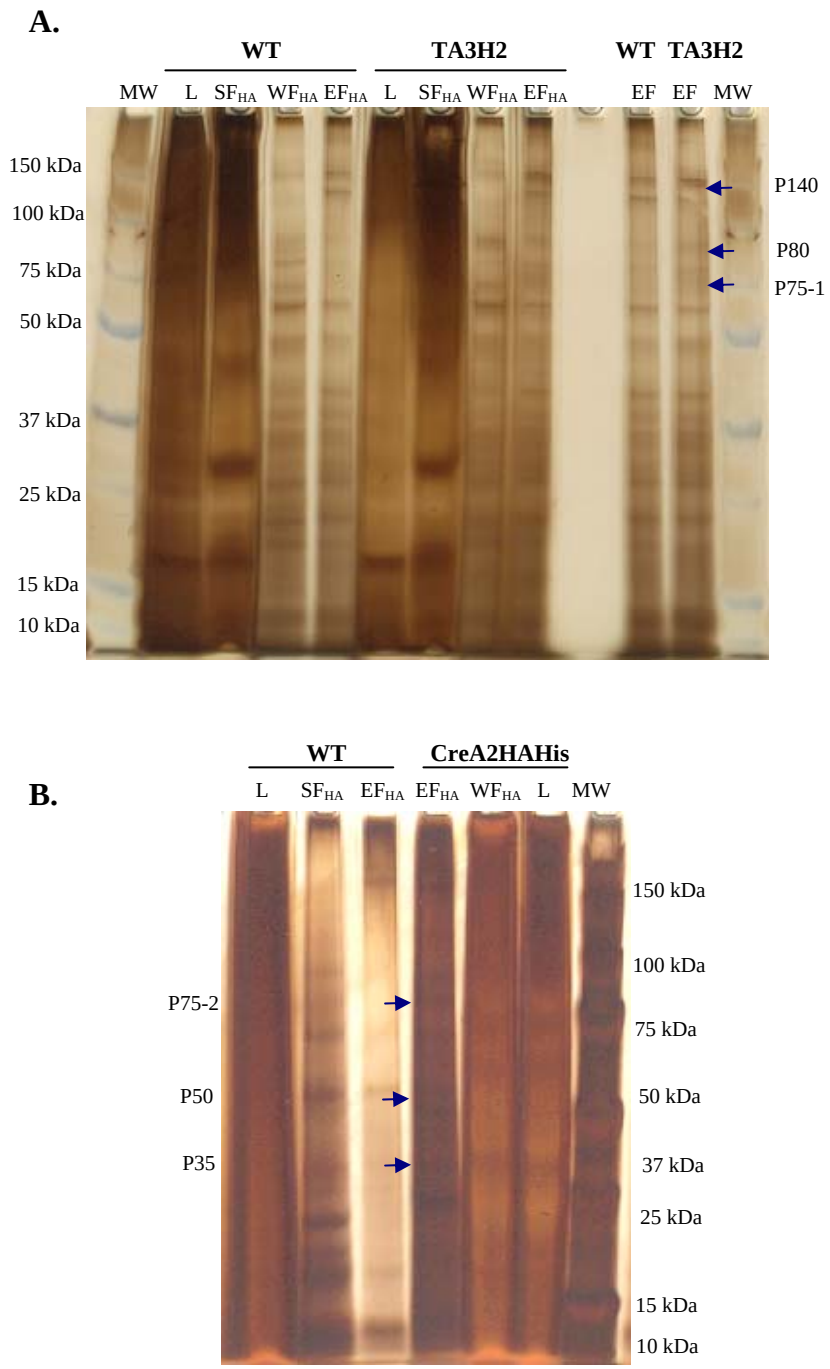


Figure 6.3 Silver staining of protein fractions of the control and TA3H2 strains using anti-HA matrix. Total protein extract from both strains were purified with HA-matrix, before gel sepatation and silver staining. Six different protein bands from TA3H2 were marked arrowhead, three were present in the first batch (A) and others three present in the second batch (B). In each strain: L, cell lysate; SF_{HA}, supernatant of HA-matrix; WF_{HA}, washed fraction of HA-matrix; EF_{HA}, eluted fraction of HA-matrix.

6.4 Proteomic analysis

The six bands that appeared stronger in TA3H2 in silver staining were recovered and analysed. The result is shown in Table 6.1. Sample P50, P75-1 and P75-2 produced peptide sequence results that showed no match to any *A. nidulans* protein. P35 was *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase, and the analytical result with 5 matching peptides is shown in Figure 6.4. P80 was human keratin, indicating the possibility of contamination during processing, and P140 did not contain sufficient protein to analyze.

Glyceraldehyde-3-phosphate dehydrogenase (GPD, EC 1.2.1.12) is one of the key enzymes in the glycolytic and gluconeogenesis pathways (Piechaczyk *et al.*, 1984). This gene, encoded by the *gpdA* gene, had been reported to be highly expressed in different organisms including *A. nidulans* (Osiewacz and Ridder, 1991; Punt *et al.*, 1988; Punt *et al.*, 1990). CreA is a DNA binding protein. The finding of GPD in the protein fraction when CreA was purified indicated that GPD might interact with the CreA protein, when the mycelia was grown in glucose medium, but it is perhaps more likely that this is just a highly expressed protein. P35 ran in a region of the containing significant silver staining, and it is possible that GPD could be a band that was corecoverd with the band of interest.

The purification of CreA prior to separation with SDS-PAGE enriched the CreA protein, but this approach did not result in the detection of other proteins which interact with CreA in the cell. This may be due to the low stability of CreA in the cell resulting in the low level of the complex between CreA and other proteins for MS&MS/MS analysis. Further, although CreA was overexpressed, potential interacting proteins were not overexpressed and thus they may only be present in very low amounts.

Table 6.1 Combined MS and MS/MS MASCOT results summary of protein samples.

Sample	**Protein Identification**	**Organism**	**Peptides matched**	**% seq. MS/MS**	**Observed MW**	**Predicted MW**
P140	Low protein in sample				140kD	
P80	Human keratin	Human			80kD	
P75-1	No match				75kD	
P75-2	No match				75kD	
P50	No match				50kD	
P35	Glyceraldehyde-3-phosphate dehydrogenase	*Emericella nidulans*	5	19%	35kD	36.3kD

1. DEASG3 Mass: 36403 Score: 308 Queries matched: 5

glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12) - Emericella nidulans

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
107	574.30	1146.59	1146.64	-0.05	0	58	0.0007	1	K.AGIALNSNFIK.L
108	575.81	1149.61	1149.66	-0.06	0	61	0.00027	1	R.VVDLITYISK.V
109	701.80	1401.59	1401.75	-0.16	0	38	0.054	1	R.TAATNIIPSSTGAAK.A
111	743.36	1484.71	1484.82	-0.11	0	85	8.9e-007	1	R.VPTSNVSVVDLTVR.T
112	804.83	1607.65	1607.80	-0.16	0	65	8.2e-005	1	K.GTIETYDEGLIVNGK.K

Matched peptides shown in **Bold Red**

```
  1 MAPKVGINGF GRIGRIVFRN AIEAGTVDVV AVNDPFIETH YAAYMLKYDS
 51 QHGQFKGTIE TYDEGLIVNG KKIRFHTERD PANIPWGQDG AEYIVESTGV
101 FTTQEKASAH LKGGAKKVVI SAPSADAPMF VMGVNNETYK KDIQVLSNAS
151 CTTNCLAPLA KVINDNFGII EGLMTTVHSY TATQKVVDGP SAKDWRGGRT
201 AATNIIPSST GAAKAVGKVI PSLNGKLTGM AMRVPTSNVS VVDLTVRTEK
251 AVTYDQIKDA VKKASENELK GILGYTEDDI VSTDLNGDTR SSIFDAKAGI
301 ALNSNFIKLV SWYDNEWGYS RRVVDLITYI SKVDAQ
```

Search Parameters

```
Type of search          : MS/MS Ion Search
Enzyme                  : Trypsin
Fixed modifications     : Carbamidomethyl (C)
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 0.3 Da
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages    : 1
Instrument type         : ESI-TRAP
Number of queries       : 119
```

Figure 6.4. The proteomic analysis of the P35 sample maching to glyceraldehyde-3-phosphate dedydrogenase

CHAPTER 7: GENERAL DISCUSSION

Aspergillus nidulans is able to utilize a wide range of different carbon sources other than the preferred glucose. When glucose is not present, the fungus is capable of breaking down other compounds such as ethanol, starch and cellulose, to supply a carbon source to the cell. There are many genes required for utilizing such alternative carbon sources which produce permeases to transport these compounds into the cell and enzymes to break them down into useable substrates. The expression of these genes is controlled by complex regulatory mechanisms, including carbon catabolite repression (CCR). In *A. nidulans,* this process is controlled by the product of the *creA* gene. Genetic analysis indicates that CreA is a wide domain, negatively acting repressor of transcription (Arst and Cove, 1973; Cubero and Scazzocchio, 1994; Dowzer and Kelly, 1991). This system is quite different from that in the yeast *S. cerevisiae*. Although the zinc-finger binding domains of CreA are similar to those of Mig1p, the mechanism of repression appears to be quite different. For example, there is no evidence that CreA acts via a general repressor complex such as Ssn6p/Tup1p, as occurs for Mig1p repression (Hicks *et al.,* 2001; Treitel and Carlson, 1995; Tzamarias and Struhl, 1994, 1995), and a deletion of the *TUP1* homologue in *A. nidulans* has little effect on carbon catabolite repression (Hicks *et al.*, 2001).

Molecular analysis has revealed that CreA may act directly on the promoter of genes encoding enzymes to repress their transcription, or it may act to repress the transcription of a positively acting regulatory protein required for expression, or indeed act at both these levels in a double-lock mechanism such as in the *alc* regulon. CreA is able to bind to the promoters of both the specific trans-acting gene in the *alc* regulon, *alcR*, and of the structural gene, *alcA*, encoding the alcohol dehydrogenase I (Kulmburg *et al.*, 1993). The disruption of CreA-binding sites in the *alcR* promoter overlapping the induction target for the trans-activator AlcR results in a partially derepressed *alc* phenotype and derepressed *alcR* transcription, indicating that this regulation is functional *in vivo*. When bound, CreA may act to prevent expression either by competing for DNA binding sites with a positively acting protein required for expression, in *alcA* for example (Kulmburg *et al.*, 1993), or it may recruit proteins that act as repressors of transcription, such as in the proline utilization pathway (Gonzalez *et al.*, 1997).

Two other loci, *creB* and *creC,* were identified by mutation and the mutant phenotype indicates they are involved in the CCR in *A. nidulans*; the *creB* gene encodes a functional deubiquitinating enzyme, and the *creC* gene encodes a WD40 repeat motif containing protein (Lockington and Kelly, 2001; Todd *et al.*, 2000). CreB and CreC proteins have functions in both repressing and derepressing conditions, and they form a complex *in vivo* (Lockington and Kelly, 2001, 2002). Mutations in *creB* or *creC* resulted in the derepression of a numbers of genes normally subject to repression such as *alcA*, encoding alcohol denydrogenase I, and *amdS,* encoding acetamidase. The over expression of the *creB* gene can compensate for the loss of CreC function, leading to the restoration of repression in a *creC* mutant strain (Lockington and Kelly, 2002), indicating that CreB acts downstream of CreC. In addition to derepression of a number of genes including *alcA* and *amdS*, mutations in *creB* and *creC* lead to poor growth on some substrates, including proline and quinate (Lockington and Kelly, 2001). The presence of the CreB deubiquitinating protein is not only required for maintaining carbon catabolite repression but it is also required for maintaining the activity of some enzyme systems in the cell (Lockington and Kelly, 2002). This evidence led to the proposed regulatory model of CCR involving the CreB/CreC deubiquitination complex as presented in Chapter 1, and the aim of this research was to provide experimental evidence in support of this model.

7.1 CreA interacts with CreB deubiquitination protein.

In order to experimentally evaluate the CCR regulatory model, the first aim of this study was to determine whether CreA is a substrate of the CreB/CreC deubiquitination complex. A technique that has been used to determine whether a particular protein is a substrate for a deubiquitination enzyme is co-immunoprecipitation using specific antibodies against epitope tagged versions of the enzyme and the target. Co-immunoprecipitation had been used to determine the interaction of CreB and CreC *in vivo* in *A. nidulans*. The CreC protein could be detected in the immunoprecipitate when CreBFLAG was purified using anti-FLAG::M2 agarose (Lockington and Kelly, 2002). This technique was also used to determine that Ubp2p, a deubiquitinating enzyme, could be copurified with epitope-tagged Rsp5p both *in vivo* and *in vitro* in *S. cerevisiae* (Kee *et al.*, 2005). Regulation of eye development in *Drosophila* involves another well known de/ubiquitination system. In this case, the deubiquitinating enzyme, Fat Facets, is required to stabilize Epsin, encoded by the *liquid facets* gene, which prevents a

misspecification of photoreceptor cells in the eye (Chen and Fischer, 2000; Chen *et al.*, 2002).

In order to undertak co-immunoprecipitation experiments, antibodies directed against the proteins to be tested are required. Numerous previous attempts to raise antibodies against CreA had been unsuccessful (Chamalaun-Hussey, 1996), and thus epitope-tags were added to the proteins in this study. Although a N-terminally tagged CreA, MycCreA, was functional, MycCreA was not detected by Western analysis. It was probable that the epitope tag at the N-terminus was removed during the post-translational processing of CreA. This is consistent with another CreA construct, in which GFP and HA tags were placed at the N-terminus and an HA tag was present in an internal region of CreAHA. In this construct, GFP was processed from the fusion protein, and both the N-terminal GFPHA fragment and the rest of CreA could be detected using an anti-HA antibody in Western analysis (Lockington RA., unpublished). Thus, MycCreA was not detected by Western analysis because of the N-terminal processing of the tagged protein. Internal and C-terminal tagged CreA proteins were used in this study. A C-terminal FLAG tagged version of CreB had already successfully been used in other studies (Lockington and Kelly, 2002).

The results of the co-immunoprecipitation experiment showed that CreA and CreB are present in a complex in *A. nidulans*. When CreBFLAG was precipitated using an anti-FLAG antibody, CreAHAGFP could be detected in the precipitated fraction. However, control experiments showed that CreAHAGFP was binding non specifically to the anti-FLAG::M2 agarose, and so this result could not be relied on. Thus, CreA was purified from a strain that contained a version of CreA containing both a His tag and two HA tags. This allowed CreA to under go two rounds of purification, first with a His-Trap column, and then with anti-HA matrix. This double purification of CreA was robust, and CreB was also present after 2 rounds of purification while no CreB was purified in control extracts, indicating that CreA and CreB form a part of a complex i*n vivo*. Although the extra purification step reduced the amount of CreB, this might be because of some dissociation of the CreA/CreB complex during the procedure.

The fact that CreA and CreB were found to be present in a complex in the cell suggests that CreA might be a ubiquitinated protein targetted by the CreB deubiquitinating enzyme. Experimental evidence is presented here to show that CreA is a ubiquitinated

protein as it can be precipitated from a protein extract using a matrix that traps ubiquitinated proteins. However, little is yet known about how ubiquitination affects the regulation in CreA in *A. nidulans*. At least five gene products, CreA, CreB, CreC, CreD, and HulA, play important roles in the model that was mentioned in Chapter 1. In that model, CreA is directly ubiquitinated by the CreD/HulA ubiquitinating complex, targeting CreA to the CreB/CreC deubiquitination complex to either remove the ubiquitin chain from CreA, or alter the CreA function in the cell, and thus regulate carbon catabolite repression. Additionally, Western analysis of the CreA2HAHis containing strain in Chapter 6 showed that there were two apparent molecular weight forms of CreA, and these may represent different ubiquitinated forms of CreA2HaHis. Consistent with this form of regulation, *creA* mRNA levels do not directly correlate with CreA-mediated repression, implying that protein modification and/or stability of CreA could be an important component of the carbon catabolite repression mechanism (Arst *et al.*, 1990; Shroff *et al.*, 1996; Strauss *et al.*, 1999). In addition, CreA contains a sequence similar to a consensus sequence that triggers ubiquitination in yeast (Strauss *et al.*, 1999), implying that CreA could be modified by ubiquitination in derepressing conditions, resulting in inactivity/ degradation of CreA (Roy *et al.*, 2007) have shown that production of high levels of CreA in the cell in both carbon catabolite repressing and derepressing conditions does not interfere with normal carbon catabolite repression and derepression of target genes, indicating that large scale differential turnover of CreA is not required for derepression. Further, the amount of CreA is not greatly different in a *creB* mutant strain. Thus, it is not likely that CreA is polyubiquitinated to target it to the protesome, but rather more likely that ubiquitination of CreA modifies function, or that only a modified form of CreA is active for repression.

De/ubiquitination is emerging as an important control mechanism in the regulation of many cellular systems, and affects the stability and function of proteins. Protein modifications by ubiquitin have been reported in sensing systems ranging from DNA repair to endocytosis, and protein function is dictated by the number of ubiquitin molecules attached to the proteins (Muratani and Tansey, 2003). A well-studied case of regulation via the de/ubiquitination of substrates is that of eye development in *Drosophila*. E3 ubiquitin ligases have a number of roles during *Drosophila* eye development, including control of cell proliferation, specification, differentiation and death. For the correct development of photoreceptors to occur in the developing eye of *D. melanogaster*, the Fat facets deubiquitination enzyme is required to deubiquitinate

epsin (encoded by the liquid facets gene) (Cadavid *et al.*, 2000; Wu *et al.*, 2004; Wu *et al.*, 1999), thereby stabilizing epsin and promoting endocytosis, which prevents the misspecification of photoreceptor cells in the developing eye (Cadavid *et al.*, 2000; Chen and Fischer, 2000; Chen *et al.*, 2002). In the absence of Fat facets, epsin remains ubiquitinated and is degraded, promoting cells to inappropriately adopt a photoreceptor fate. A mouse homologue of Fat facets, Fam, has been identified and it has been suggested to play a role in mouse development via its deubiquitination of epsin (Chen *et al.*, 2002; Oldham *et al.*, 2002). This is one of the few cases in muticellular eukaryotes where the deubiquitination enzyme, the ubiquitinated substrate, and the role they play are known, although the E3 ligase that targets epsin for ubiquitination is yet to be determined. Ubiquitination has also been reported to regulate the high affinity nitrate transporter protein in *Hansenula polymorpha*. This type of regulation is in response to the amount of ammonium and glutamine, targeting the nitrate transporter for degradation in the vacuole (Navarro *et al.*, 2006).

The finding that CreA is a ubiquitinated protein implies that there must be a ubiquitin ligase that specifically targets CreA. This may be HulA, a HECT ubiquitin ligase, but specific experiments have not been undertaken to determine this interaction. HulA has been reported to interact strongly with ApyA, but it also showed a weak interaction with CreD in the Bacterial-2-hybrid analysis (Boase and Kelly, 2004), and this complex had been proposed to be involved in an opposing process to deubiquitination in the carbon catabolite repression network in *A. nidulans*. Additionally, a mutation in *creD*, *creD34*, showed weak suppression of *creA204*. But the *creD* mutant was a strong suppressor of *creB* and *creC* mutant phenotypes, and also led to stronger repression of other genes such as *facA* and *alcA* leading to greater fluoroacetate resistance and allyl alcohol resistance (Kelly and Hynes, 1977), indicating that the *creD34* mutation leads to tighter repression of enzymes subject to carbon catabolite repression. To further determine any potential interaction between the CreD and CreA proteins, expression of epitope-tagged versions of CreA and CreD should be evaluated in both $creD^{+}$ and $creD^{-}$ backgrounds using the co-immunoprecipitation technique, which was used to determine the interaction of CreA and CreB in this study. However, expression of epitope-tagged CreA in a *hulA* mutant background cannot be investigated because deletion of *hulA* resulted in a lethal phenotype (Denton, J.A., unpublished data), and the deletion could only be obtained as a heterozygous diploid.

Proteomic analysis of purified CreA had also been used to identify other proteins which may interact with CreA, but no clear interacting proteins were identified by using this approach. This may be due to instability of CreA in the cell lowering the amount of any complex to below the detection limit of the analytical steps. A microscopic study of a GFP-tagged CreA containing strain using cyclohexamide to stop protein synthesis showed that the tagged CreA protein was rapidly degraded in the cytoplasm but was fairly stable in the nucleus (Roy P., personal communication). Although overexpression of *creA* was driven from a highly active *gpdA* promoter and CreA was easily detected by Western analysis, this does not mean that the expressions of other proteins that interact with CreA will be elevated, when those proteins are still expressed at low levels from their own promoters. This will result in a different molecular ratio of the complex between CreA and other proteins, and may also be reason for the failure to detect interacting proteins. However, an identical approach using over expression *creBFLAG* was successful in identifying proteins interacting with epitope-tagged CreB (Lockington R. A., personal communication).

7.2 Quinate Permease interacts with the CreB deubiquitination protein.

Mutations in *creB* and *creC* also lead to the failure to express enzymes for the utilization of proline and quinic acid in both the presence and absence of glucose (Hynes and Kelly, 1977; Lockington and Kelly, 2001). In derepressing conditions, *creB* mutant strains are unable to utilize quinate as a carbon source, suggesting a role for *creB* in quinate utilization. It is likely that the failure to grow on quinic acid as a sole carbon source is due to the absence of the quinic acid permease. Thus, the second aim of this study was to determine whether the quinate permease is a ubiquitinated protein and a target of CreB.

As was found for CreA, the positioning of the epitope-tag on the permease was important and needed to be determined experimentally. Epitope tags at the amino (N)-terminus resulted in a nonfunctional protein. However, the C-terminal epitope tagged QutD was functional and could easily be detected in immunoprecipitation experiments. A proportion of QutD was co-precipitated with CreB, indicating that the CreB deubiquitinating enzyme is present in a complex with QutD *in vivo*. This result confirms that the QutD protein is likely to be a direct target of the CreB deubiquitination enzyme. This result was strengthened by the finding that the QutD

permease was a ubiquitinated protein, and able to be precipitated using a ubiquitin specific capture matrix.

These experimental results with QutD provide support for the regulatory model of CCR involving the CreB/CreC deubiquitination complex as shown in Chapter 1. The expression of QutDHA was detected only in mycelia grown in derepressing and inducing conditions, indicating that the QutD permease is strongly regulated by both carbon catabolite repression and induction by quinic acid. This is consistent with a report that showed that the *qut* cluster genes are induced by the uptake of quinate into the mycelia by the *qutD* gene product, and gene regulation is regulated by carbon catabolite repression rather than substrate exclusion (Whittington *et al.*, 1987).

The function and stability of the quinate permease is affected by the presence of CreB protein in the cell. Western results showed that the concentration of QutD in the cell is lower in the *creB* null mutant background than in the wild-type background, indicating that deubiquitination is required to prevent protein turnover. The low amount of QutD is not due to a low amount of messenger RNA in the *creB* mutant background, as this was actually higher in the *creB* mutant strain than in the wild-type strains, indicating that this result was not caused by a failure of transcriptional induction of *qutD*. This transcriptional overexpression of *qutD* mRNA may to some extent compensate for the loss of the ubiquitinated QutD via degradation by the 26S proteasome. These results show that the CreB protein acts through stabilizing the QutD protein rather than affecting the level of *qutD* mRNA. The regulation of QutD might involve both the induction of the permease messenger RNA by quinate in the mycelia and regulation of the QutD stability through the CreB deubiquitinating enzyme. These findings suggest that quinate permease is regulated through a ubiquitination and deubiquitination network, involving the CreB deubiquitination protein.

The CreB/CreC regulatory deubiquitination complex may act both on CreA to affect carbon catabolite repression, and directly to stabilize permeases, transporters or glucose sensing proteins by removing ubiquitin from those proteins, thereby altering the cellular concentration or localization of signaling molecules such as glucose and quinate ions. Examples of permease and transporter ubiquitination are found in *S. cerevisiae*, in which the general amino acid permease, Gap1p, is targeted by the ubiquitin ligase, Npi1p, for ubiquitination and subsequent degradation in response to

the addition of ammonium to the media (Rotin *et al.*, 2000; Springael and Andre, 1998; Springael *et al.*, 1999a; Springael *et al.*, 1999b). The role of Hxk2p has been reported in *S. cerevisiae*, where Hxk2p was localized in the nucleus and required for glucose repression of several genes (Herrero *et al.*, 1995; Herrero *et al.*, 1998). Subsequently, it was shown that Hxk2p can bind directly to a complex formed by Mig1p and the *MIG1* element of the *SUC2* promoter (Ahuatzi *et al.*, 2007; Moreno *et al.*, 2005). This indicates that the regulatory mechanism of CCR in yeast involves both transcription and post-transcriptional regulation of the levels of specific substrate in the cell.

Catalytic hexokinases from yeast and higher organisms have been shown to possess a regulatory function and are believed to be involved in the glucose sensing mechanism (Rolland *et al.*, 2002). Evidence has shown that HxkA and GlkA, hexose phosphorylating proteins in *A. nidulans*, are involved in a glucose sensing mechanism that leads to carbon catabolite repression (Ruijter *et al.*, 1996). Later, double mutant strains (*hxkA*/*glkA4*) were found to show considerable transcriptional derepression in the presence of either glucose or fructose (Flipphi *et al.*, 2003). The double mutants have glucose uptake characteristics similar to those of wild type. This indicates that the unphosphorylated sugar is unlikely to play a direct role in signaling carbon catabolite repression in *A. nidulans* (Flipphi *et al.*, 2003). Thus, CCR could be signaled independently for individual carbon sources and CreA might be the ultimate receptor of multiple converging signaling routes in *A. nidulans*

The QutD permease is present only in cells grown in medium containing quinic acid as the sole carbon source, but not when glucose is also present. The transcriptional repression of *qutD* can be explained by the relationship between the CreA DNA-binding protein, and the hexose transporter and sugar phosphorylation in *A. nidulans* as mentioned by Flipphi *et al.* (2003). It is possible that the phosphorylation of glucose, which was imported into the mycelia by the HxkA protein, induces active CreA to bind to the promoter element of *qutD*, even though the inducer, quinic acid, was also present in the medium. This will result in the repression of the *qutD* gene in glucose containing medium. However, this regulatory control has not been tested and it needs further verification with the appropriate experimental approach to determine the level and localization of HxkA and CreA under carbon catabolite de/repression conditions.

Another transporter protein, PrnB, which the genotypic and phenotypic evidence suggested was regulated by CreB, was also studied using the same approach that was used for the quinate permease. The regulation of the proline utilization cluster would suggest that the action of CreB is likely to be directly on the permeases or enzymes themselves rather than via the pathway specific regulatory proteins involved in induction, as the proline permease encoding gene, *prnB,* is directly regulated by CreA (Cubero *et al.*, 2000), whereas the pathway specific transcriptional activator PrnA has constitutive nuclear localization and is not dependent on proline induction or carbon catabolite repression (Pokorska *et al.*, 2000). Experiments were undertaken to determine the expression of epitope-tagged versions of PrnB before determining whether the proline permease is a target of CreB. However, epitope-tagged versions of PrnB, either N- or C- terminal tagged, could not be detected although they were functional as they complemented the *prnB6* mutation for proline utilization. Because of these difficulties, it was not possible to determine whether PrnB and CreB interact *in vivo* using co-immunoprecipitation. However, this finding is consistent with a study on GFP-tagged PrnB constructs that also did not show full complementation (Tavoularis *et al.*, 2001), and some *prnB* mutations affect proper translocation of PrnB in the membrane, exhibiting physiological cellular expression of PrnB (Tavoularis *et al.*, 2003). Current work, however, has shown that a C-terminal tag of PrnB, BAD (biotin accepter domain) and LacY sequences, could be detected by Western analysis using antibodies against the epitope tags, avidin-HRP and anti-Y antibodies, respectively. The tagged PrnB pemease also could be efficiently pulled down using avidin-Sepharose beads, through the biotinylated BAD domain (Kafasla *et al.*, 2007). Thus these epitope-tagged PrnB proteins could be used to study the interaction of CreB and PrnB in the future.

7.3 Conclusion

Ubiquitin is covalently added to target proteins by an ubiquitination enzyme complex, usually including activating enzyme, conjugating enzyme, and ligating enzyme (Wilkinson, 2000). The addition of four or more ubiquitin molecules target proteins for degradation by 26S protesome, whereas the number of ubiquitin less than that can alter protein intracellular location or function. Many cases are known in which the role of a specific ubiquitin ligases is known, but there are few examples where a specific deubiquitination enzyme has been shown to rescue a protein from ubiquitin mediated-degradation, or alter its functon. These experiments have provided direct experimental

evidence that the regulatory protein CreA and the quinate transporter protein, QutD, are substrates for the CreB deubiquitinating protein *in vivo* in *A. nidulans*. Moreover, independently of this, experimental evidence showed also that both CreA and QutD are ubiquitinated proteins. These findings provide strong experimental support for the carbon catabolite regulatory model in *A. nidulans* outlined in the Introduction chapter, and have confirmed the interaction of some gene products involved in this model.

The discovery that deubiquitination by CreB, possibly the CreB/CreC complex, regulates the function of the CreA DNA-binding protein, and the amount of quinate permease, QutD, should make it possible to reveal the functions of these orthologues in other systems. A CreB homologue is present in humans, UBH1 (Hansen-Hagge *et al.*, 1998; Lockington and Kelly, 2001), and the severity of the Myotonic Dystrophy phenotype is affected by mutations in the myotonic dystrophy-1 gene, which is similar to CreC in *A. nidulans* (Groenen and Wieringa, 1998). Thus, it is likely that the myotonic dystrophy phenotype is modulated by a ubiquitination/deubiquitination pathway. It had been reported that the Ubiquitin-specific protease (UNP) protein, Usp4, features motifs common to oncoproteins, and interaction with the retinoblastoma (pRb) tumor suppressor protein and the related “pocket proteins’, p107 and p130 in the cell cycle could be detected *in vitro* (Blanchette et al., 2001). pRb has the same type of zinc-finger motif as CreA, and Usp4 protein has 75% sequence similarity to Usp15 (Singhal and Baker, 2007). Usp15 is a deubiquitination enzymr similar to the CreB deubiquitination protein, indicating that the turnover and function of these proteins in the cell cycle might be regulated via a ubiquitination and deubiquitination systems. However, the role of CreB/CreC deubiquitination complex may not act directly on the target proteins, such as CreA and QutD, in this system. It may regulate the function or stability of proteins by controlling the function of a specific ubiquitin-ligase enzyme complex. For example, Rsp5p, the HECT ubiquitin ligase in *S. cerevisiae*, is regulated by and has an antagonistic relationship with the Ubp2p/Rup1p deubiquitination complex (Kee *et al.*, 2005).

The roles of other gene products need to be explored experimentally to fully validate the CCR regulatory model. The roles of CreD, AcrB and HulA need to be confirmed. Although some of these proteins showed interaction using the bacterial-2- hybrid system, there have not any experimental evidence to show the interaction of those proteins (or other proteins) *in vivo* in *A. nidulans*. Now that experimental evidence

confirms the interaction of CreA and QutD with CreB in the cell, the exploration of the ubiquitin ligase enzyme complexes in *A. nidulans* should be the next discovery objective. It would be possible to use the same approach to provide evidence to support the CCR regulatory model. More understanding of the link between deubiquitination and the regulation of carbon metabolism, may allow us to understand the key regulatory role of de/ubiquitination pathway beyond carbon catabolite repression in *A. nidulans*.

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