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Research paper

KAEA (SUDPRO), a member of the ubiquitous KEOPS/EKC protein complex, regulates the arginine catabolic pathway and the expression of several other genes in *Aspergillus nidulans*

Agnieszka Dzikowska [a,b,*], Anna Grzelak [a], Joanna Gawlik [c], Edyta Szewczyk [a], Paweł Mrozek [a], Piotr Borsuk [a], Michał Koper [a], Joanna Empel [a,1], Paweł Szczęsny [b,d], Sebastian Piłsyk [b], Małgorzata Pękala [a], Piotr Weglenski [b,e]

[a] Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, ul. Pawińskiego 5A, 02-106 Warsaw, Poland
[b] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawińskiego 5A, 02-106 Warsaw, Poland
[c] College of Inter-Faculty Individual Studies in Mathematics and Natural Sciences, University of Warsaw, Poland
[d] Institute of Experimental Plant Biology, Faculty of Biology, University of Warsaw, ul. Miecznikowa 1, 02-096 Warsaw, Poland
[e] Centre of New Technologies, University of Warsaw, ul. Żwirki i Wigury 93, 02-089 Warsaw, Poland

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ABSTRACT

The *kaeA*^{KAE1} (*suDpro*) gene, which was identified in *Aspergillus nidulans* as a suppressor of proline auxotrophic mutations, encodes the orthologue of *Saccharomyces cerevisiae* Kae1p, a member of the evolutionarily conserved KEOPS/EKC (Kinase, Endopeptidase and Other Proteins of Small size/Endopeptidase-like and Kinase associated to transcribed Chromatin) complex. In yeast, this complex has been shown to be involved in tRNA modification, transcription, and genome maintenance. In *A. nidulans*, mutations in *kaeA* result in several phenotypic effects, the derepression of arginine catabolism genes, and changes in the expression levels of several others, including genes involved in amino acid and siderophore metabolism, sulfate transport, carbon/energy metabolism, translation, and transcription regulation, such as *rcoA*^{TUP1}, which encodes the global transcriptional corepressor.

1. Introduction

Kae1p is a subunit of the KEOPS/EKC complex, which is highly conserved in Archaea, Bacteria and Eukaryota (Galperin and Koonin, 2004, 2010). In *Saccharomyces cerevisiae*, the complex is composed of five subunits. In addition to Kae1p, it includes Bud32p, Cgi121p and Pcc1p, which are conserved only in Archaea and Eukaryota, and Gon7p/Pcc2p, which is specific to Fungi (Downey et al., 2006; Kisseleva-Romanova et al., 2006; Mao et al., 2008; Daugeron et al., 2011). Kae1p is a metal-binding protein belonging to the ASKHA (Acetate and Sugar Kinase, Hsp70 chaperone proteins and Actin) protein superfamily (Hurley, 1996; Aravind and Koonin, 1999; Hecker et al., 2008; Mao et al., 2008). It is encoded by one of the very few genes present in all of the genomes that have been sequenced to date with the exception of some highly reduced genomes (Galperin, 2008; Galperin and Koonin, 2010). Eukaryotic genomes also express mitochondrial Qri7p (OSGEPL in humans), a paralogue of Kae1p and the orthologue of bacterial YgjD (Oberto et al., 2009; Wan et al., 2013). Archaeal PaKae1 has been shown to have DNA-binding and apurinic site endonuclease activities (Hecker et al., 2007).

Bud32p is an atypical serine/threonine protein kinase (Facchin et al., 2002) that is homologous to human TP53RK/PRPK, which has been shown to phosphorylate p53 (Abe et al., 2001). In the KEOPS/EKC complex, Kae1p and Bud32p are closely associated (Mao et al., 2008). Similarly as Kae1p, Bud32p is also highly conserved; in several archaeal genomes, these two proteins are fused into a single polypeptide. In contrast to Kae1p, Bud32p has no bacterial homologue (Hecker et al., 2008, 2009).

Recent data have shown that the KEOPS/EKC complex participates in a universal tRNA modification: converting adenosine A_{37} to N6-threonylcarbamoyl adenosine ($t6A_{37}$) (Daugeron et al., 2011; El Yacoubi et al., 2011; Srinivasan et al., 2011; Perrochia et al., 2013a). The modification of A_{37} immediately 3′ of anticodons decoding ANN codons is one of the few tRNA modifications present in all organisms (de Crecy-Lagard et al., 2007). $t6A_{37}$ strengthens the interaction of the A–U codon–anticodon base pair of ANN codons. This prevents frameshifting during translation and enables proper translation initiation at the AUG start codon, as the initiator tRNA^{Met} is also modified by $t6A_{37}$ (Yarian et al., 2002). Both Kae1p and its bacterial orthologue, YgjD,

Abbreviations: DAB, 1,4-diamino-2-butanone; GSA, glutamic-5-semialdehyde; OAT, ornithine aminotransferase; RT-qPCR, reverse transcription-quantitative PCR.

* Corresponding author at: Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, ul. Pawińskiego 5A, 02-106 Warsaw, Poland.
E-mail addresses: adzik@igib.uw.edu.pl, adzik@ibb.waw.pl (A. Dzikowska).
[1] Present address: Department of Molecular Microbiology, National Medicines Institute, ul. Chełmska 30/34, 00-725 Warsaw, Poland.

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were shown to be required for $t6A_{37}$ modification. In yeasts, modification of cytoplasmic and mitochondrial tRNAs requires Kae1p and Qri7p, respectively. It is highly probable that Kae1p and its orthologues participate in $t6A_{37}$ modification in all organisms. Modification of both cytoplasmic and mitochondrial tRNA pools also requires Sua5p (YrdC in bacteria), a protein that is highly conserved in all organisms, but is not part of the KEOPS/EKC complex. It has been proposed that Kae1p functions as a protein or a tRNA chaperone (El Yacoubi et al., 2009, 2011; Srinivasan et al., 2011). Archaeal KEOPS has been demonstrated to bind tRNA in vitro, and it was suggested that the complex might form a platform for the $t6A_{37}$ modification of tRNA (Perrochia et al., 2013a). Sua5p and Qri7p, the mitochondrial paralogue of Kae1p, were found to be sufficient for $t6A_{37}$ biosynthesis in vitro (Wan et al., 2013). However, the analysis of archaeal KEOPS showed that while Kae1, Bud32 and Pcc1 are required for the reaction, Cgi121 increases its efficiency (Perrochia et al., 2013b). Recently, a crystal structure-based model of the yeast KEOPS complex has also been proposed (Zhang et al., 2015).

The involvement of the KEOPS/EKC complex in $t6A_{37}$ modification explains its exceptional evolutionary conservation because this modification is essential for accurate mRNA decoding. This appears to be the primary function of the complex. However, several effects of mutations in genes encoding subunits of the complex have also been described, and their association with $t6A_{37}$ modification is difficult to explain, suggesting that KEOPS/EKC may be multifunctional. Downey et al. (2006) have shown that the KEOPS/EKC complex is implicated in telomere elongation and uncapping, and proposed that it promotes an "open" telomere conformation, allowing an access of both telomerase and exonuclease. The KEOPS/EKC complex was also found to be necessary for telomere recombination (Hu et al., 2013). In yeast, the Pcc1p subunit of the complex is required for efficient transcription from promoters induced with α factor and galactose. ChIP data have demonstrated that Pcc1p is associated with transcribed genes. It is not required to bind the specific activator Gal4p, however, it is necessary for the efficient recruitment of TBP, SAGA and Mediator complexes to their respective promoters. Genetic interactions suggest that Pcc1p interacts with RNAPII and other general transcription factor co-activators. Similarly, Kae1p was shown to be associated with promoters induced with α factor and galactose (Kisseleva-Romanova et al., 2006). ChIP data have also shown that OSGEP and LAGE3, the human homologues of Kae1p and Pcc1p, are localized in chromatin (Costessi et al., 2012). Kisseleva-Romanova et al. (2006) suggested that the KEOPS/EKC complex was responsible for regulation of chromatin structure, possibly due to the ATP-dependent remodeling activity of Kae1p.

In this paper, we describe the *kaeA* gene (formerly known as *suDpro*), which encodes a member of the KEOPS/EKC complex in the filamentous fungus *Aspergillus nidulans*. *kaeA* was identified as a suppressor of proline auxotrophic (*pro*$^-$) mutations. We characterized two mutations in *kaeA* that result in the derepression of arginine catabolic enzymes: arginase and ornithine aminotransferase (OAT). We showed that *kaeA* is involved in the regulation of not only arginine catabolic gene expression but also several other genes that control various domains of cellular metabolism. We identified genes encoding the remaining proteins of the KEOPS/EKC complex in the *A. nidulans* genome. Our results support the hypothesis that in addition to its conserved role in tRNA modification in Eukaryota, the KEOPS/EKC complex may be involved in transcription regulation.

2. Materials and methods

2.1. A. nidulans strains, transformation, and growth conditions

proA6, *suD25pro*, *adF9*, *yA2*; *metG55* was used as a recipient strain in transformation experiments. *proA6*, *suD25pro*, *adF9*, *yA2*, *phenA2* and *proA6*, *suD19pro*, *pabaA12*, *biA1* (also referred to as *suD25pro* and *suD19pro*) were used for growth tests, northern blot analysis, and transcriptomics. *proA6*, *pabaA9*, *biA1* or *proA6*, *adF9*, *yA2*, *phenA2* or *biA1* were used as control *suD*$^+$ strains. Transformation of *A. nidulans* was performed as described by Tilburn et al. (1983) and modified according to Johnstone et al. (1985). The *A. nidulans* minimal chromosome-specific cosmid pWE15 and pLORIST gene libraries (Brody et al., 1991) were used for transformation.

The mycelia used in the OAT and arginase assays, and for northern blot analysis, were grown in minimal medium (10 mM nitrate and 1% glucose) for 8–10 h at 37 °C and then induced with 10 mM arginine for 2.5 h. Proline was used at a 0.4 mM concentration. Metabolites necessary to supplement other auxotrophies were added where required (Pontecorvo et al., 1953).

Sensitivity to 1,4-diamino-2-butanone (DAB) was tested on minimal medium with 1 mM DAB. Putrescine or spermidine was added at concentration of 1 mM. Sensitivity to ethidium bromide (EtdBr) was tested on complete medium with 3 μg/ml EtdBr; sensitivity to selenate was tested on minimal medium with 5 mM methionine as a sole sulfur source and 0.1 mM sodium selenate. One percent glucose or 2% glycerol was used as a sole carbon source.

2.2. DNA sequencing, promoter and protein analysis

Subclones of the *kaeA* gene in pBluescript KS + were sequenced using the vector and *kaeA*-specific primers. PCR-amplified fragments from mutant alleles were sequenced using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Kit and *kaeA*-specific primers. For each *kaeA* allele, three fragments covering the whole *kaeA* locus were obtained and sequenced. The promoter region was analyzed using the MatInspector and MatBase Genomatix software suite (www.genomatix.de). KAEA protein structure was modeled using Modeller software (Eswar et al., 2006), based on the resolved Kae1 structure from *Pyrococcus abyssi* (Hecker et al., 2007).

2.3. Cloning and sequencing of kaeA cDNA

The *A. nidulans* λZAPII 24 cDNA library (Aramayo and Timberlake, 1990) was screened using plaque hybridization (Sambrook and Russell, 2001). A radioactively labeled 1039 bp *ClaI–NcoI* (see Fig. 6) fragment of the *kaeA* gene was used as a probe. Phagemids were excised from several hybridizing plaques and cDNA was sequenced from both strands.

2.4. Mapping of the 5′ and 3′ mRNA ends of the kaeA gene

The *kaeA* transcription initiation sites were determined by primer extension analysis using 6-P-suD primer (Supplementary Table 1). The total RNA from the *proA6*, *pabaA9*, *biA1* strains grown on minimal medium (10 mM nitrate and 1% glucose) or on minimal medium with 10 mM arginine was used for the primer extension reaction, which was performed as described by de Graaff et al. (1994). The 3′ end of the *kaeA* transcript was determined by the sequencing of the 3′ end of the *kaeA* cDNA clone.

2.5. Northern blot hybridization analysis

Northern blot hybridization was carried out as described by Macios et al. (2012). Northern blots were quantified using the STORM PhosphorImager™ or FLA-7000 system, and Multi Gauge v.3.0 software (FujiFilm).

2.6. Arginase and OAT activity assays

Arginase and OAT activities were assayed as previously described (Albrecht and Vogel, 1964; Cybis and Weglenski, 1972; Bradford, 1976; Dzikowska et al., 2003). One specific unit of activity was defined as: 1) the OAT activity that produces 1 μmole of glutamic 5-

semialdehyde per minute; 2) the arginase activity that produces 1 μmole of urea per minute, under standard conditions. Average specific activities (±SD) were calculated from the results of at least three independent experiments.

2.7. Sulfate uptake analysis

Sulfate uptake analysis was performed as described in Pilsyk et al. (2015) with small modifications. Cultures were grown in MM-S liquid medium supplemented with 5 mM methionine (repressing conditions), and 15 μCi of [^{35}S]-labeled H_2SO_4 (Hartmann Analytic) was added to each culture with cold sodium sulfate to a final concentration of 0.1 mM. The cultures were maintained in a rotary shaker (215 rpm) at 37 °C, and 10-ml aliquots were taken after 10, 30 and 60 min of incubation. For sulfate incorporation measurements, cultures were grown for 18 h at 37 °C in MM-S liquid medium supplemented with 5 mM methionine and 5 μCi of [^{35}S] 0.1 mM sulfate. Sulfate uptake was stopped, mycelia samples were prepared and as described previously (Pilsyk et al., 2015). Dried samples were soaked with 1 ml of liquid scintillator (Ultima Gold™, Perkin Elmer) and radioactivity was measured in a 1209 Rackbeta Vallac scintillation counter (LKB). Transport rates [nmol/mg dry weight (DW)] and assimilation data (±SD) were estimated on the basis of at least three measurements.

2.8. RNA isolation, transcriptomics, and RT-qPCR analysis

For transcriptome analysis, *suD25pro* and the control (*proA6*, *adF9*, *yA2*, *phenA2*) strains were grown on minimal medium and total RNA was isolated as described by Chomczynski and Sacchi (1987). Total RNA samples from three independent biological replicates were combined, treated with RQ1 RNase-Free DNase I (Promega), and the poly A$^+$ RNA was purified using a Poly(A)Purist™ MAG kit (Ambion). cDNA was synthesized from 4 μg of polyA$^+$ RNA using the cDNA Synthesis System (Roche), according to the manufacturer's instructions. The synthesis of the first strand was carried out with MIXcDNA primer 5′-$(T)_{22}$V-3′, where V stands for any base but T. Shotgun libraries were prepared according to rapid library protocols (Roche). Sequencing was performed on a Genome Sequencer GS FLX Instrument (Roche), using the GS FLX Titanium chemistry and following standard protocols.

Reads had a total length of 5.6×10^7 and 4.8×10^7 bp for the control strain and the *kaeA* (*suD25pro*) mutant, respectively. The reads were cleaned with PRINSEQ (Schmieder and Edwards, 2011) to eliminate short and low quality reads. Then, they were mapped to the *A. nidulans* genome (version FGSC A4), provided by the AspGD consortium (Cerqueira et al., 2014), using STAR aligner (Dobin et al., 2013). Finally, 50826 and 47177 reads were mapped to the control strain and the *kaeA* (*suD25pro*) mutant, respectively. Hits to genomic features were counted using HTSeq-count software from the HTSeq package (www-huber.embl.de/users/anders/HTSeq). As there was only one sequence library for each strain (although it consisted of three pooled biological replicates), we analyzed the data using the DEGSeq package, which comprises implemented methods based on MA-plots for the identification of differentially expressed genes in the absence of replicates (Wang et al., 2010). We used the MARS method from the DEGSeq package with LOESS normalization and other parameters set on default. Output from the DEGSeq analysis is shown in Suppl. Materials 2. Gene expression was regarded as significantly changed if the *P*-value was <0.001 and the fold change was >2. Protein function annotation was performed using the AspGD (www.aspergillusgenome.org), SGD (www.yeastgenome.org) and PomBase (www.pombase.org) databases (Cherry et al., 2012; Wood et al., 2012; Cerqueira et al., 2013).

For RT-qPCR analysis, *suD25pro* and the control (*proA6*, *adF9*, *yA2*, *phenA2*) strains were grown on minimal medium, and total RNA was isolated as described by Schmitt et al. (1990), using a FastPrep®-24 instrument (MP Biomedicals) for mycelium homogenization. RNA was treated with DNase I (Roche Diagnostics), phenol-chloroform extracted and ethanol precipitated. RNA quality and concentration was measured using RNA Nano chip on the 2100 Bioanalyzer instrument (Agilent Technologies) and only RNA samples with RIN value (RNA Integrity Numbers) not lower than 9.0 were selected for reverse transcription. cDNA was synthesized from 2 μg of total RNA using SuperScript® III Reverse Transcriptase (Invitrogen, Life Technologies) and a mixture of oligo-dT and random hexamer primers, according to the supplier's protocol. Real-time RT-PCR was performed using the LightCycler® 480 II System (Roche Diagnostics) with specific primers for *agaA*, *otaA*, *mirB*, *puA*, *sB*, *rcoA* genes and SYBR Green detection. Four biological replicates were analyzed for both strains with two technical replicates for each. RT-qPCR was performed in triplicate, in 384-well plates with each 10-μl reaction mixture containing 5 μl of LightCycler®480 SYBR Green I Master mix (Roche Laboratories), two primers (3 pmol of each) and 2 μl of diluted template cDNA. Reactions were pipetted using JANUS® Extended Integrator 8-tip Automated Workstation (PerkinElmer). Efficiency (E) and specificity of each pair of primers were tested in RT-qPCR reactions using 6-point standard curves of 5-fold diluted cDNA of the control strain. Subsequently, the melting curve analysis was performed. E value for all primer pairs used was in the range of 1.93–2.00. Cp values were calculated using LightCycler®480 Software 1.5 (Roche Diagnostics), based on the Second Derivative Maximum Method. The qPCR data were analyzed on the R statistical platform, using the HTqPCR package (Dvinge and Bertone, 2009). Cp values were normalized using 18S as an endogenous control. Differential expression was assessed using a t-test with a Benjamini & Hochberg correction, using the wild type as a reference.

2.9. Confocal microscopy

Conidia were grown in complete medium in 60 μ-Dish ibiTreat (ibidi) for 13 h at 30 °C. For the visualization of the mitochondria, hyphae were incubated with MitoTracker® Red CMXRos (Life Technologies) for 1 h at 30 °C. Imaging of hyphae was performed using an Olympus microscope FluoView FV1000 with 60x/NA = 1.4 objective.

3. Results and discussion

Arginine catabolism in *A. nidulans* is a good model to study the regulation of gene expression in lower Eukaryotes, at both the transcriptional (Dzikowska et al., 2003; Macios et al., 2012) and posttranscriptional level (Olszewska et al., 2007; Krol et al., 2013). Several non-allelic regulatory mutations resulting in the derepression of the arginine catabolic enzymes, arginase and OAT, were identified as suppressors of proline auxotrophic (*pro*$^-$) mutations (Weglenski, 1967; Piotrowska et al., 1969; Klimczuk and Weglenski, 1974). In *A. nidulans* as in many other microorganisms, proline is synthesized from glutamate via glutamic-5-semialdehyde (GSA) (Fig. 1). Because proline can also be synthesized by an alternative pathway from exogenous arginine, via ornithine and GSA, in two reactions catalyzed by arginase and OAT, and encoded by *agaA* and *otaA*, respectively, the growth of *pro*$^-$ mutants blocked prior to GSA can be restored by the addition of either proline or arginine to the growth medium. However, this alternative proline synthesis pathway is apparently not functional when the *pro*$^-$ mutant is grown on arginine-free medium. The addition of arginine results in the induction of arginase and OAT activities, allowing the utilization of arginine for proline synthesis (Bartnik and Weglenski, 1974; Borsuk et al., 1999; Dzikowska et al., 1999). This induction depends on the pathway-specific activator ARCA (Empel et al., 2001).

3.1. Pleiotropic effects of the suDpro mutants

Using the classic UV irradiation method, *suD19pro* and *suD25pro* mutants were obtained as suppressors of mutations in the *proA* locus (Weglenski, 1967; Piotrowska et al., 1969; Klimczuk and Weglenski, 1974). Both suppressor mutations are recessive and resulted in ca. 10-

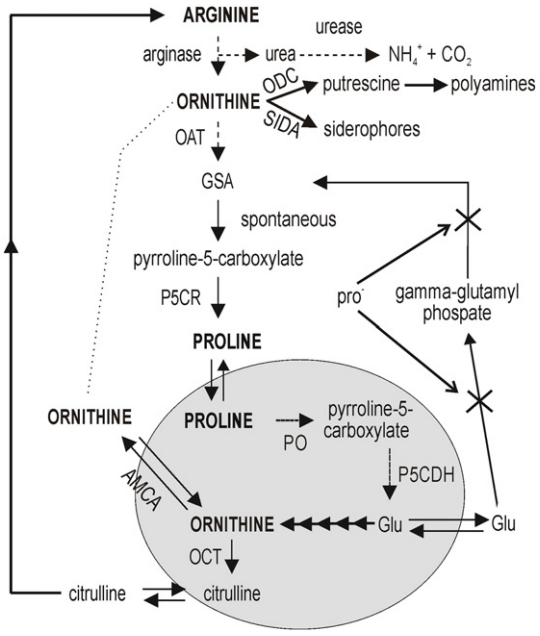


Fig. 1. Arginine and ornithine metabolism in fungi. The continuous lines represent biosynthetic pathways, and the broken lines represent catabolic pathways. The position of metabolic blocks resulting from *pro*$^-$ mutations is indicated. The mitochondrion is shown in gray. Abbreviations: OAT — ornithine aminotransferase; PO — proline oxidase; P5CR — pyrroline-5-carboxylate reductase; P5CDH — pyrroline-5-carboxylate dehydrogenase; OCT — ornithine carbamoyltransferase; ODC — ornithine decarboxylase SIDA — ornithine N5-oxygenase; AMCA — mitochondrial ornithine transporter.

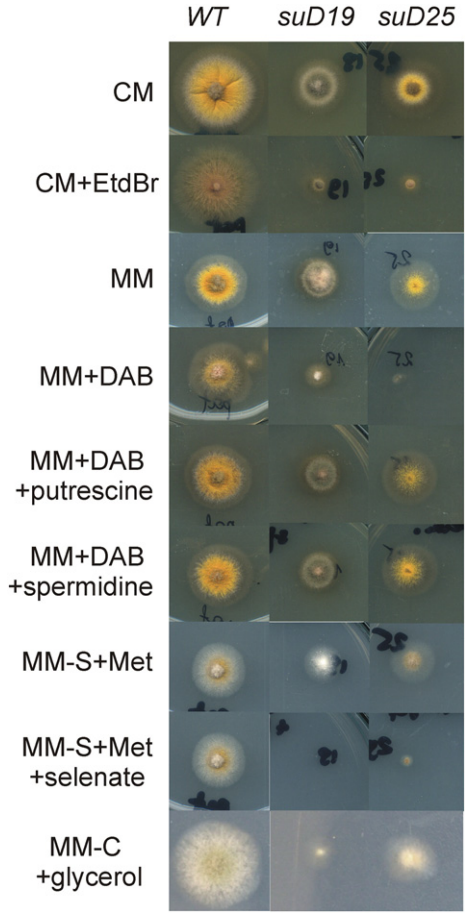
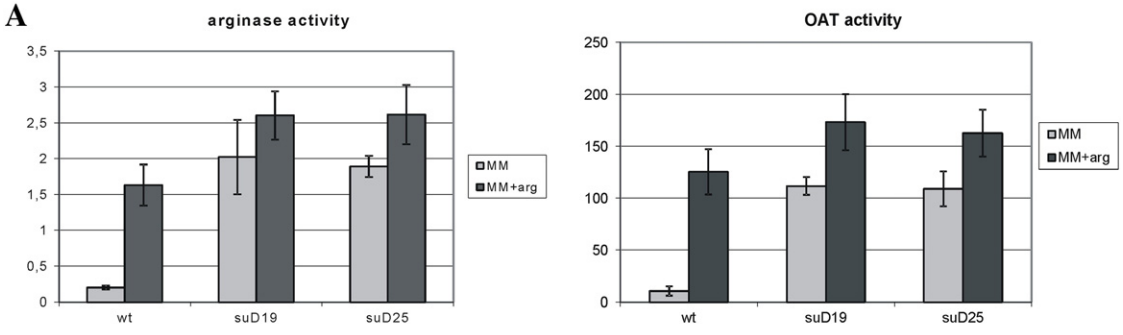
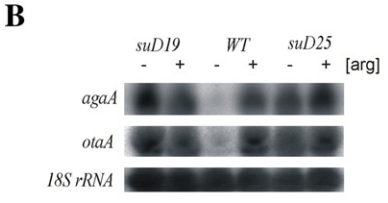


Fig. 3. Pleiotropic effects of *suD19pro* and *suD25pro* mutations. The growth of the *suD19pro*, *suD25pro* and the wild-type control strain (WT) was compared on complete (CM) and minimal (MM) medium with ethidium bromide (EtdBr), spermidine, putrescine and/or DAB, on minimal medium with methionine as a sulfur source and with or without selenate (MM-S + Met), and on minimal medium with glycerol as a carbon source (MM-C + glycerol).

fold increase of uninduced arginase and OAT activity levels, compared to that observed in mycelium of the *suD*$^+$ strain (Fig. 2A). The enzyme activities correlate well with levels of *agaA* and *otaA* mRNA measured by northern hybridization (Fig. 2B), RT-qPCR (Fig. 9 and Sup. Fig. 7), and transcriptome analysis (see part 3.4).

Apart from the elevated basal level of arginine catabolic enzymes, *suDpro* mutations caused several other phenotypic effects (Fig. 3). Both *suD19pro* and *suD25pro* mutants grew more slowly than the WT





C

	WT	*suD19pro*	*suD25pro*
agaA	1x	17.2x↑	14.2x↑
otaA	1x	4.8x↑	6.8x↑

Fig. 2. *suDpro19* and *suDpro25* mutations result in elevated expression level of arginine catabolism genes. (A) Average arginase and OAT specific activity (±SD) in mycelium grown in minimal medium with or without arginine. (B) *agaA* and *otaA* transcript level on minimal medium without (−) or with (+) arginine. (C) Relative amounts of *agaA* and *otaA* mRNAs on medium without arginine (uninduced conditions).

on the complete and minimal medium. The growth of suppressor mutants is much more strongly inhibited than that of the WT by selenate which is a toxic analog of sulfate. Selenate is transported into the cell by sulfate permease, a product of the *sB* gene (Arst, 1968; Pilsyk et al., 2007; Pilsyk and Paszewski, 2009). Sulfate uptake and incorporation are derepressed in *kaeA* mutants (Fig. 4), resulting in selenate sensitivity. RT-qPCR analysis indicates that this derepression most likely does not result from an increased level of *sB* transcription (Fig. 9 and Sup. Fig. 7), suggesting that KAEA protein may be involved in the regulation of sulfate permease activity at the posttranscriptional level.

Suppressor mutants are also much more sensitive than the WT to the ornithine decarboxylase (ODC) inhibitor DAB, which prevents the conversion of ornithine to putrescine (Fig. 1). The effect of DAB can be reversed by the addition of putrescine or spermidine (Fig. 3). RT-qPCR (Fig. 9 and Sup. Fig. 7) and transcriptome analysis (data not shown) indicate that the DAB sensitivity of the suppressor mutants does not result from changes in the mRNA level of *puA*, which encodes ODC. However, this sensitivity may result from the lowered expression of the putative polyamine efflux transporter revealed by transcriptomics (see part 3.4).

Growth of *suD19pro* and *suD25pro* is even more impaired when glucose is replaced as the sole carbon source in the minimal medium by glycerol, suggesting a defect in respiration. This result correlates with a changed mitochondrial structure, which aggregate in clumps in both mutants studied, especially in *suD25pro* (Fig. 5). A defect in mitochondrial function may also explain the higher sensitivity of these mutants to ethidium bromide (Fig. 3), which is known to preferentially inhibit mitochondrial DNA replication. In *S. cerevisiae*, mutations in *ABF2*, which is essential for mtDNA maintenance, are sensitive to ethidium bromide (Chen et al., 2005). Similar phenotypes were reported in *S. cerevisiae* and *Caenorhabditis elegans* with mutations in *QRI7/OSGEPL*, the mitochondrial paralogue of *KAE1* (Oberto et al., 2009). However, to our knowledge, no mitochondrial defects have been reported for *KAE1* mutants in *S. cerevisiae*. We have identified a *QRI7* orthologue in *A. nidulans* (AN8780), but the sensitivity of the *suDpro* mutants to ethidium bromide suggests that KAEA may also be important for mitochondrial function and maintenance in this organism.

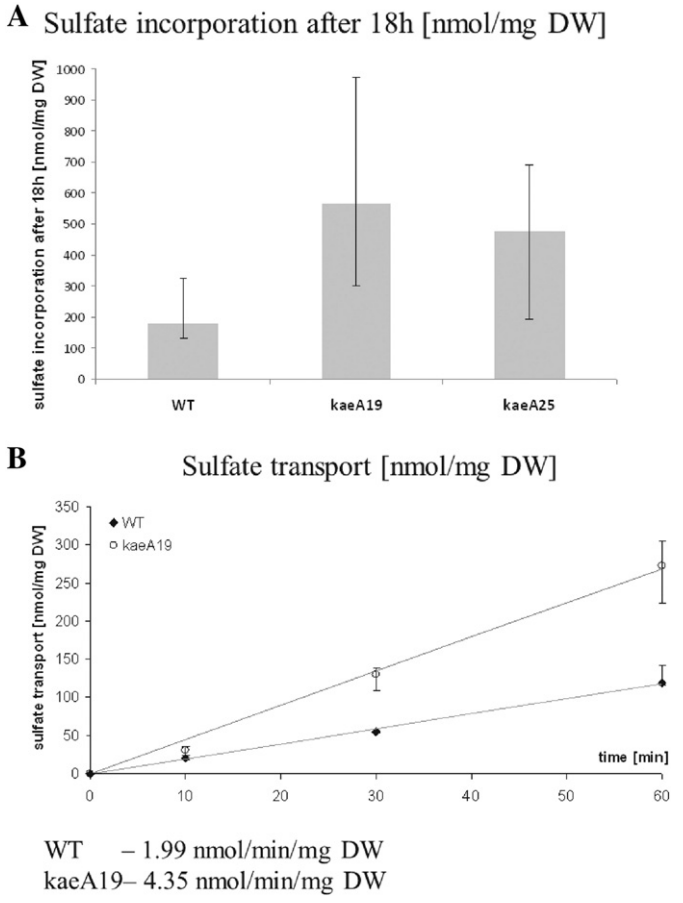


Fig. 4. Sulfate incorporation and uptake in *kaeA* mutants and wild-type strains. (A) Average sulfate incorporation level in mycelium grown for 18 h in minimal medium supplemented with 5 mM methionine and [^{35}S] sulfate. (B) Kinetics of sulfate uptake (see Materials and methods section for details). DW — dry weight.

3.2. suDpro codes for a protein of the Kae1 family

We have cloned the *suDpro* gene by co-transformation of the *suD25pro* strain with the *A. nidulans* minimal chromosome-specific cosmid library and the plasmid pUG11-41, bearing the *metG* gene (Sienko and Paszewski, 1999). The frequency of co-transformation in *A. nidulans* is sufficiently high (Dzikowska and Weglenski, 1989) to assume that among *met*$^+$ transformants the *pro*$^-$ phenotype would appear. We selected *met*$^+$ transformants that had lost the suppressor phenotype, i.e., in the *proA*$^-$ background they did not grow on the proline-free medium (for details see Suppl. Materials 1). The *suDpro* gene was sequenced and identified as AN6569 in the *A. nidulans* genome.

A cDNA copy of the *suDpro* gene was isolated from the *A. nidulans* cDNA library and sequenced. It contains an open reading frame coding for a protein of 363 amino acids. The coding sequence of the *suDpro* gene was submitted to GenBank (http://www.ncbi.nlm.nih.gov) as *Emericella nidulans* partial *suDpro* gene, intronic sequence, wild type (accession no. AJ293803). The gene contains one typical, short fungal intron of 66 bp in the coding sequence. Interestingly, the intron in *suDpro* published in the *A. nidulans* genome sequence database (www.broadinstitute.org/annotation/genome/aspergillus_group) is 12 bp shorter at its 3′ end (Fig. 6), suggesting differential splicing. The transcription start site was identified at position −109 in relation to ATG (Suppl. Fig. 1), and the polyadenylation site was 46 bp downstream of the stop codon.

The promoter region of *suDpro* is shown in Fig. 6. It contains two TATA-like sequences and potential binding sites for GATA factors (Scazzocchio, 2000), BRLA (Chang and Timberlake, 1993), ABAA (Andrianopoulos and Timberlake, 1994) and AnCF (Brakhage et al., 1999). We have also identified a sequence identical to the yeast sterol regulatory element (Vik and Rine, 2001). It is worth noting that the distance between the *suDpro* transcription start site and the ATG of the 5′ neighbor gene (AN6568) is very short (351 bp), suggesting that this region may function as a bidirectional promoter.

The *suDpro* gene potentially codes for a 363-aa-long protein predicted to have M_W = 39.3 kDa. A BLAST search showed extremely high similarity between this protein and the eukaryotic Kae1 protein family (Hecker et al., 2007; Mao et al., 2008). Therefore, we renamed from *suDpro* to *kaeA*. Similar to other proteins of this family, KAEA protein contains all motifs specific to the ASKHA chaperone superfamily, i.e., α/β structures, a metal-binding domain, and a specific ATP-binding site, as well as two unique inserts characteristic to the Kae1 family (Hurley, 1996; Aravind and Koonin, 1999; Hecker et al., 2008; Mao et al., 2008). Interestingly, the potential differential splicing could yield a protein with four additional and one changed amino acid within the conserved α3 domain (Fig. 7).

As KAEA homologues are elements of the EKC/KEOPS complex, we searched the *A. nidulans* genome for other members of this complex using Position-Specific Iterative BLAST (PSI-BLAST) (Altschul and Koonin, 1998). AN2513 was identified as a homologue of Bud32p (Suppl. Fig. 2), AN11910 — Cgi121p (Suppl. Fig. 3), and AN2845 — Pcc1p (Suppl. Fig. 4). AN11901 was recognized as a homologue of the fungi-specific Gon7p/Pcc2p subunit (Suppl. Fig. 5).

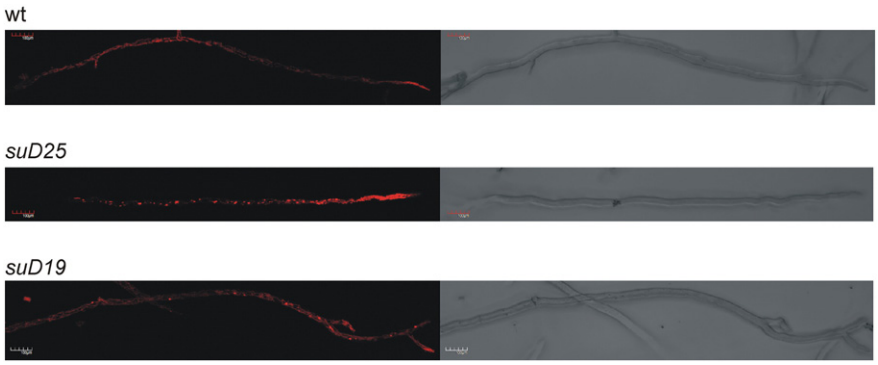


Fig. 5. The structure of the mitochondrial network is changed in *suD19pro* and *suD25pro*. The mitochondrial structures in *suD19pro*, *suD25pro* and the wild-type control strain (wt) were compared using a confocal microscope. The mitochondria were visualized with MitoTracker® Red CMXRos.

3.3. Characterization of suD19pro and suD25pro mutations

The *suD19pro* and *suD25pro* alleles of *kaeA* were sequenced. Both mutations are short deletions in the coding region, which do not change the reading frame (Fig. 6). A model of the KAEA structure shows that amino acids from both deleted regions are localized on the protein surface (Suppl. Fig. 6); it is possible that these deletions influence the interaction of KAEA with other proteins. The deletion in *suD19pro* encompasses twelve base pairs, resulting in a lack of four amino acids (STPQ) in the non-conserved linker between the β2 and β3 sheets (Fig. 7). However, shortening this linker might influence the interaction with Bud32p, as it has been shown that the structure of the next β3-α1 linker of Kae1p is changed after the binding of this kinase (Mao et al., 2008). The deletion in *suD25pro* is shorter (nine base pairs) and results

GAGTTGATCAAGGTCCTGCGAGAGCAAATCCTTTAGTTTCGAATTGTGGGTTGAGAGGACGA
GATCTCGGGGGATGGCGAAAAGTTCTTCGTCTTCGTC**GATA**TCGGCTTGAGCCACTGGAACC
CGCGAGGCAAGCG**TATCAGAATG**GAATCACTTCTTCGTAAAG**GATA**CAACTTAATAAGGTCG
AGGACAGATTGCTAGACGAGGAAAAACTCGTCCGGGTGGAGTTAAAACATACCGACACCTCG
CCCAGCAGCATTGGCTCTTAGATCCGCAATACGAATCTTGGAGTTGACTTTGACGCCAGGCC
TAGAT**GATA**GCCAGGTAGTAAACTCGTCTGACCGGCATTGGAAATTGTCACGGTCGGGGAAG
TGAGCCGAAGAGGA**CAT**GACTTTTGACAACCTTGCGGGATTGTGGGCGGGCACAAAACGAAG
TTAGCACCTCGGAGGATGGATTCAGGGGTGAAAAAGGACAACCCGCAATTG**TATAA**T**TATAA**
AAAGAAGGTTGGAGCCAGGAGAGGAGGAATCTGCCGAAGTGGGTAAAAAGCAGAGCAGTAGG
GAGGGGAGGGGAGGAGAGAGTAGTGGGCTGTTGAGTTGTCGCTGTAGCTTACTTAGTTTAGT
AGGGTCTTGTATACCGTCG**GATA**AACGCAGCGGCGGAACCTAACCTGGAACTGCCGGGACCG
ClaI
GATCCAAAGCAGACGAG**CCAAT**C**GATA**ACGTTGCT**GATA**AAAAAAAAAGGATCTCCGACGCT
G**CCCCTC**CATACAGCACTCACACTACCTATAGCTT**CATTCC**GCA**TCGTATAAT**ACTATCTTA
ATGAGTTTTTCCCCCTAATTGTCAGCAGAAAATACTACCC**AGAATG**ATTGCCATCGGTCTTG
AAGGTTCCGCCAACAAACTCGGCGTGGGTATCATGTTACACCCTAAGGACGG*TAGCACCCCG*
*CA*AGTCCTCGCCAACATCCGCCACACATATGTCTCCCCTCCCGGCGAGGGATTTCTCCCCAA
A**GATA**CAGCAAGGCACCACCGGTCCTGGGTCGTCAGTCTCGTGAAGAAAGCACTCAAGGAAG
CGCGTATCTCCGTTGACGATGTGGACTGTATATGCTACACCAAGGGACCAGGAATGGGAGCT
CCCCTTCAGAGTGTGGCAGTGGCTGCACGGACGCTAAGCTTACTATGGGGGAAGGAACTAGT
TGGTGTTAACCACTGTGTTGGACgtatgctcccaagtcccgattttatgatatagaataata
gtaggtacctaatgcggatcgctccagATATCGAAATGGGTCGTCTAATTACCGGCGCATCG
AACCCGGTCGTCCTATACGTGTCTGGAGGGAATACACAAGTAATCGCTTACAGCTCGCAACG
GTATCGCATCTTCGGTGAGACTCTCGATATCGCGGTGGGCAACTGTCTTGACCGATTCGCGC
GAACGCTACATATCTCTAATGACCCGGCTCCGGGGTACAATATTGAGCAACTCGCCAAAAAG
GGCAAGCAACTGGTCGATTTGCCATACACAGTAAAAGGCATGGACTGCTCTATGTCAGGCAT
TCTTGCAGCCATTGATGCTCTCGCCGCAACGTACGGACTAAACGGAGAACAACCGGATGAGG
AGGAAGACGTAACAGATGTTACGCCCGTTTCAGACGGGGCTTTAGAAAGCCGGAAACCAACC
CGGGCAGATCTGTGCTTTTCGCTGCAGGAGACGGTATTCTCGATGTTGGTGGAAATTACAGA
NcoI
GCGCGCCATGGCACATGTTGGGTCGAAGGAAGTTTTGATTGTTGGCGGAGTCGGATGTAATG
AGAGGCTGCAGGAGATGATGGGGATCATGGCGCGGGATCGCGGAGGCAGTGTGCATGCCACG
GATGAGAGGTTTTGTATTGACAACGGGATTATGATTGCCCAGGCTGGTATGCTCGCATATAA
*GACAGGCTT*CCGGACGCCGCTCAAGGAATCTACGTGCACGCAGCGCTTCCGGACGGATGATG
TATTTGTGCAATGGAGAGAT**TAG**ACTTATTCAGCCATATCCTGCTAATGTAATGAAGATTCA
ATTTGAA*

Fig. 6. Sequence of the *kaeA* (*suDpro*) gene. The transcription start site is marked in dark gray. Putative TATA box, start and stop codons of *kaeA* and start codon (CAT) of a neighboring divergently transcribed AN6568 gene, are bolded and underlined. Putative transcription factor-binding sites in both orientations are bolded; GATA — GATA factor-binding site; CATT CC — ABAA-binding site; CCAAT — AnCF-binding site; GAGGGG — BRLA-binding site; TCGTATAAT — yeast sterol regulatory element. * — polyadenylation site. Intron — small letters (small underlined — nucleotides not present in the intron identified by the Broad Institute). Italic light gray — deletion in *suD19pro*, italic light gray and underlined — deletion in *suD25pro*. Recognition sites for ClaI and NcoI are indicated over the sequence.

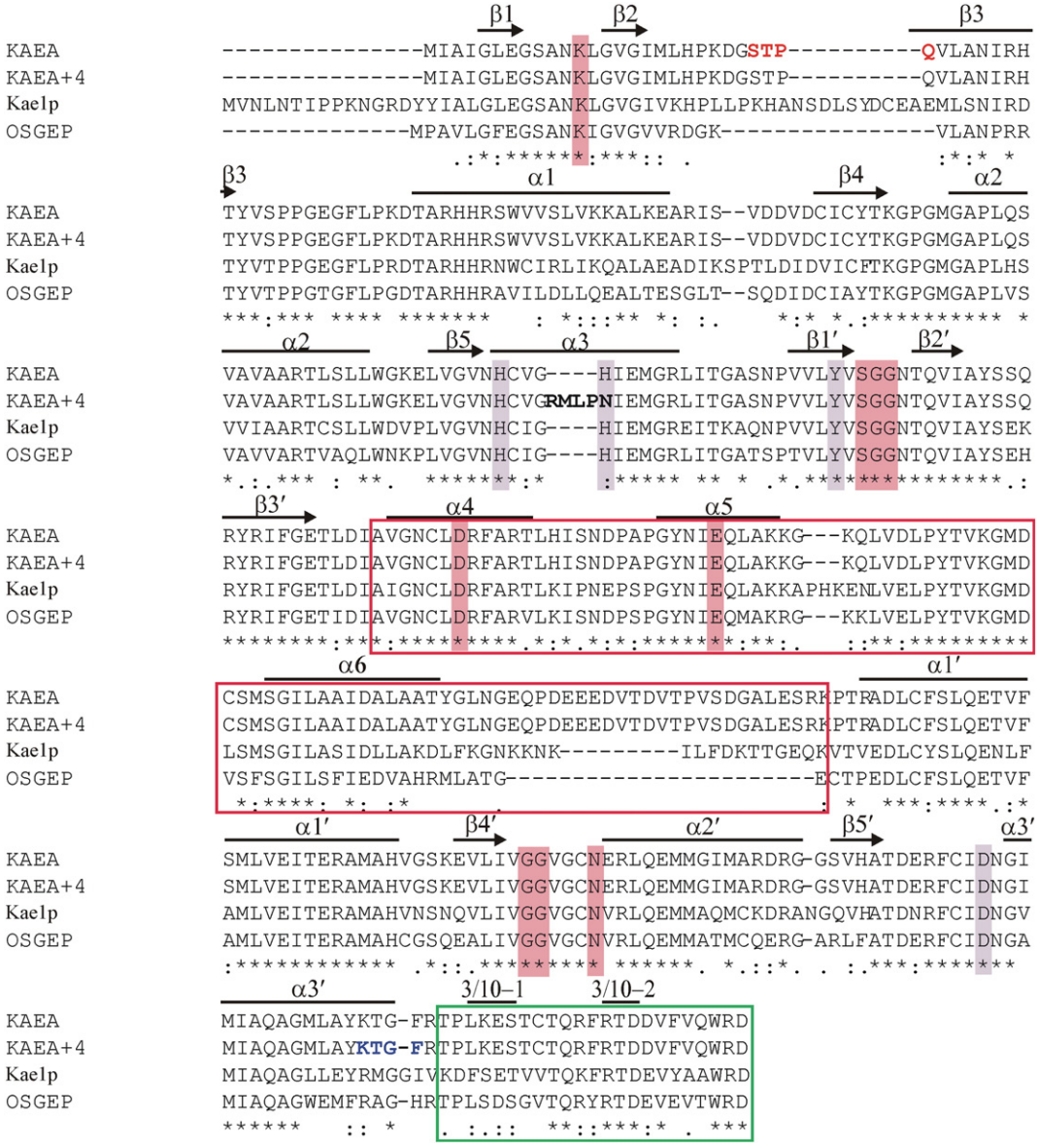


Fig. 7. Sequence alignment of *A. nidulans* KAEA, its potential splicing variant (KAEA + 4), *S. cerevisiae* Kae1p and human OSGEP. β sheets, α- and 3_{10} helices (3/10-1 and 3/10-2) are indicated over the sequence, according to Mao et al. (2008). Violet — residues of KAEA coordinating metal binding. Pink — residues of KAEA coordinating ATP binding. Red — deletion in *suDpro19*, blue — deletion in *suD25pro* (KTGF replaced by N). Inserts unique to the Kae1 family are framed: in red — insert 1, in green — insert 2. In bold — additional amino acids in KAEA resulting from the potential differential splicing. Alignment was obtained using Clustal Omega software (Sievers et al., 2011).

in a replacement of four amino acids (KTGF) by one (N) in a C-terminal part of the α3′ helix. This deletion encompasses conserved amino acids (bases K/R and G), and may change the structure of the linker between the α3′ and 3/10-1 helix (Fig. 7). The latter was shown to interact with Pcc1p (Mao et al., 2008). It is also possible that both *suDpro* mutations could influence the binding of KAEA to other proteins that are not part of the KEOPS/EKC complex. It has been reported that subunits of the complex may have additional, individual functions. For example, Bud32p interacts with Grx4p glutaredoxin, suggesting additional functions of this kinase in yeast (Peggion et al., 2008).

3.4. Mutation in *kaeA* affects expression of several genes

We have shown that mutations in *kaeA* result in pleiotropic effects. This was to be expected, given the multiple functions ascribed to the EKC/KEOPS complex in other organisms. We have compared the transcriptomes of a *kaeA* mutant (*suD25pro*) and the control isogenic strain grown on minimal medium, to identify genes whose transcription is affected by the mutation. The *kaeA* mutant shows significant changes in gene expression, with 36 genes upregulated and 107 genes downregulated in the mutant compared to the wild type (*P*-value <0.001 and at least two-fold change in the level of expression) (Fig. 8 and Supplementary Table 2). As expected, *agaA* and *otaA* were identified among the upregulated genes, in agreement with the northern hybridization results (Fig. 2B) and RT-qPCR (Fig. 9 and Suppl. Fig. 7). This confirms the reliability of the transcriptomic analysis.

The expression profile of the *kaeA* mutant (*suD25pro*) is enriched for genes involved in amino acid/siderophore metabolism and carbon/energy metabolism (Fig. 8). It is very probable that at least some effects of the *kaeA* mutation on the transcription level of several genes are indirect, as was shown in yeast. Transcriptome analysis of the *S. cerevisiae KAE1-18* thermosensitive mutant revealed that the mutation resulted in upregulation of several genes coding for enzymes of the amino acid biosynthetic pathways (Daugeron et al., 2011). Derepression of these genes seems plausible, as this mutation results in the upregulation of *GCN4*, which encodes the global transcriptional activator of amino acid

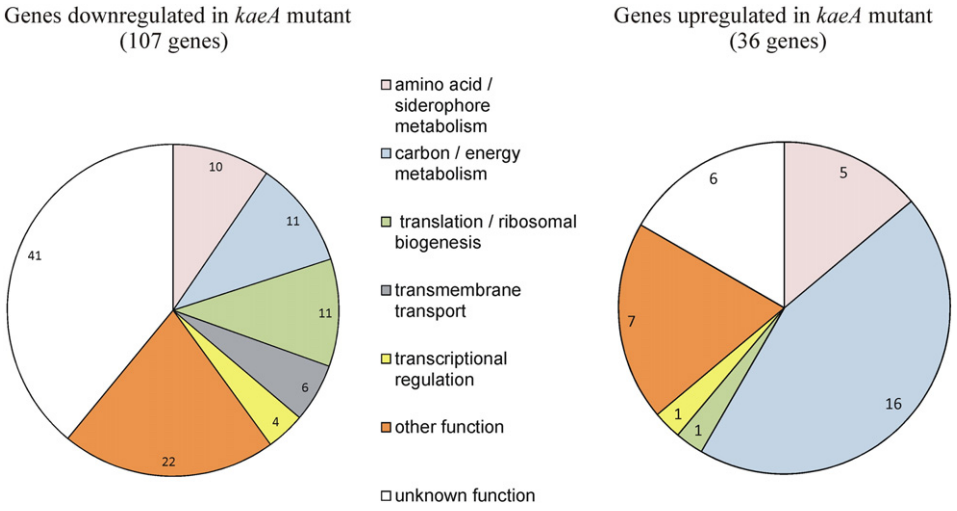


Fig. 8. Genes up- and downregulated in the *kaeA* (*suD25pro*) mutant (see Suppl. Table 1 for details).

biosynthesis. *GCN4* expression is regulated at the translation level (Hinnebusch, 2005). Daugeron et al. (2011) proposed that overexpression of Gcn4p in the *KAE-18* mutant results from the misregulation of *GCN4* mRNA translation. In *A. nidulans*, the accumulation of the CPCA protein, the homologue of Gcn4p, might also be due to a lower rate of degradation. In yeast, the degradation of Gcn4p was shown to depend on Pcl5p cyclin (Aviram et al., 2008); we observed that the expression of the *PCL5* homologue (AN9500) is downregulated in the *kaeA* mutant (see Supplementary Table 2). The mutation of *kaeA* also results in slight downregulation of *jlbA*, which codes for another transcription factor proposed to be involved in regulation of amino acid biosynthesis (Strittmatter et al., 2001). This might explain the downregulation of some of these genes in the *kaeA* mutant.

As described above, both arginine catabolism genes, *agaA* and *otaA*, are upregulated in the *kaeA* mutant. Ornithine is not only a precursor to arginine metabolism but also a substrate for polyamine and siderophore biosynthesis (Fig. 1). This suggests that changes in arginine/ornithine metabolism could influence the concentration of these compounds. Several genes' expression level is much lower in the *kaeA* mutant compared to the wild type, including AN7295, the homologue of *S. cerevisiae TPO1* and *Candida albicans FLU1*, which encodes a major plasma membrane polyamine efflux transporter, a member of the major facilitator superfamily (MFS) of transporters (Albertsen et al., 2003; Kashiwagi and Igarashi, 2011; Li et al., 2013). Both proteins were shown to be involved in the excretion of excess intercellular polyamines and might be involved in the efflux of DAB, the toxic analog of putrescine. The lower expression of AN7295 in the *kaeA* mutant strains could explain their higher sensitivity to DAB.

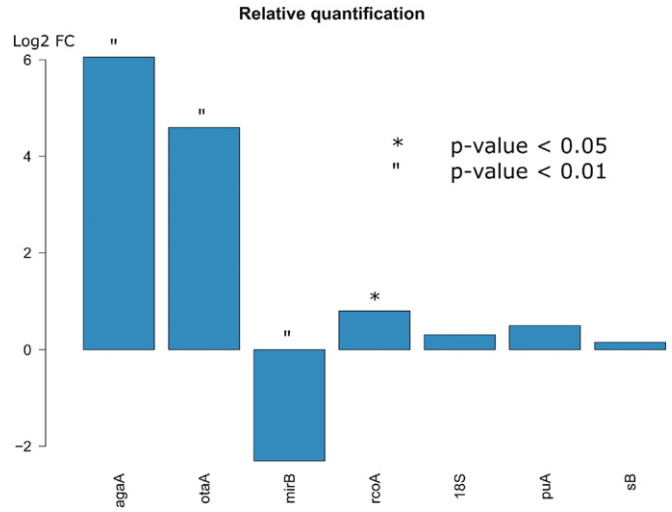


Fig. 9. Quantitative transcriptional analysis of selected genes. Relative expression of *agaA*, *otaA*, *mirB*, *puA*, *sB* and *rcoA* genes in the *kaeA25* mutant in comparison with the control strain was calculated by RT-qPCR analysis (see Materials and methods section for details). For *18SrRNA*, *puA* and *sB* the difference between the two strains is not significant (*P*-value > 0.05). FC — fold change (*kaeA*/control).

Siderophores are ferric iron-specific chelators excreted by most fungi to mobilize extracellular iron or employed for intercellular iron storage (Haas, 2003, 2012). In the *kaeA* mutant, among genes participating in siderophore biosynthesis and uptake, *sidA*, which encodes ornithine monooxygenase (Eisendle et al., 2003), is upregulated, while *sidG* and *mirB*, which encode fusarinine C transacetylase and the trisacetylfusarinine C transporter (Schrettl et al., 2007) (Haas et al., 2003), respectively, are downregulated. For the *mirB* transcript, this result was confirmed by RT-qPCR (Fig. 9 and Suppl Fig. 7). It is possible that the misregulation of these genes leads to a change in the cellular iron concentration and results in changes in several metabolic processes, including ribosomal biogenesis and translation, as was suggested by Schrettl et al. (2010).

rcoA is among genes upregulated in the *kaeA* mutant; this result was confirmed by RT-qPCR (Fig. 9 and Suppl Fig. 7). *TUP1*, which encodes the global corepressor of transcription, is a homologue of *rcoA* in *S. cerevisiae*. Tup1p forms a complex with Cyc8p/Ssn6p (Varanasi et al., 1996) and inhibits transcription by various mechanisms. These include interaction with the N-terminal tails of histones H3 and H4 (Edmondson et al., 1996; Davie et al., 2002) and/or different histone deacetylases (Watson et al., 2000; Wu et al., 2001; Davie et al., 2003), the establishment of repressive chromatin structure (Fleming et al., 2014), or direct interaction with subunits of the mediator complex (Gromoller and Lehming, 2000; Lee et al., 2000; Papamichos-Chronakis et al., 2000). On the other hand, Tup1p appears to enhance the expression of some genes (Zhang and Guarente, 1994; Conlan et al., 1999). Recently, it was proposed that Tup1p masks the activation domain of activators and blocks the recruitment of coactivators (Wong and Struhl, 2011). Tup1p is recruited to target promoters by specific DNA-binding factors and regulates the expression of several genes involved in mating, glucose and oxygen use, stress response, and DNA damage (Smith and Johnson, 2000). In *A. nidulans*, *rcoA* is involved in the regulation of fungal growth and development and, to lesser extent, in carbon regulation (Hicks et al., 2001; Todd et al., 2006). *rcoA* has been demonstrated to be essential for the maintenance of the closed chromatin structure of some promoters (Garcia et al., 2008). It is possible that changes in the expression of some genes observed in *kaeA* mutant result from the upregulation of the *rcoA* gene in this strain. In fact, yeast

homologues of several genes up or downregulated in the *kaeA* mutant, have been shown to be regulated by Tup1p (see Supplementary Table 2) (Hu et al., 2007; Venters et al., 2011).

Although the involvement of the KEOPS/EKC complex in tRNA modification is well documented, the role of this complex in other cellular processes is still unresolved. Moreover, the presence of the fifth Pcc2p/Gon7p additional subunit in fungi suggests a specific function of the complex in this organismal group. It was proposed that KEOPS/EKC regulates the accessibility of chromatin and recruits transcription factors to specific promoters (Downey et al., 2006; Kisseleva-Romanova et al., 2006; Costessi et al., 2012). PIPA, the Bud32p orthologue in *A. nidulans*, was shown to interact with the Cdk9 kinase homologue (PTKA) (Kempf et al., 2013). Cdk9 phosphorylates serine 2 in repeating heptapeptides of the C-terminal domain of the largest subunit of RNA polymerase II. This modification is necessary to switch from transcription initiation to elongation. PIPA/PTKA interaction is restricted to the head of the conidiophore, indicating that Bud32 (PIPA), and possibly the whole KEOPS/EKC complex, is involved in transcriptional regulation during *A. nidulans* development. Genetic data suggest that KAEA may also be involved in the transcriptional regulation of the arginine catabolic genes. Both *kaeA* mutations, *suD19pro* and *suD25pro*, result in the derepression of *agaA* and *otaA* transcription under non-inducing conditions, i.e., in the absence of exogenous arginine. The *arcA* gene, which codes for the pathway-specific activator, is indispensable for *agaA* and *otaA* induction by arginine. A recessive $arcA^r3$ mutation results in non-inducibility of these genes and the inability to utilize arginine as a proline or nitrogen source (Bartnik and Weglenski, 1974; Empel et al., 2001). The $arcA^r3$ mutation was shown to be epistatic against *kaeA* mutations; in the double $arcA^r3$; *suDpro* mutant, arginase and OAT are not inducible by arginine (Bartnik and Weglenski, 1974). This genetic interaction suggests that KAEA collaborates with the ARCA activator.

In conclusion, it is possible that several of the pleiotropic effects of the *suDpro* mutations are indirect and result from the changed expression levels of global regulators such as $cpcA^{GCN4}$ and $rcoA^{TUP1}$ However, to the best of our knowledge, only GCN4, but not TUP1, has been shown to be regulated at the translational level. We postulate that the expression of some genes, e.g., arginine catabolism genes, may be directly regulated at the level of transcription by the KAEA, and possibly the KEOPS/EKC complex.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.07.066.

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