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# CARATTERIZZAZIONE AVANZATA DEL COMPLESSO DI LIEVITO KEOPS/EKC

# ADVANCED CHARACTERIZATION OF THE YEAST KEOPS/EKC COMPLEX

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*«Tutti sanno che una cosa è impossibile da realizzare,*
*finché arriva uno sprovveduto che non lo sa e la inventa.»*
*Albert Einstein*

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# Abstract

During my PhD I have studied the properties of two yeast proteins, the protein kinase Bud32 and the putative protease Kae1, that take part in a nuclear complex named KEOPS/EKC. Actually, while Kae1 is associated uniquely to the proteins of the complex, Bud32 has many other partners in the cell; in fact, I have also studied its strong relationship with the Grx4 glutaredoxin.

The yeast KEOPS/EKC complex has been isolated in 2006 by two different groups and has been shown to be involved in both telomere homeostasis and transcription regulation. The complex is evolutionarily conserved and is composed of five proteins: the protein kinase Bud32, the hypothetical metallo-protease Kae1, the still uncharacterized Cgi121, and two other proteins of small size, Pcc1 and Pcc2/Gon7. For many years, our attention has been focused on the atypical protein kinase Bud32, which interacts with many other yeast proteins, suggesting that it may play several roles in the cell. Among these Bud32 partners, we demonstrated that the glutaredoxin Grx4 is a substrate of the protein kinase, being readily phosphorylated by Bud32 mainly at Ser 134. Also, this modification is upregulated by the previous phosphorylation of Bud32 at its Ser258 residue by the Sch9 protein kinase (the yeast homologue of mammalian Akt/PKB). During the first part of my PhD I deepened the study of the physiological significance of this new phosphorylation cascade. By the phenotypic analysis of yeast strains expressing mutagenized forms of Grx4, I demonstrated that the phosphorylation of Grx4 by Bud32 is important for Grx4 functionality *in vivo*. However I could not identify a specific effect of the Bud32-mediated phosphorylation of Grx4 on the known activities of the glutaredoxin, wich is involved in iron cellular homeostasis and in the survival under oxidative stress conditions. This result suggests that the Bud32-mediated phosphorylation of Grx4 play a role in different, uncharacterized functions of the glutaredoxin. I also checked if the phosphorylation of Bud32 by Sch9 could modulate the activity of the whole KEOPS complex, but the analysis of telomeres length and of the activation rate of the galactose-inducible *GAL1* gene (one of the main transcriptional targets of KEOPS) showed that these functions are unaffected in a Bud32 unphosphorylatable mutant (S258A). These results suggest that the phosphorylation of Bud32 at Ser258 is unrelated to its function within the KEOPS complex.

I then addressed my attention to the Kae1 subunit of the complex. By using two strains expressing mutagenized forms of Kae1, I could demonstrate that the activity of this protein is essential for the complex, both at the telomere and at the transcriptional level. The biochemical function of Kae1 is however still unknown. It was initially classified as a protease, and, in effect, in 2006 an endopeptidase activity was indirectly demonstrated for the human homologue of Kae1, OSGEP. On the contrary, in 2007 Hecker *et al*. demonstrated that an archaeal orthologue of Kae1 is an AP-endonuclease . During my PhD I tried to define the activity of yeast Kae1, but the results obtained are not sufficient to clarify this point.

Finally, I decided to verify the hypothesis, coming from a recent work that describes the atomic structure of an archaeal-derived KEOPS complex, that Kae1 could be a substrate of Bud32. Using the yeast Bud32 and Kae1 proteins, co-expressed and purified from *E.coli*, I observed that the also the recombinant proteins are tightly associated, forming a kind of catalytic KEOPS subcomplex. Using several mutagenized forms of these proteins I demonstrated, by *in vitro* phosphotranspherase assays, that Bud32 is able to phosporylate Kae1 and that the binding of Kae1 has an inhibitory effect on the catalytic activity of the kinase. An important confirmation comes from the MS analysis of phosphorylated Kae1, that identified Ser 367 as a target of Bud32. However this might not be the only phosphorylated residue.

Altogether these results indicate that, at least *in vitro*, a regulatory relationship exists between Bud32 and Kae1. This is interesting as the two proteins are liable to carefully modulate the functions of the entire KEOPS complex.

# Riassunto

Durante il Dottorato di ricerca, mi sono occupata dello studio di due proteine di lievito, la proteinchinasi Bud32 e l'ipotetica proteasi Kae1, che fanno parte di un complesso nucleare denominato KEOPS/EKC. Mentre Kae1 è associata unicamente alle proteine del complesso, Bud32 ha molti altri partner all'interno della cellula; oggetto del mio studio è stata infatti anche la sua forte relazione con la glutaredoxina Grx4.

Il complesso di lievito KEOPS/EKC, isolato nel 2006 da due diversi gruppi di ricerca, è coinvolto nell'omeostasi telomerica e nella regolazione della trascrizione. Il complesso è evolutivamente conservato ed è composto da cinque proteine: la chinasi Bud32, l'ipotetica proteasi Kae1, Cgi121, proteina non ancora caratterizzata, e altre due piccole subunità, Pcc1 e Pcc2/Gon7. Per molti anni la nostra attenzione è stata rivolta alla proteinchinasi atipica Bud32, che interagisce con molte altre proteine di lievito, suggerendo come essa possa avere altri ruoli all'interno della cellula. Tra i vari partner di Bud32, abbiamo dimostrato che la glutaredoxina Grx4 risulta essere un substrato della chinasi sia *in vivo* che *in vitro*, ed è infatti fosforilata da Bud32 principalmente nella Ser 134. Questa relazione è inoltre a sua volta modulata dalla precedente fosforilazione di Bud32 nella Ser258 da parte della chinasi Sch9 (l'omologa in lievito della chinasi di mammifero Akt/PKB). Durante la prima parte del mio dottorato mi sono concentrata sulla ricerca di un possibile significato fisiologico di questa nuova cascata di fosforilazioni. Analizzando ceppi di lievito che esprimono forme mutagenizzate di Grx4, ho dimostrato come la fosforilazione di Grx4 da parte di Bud32 sia importante per la funzionalità della proteina *in vivo*. Sfortunatamente, non ho potuto identificare un effetto di questa fosforilazione sulle attività della glutaredoxina, coinvolta nella regolazione dell'omeostasi cellulare del ferro e nella sopravvivenza in condizioni di stress ossidativo. Questi risultati portano all'ipotesi che la fosforilazione di Grx4 da parte di Bud32 giochi un ruolo in qualche funzione diversa e ancora non caratterizzata della glutaredoxina.

Ho inoltre verificato se la fosforilazione nella Serina 258 di Bud32 da parte di Sch9 potesse modulare l'attività dell'intero complesso KEOPS, ma l'analisi della lunghezza dei telomeri e del livello di attivazione del gene inducibile *GAL1* (uno dei maggiori target trascrizionali di KEOPS) ha rivelato che queste funzioni non erano colpite nel mutante

*BUD*$^{S258A}$. Questi risultati suggeriscono che la fosforilazione della serina 258 di Bud32 non sia collegata alle funzioni della chinasi nel complesso KEOPS.

Ho successivamente indirizzato la mia attenzione alla proteina Kae1. Attraverso l'utilizzo di due ceppi, esprimenti forme mutagenizzate di Kae1, ho potuto dimostrare come l'attività di questa proteina sia essenziale per l'intero complesso, sia a livello dei telomeri che della trascrizione. La funzione biochimica di Kae1 è tuttavia ancora sconosciuta. Inizialmente la proteina è stata classificata come una proteasi, e in effetti nel 2006 è stata indirettamente dimostrata un'attività endopeptidasica per l'omologa umana di Kae1, OSGEP. Al contrario, nel 2007 Hecker *et al.* hanno dimostrato che l'omologa di Kae1 in Archea è un'AP-endonucleasi. Durante il mio dottorato ho provato a definire l'attività della proteina Kae1 di lievito, ma i risultati ottenuti non sono stati sufficienti per chiarire questo punto.

Infine, ho deciso di verificare l'ipotesi, derivante da un recente lavoro in cui viene descritta la struttura atomica del complesso KEOPS negli Archaea, che Kae1 possa essere un substrato di Bud32. Utilizzando le proteine di lievito Bud32 e Kae1, espresse in *E.coli*, ho osservato che, come accade nelle cellule di lievito, le proteine ricombinanti sono strettamente associate e formano quindi una sorta di sub-complesso catalitico di KEOPS.

Utilizzando diverse forme mutagenizzate delle due proteine, in test chinasici *in vitro*, ho dimostrato che Bud32 è in grado di fosforilare Kae1 e che il legame di Kae1 ha un effetto inibitorio sull'attività catalitica della chinasi. Un'importante conferma è derivata dall'analisi di spettrometria di massa su Kae1 fosforilata, in cui la Ser 367 è stata identificata come target di Bud32. Tuttavia questo potrebbe non essere l'unico residuo fosforilato.

Nel complesso, questi dati indicano che, perlomeno *in vitro* Bud32 e Kae1 vengono reciprocamente regolate. Questo dato è interessante, dal momento che le due proteine potrebbero modulare le funzioni dell'intero complesso KEOPS.

# Introduction

## YEAST AS A MODEL EUKARYOTE

The yeast *Saccharomyces cerevisiae* is an eukaryotic micro-organism classified in the kingdom of Fungi. It is perhaps the most useful yeast, owing to its use since ancient times in baking and brewing. It is also one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *Escherichia coli* as the model prokaryote. *S.cerevisiae* cells are round to ovoid, 5–10 μm in diameter. It reproduces by a division process known as budding. Yeasts have asexual and sexual reproductive cycles, however the most common mode of vegetative growth in yeast is asexual reproduction by budding or fission. In the budding yeast *S.cerevisiae* a small bud, or daughter cell, is formed on the parent cell. The nucleus of the parent cell splits into a daughter nucleus and migrates into the daughter cell. The bud continues to grow until it separates from the parent cell, forming a new cell. Under high stress conditions haploid cells will generally die, however under the same conditions diploid cells can undergo sporulation, entering sexual reproduction (meiosis) and producing four haploid spores, which can go on to mate (conjugate), reforming the diploid. Yeast has two mating types, a and α, which show primitive aspects of sex differentiation. Unlike most other microorganisms, in *S.cerevisiae* both the haploid and diploid state are stable. Thus, recessive mutations can be conveniently isolated and manifested in haploid strains, and complementation tests can be carried out through the formation of diploid strains. Although yeasts have greater genetic complexity than bacteria, containing 3.5 times more DNA than *E.coli* cells, they share many of the technical advantages that permitted rapid progress in the molecular genetics of prokaryotes and their viruses. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, and most important, a highly versatile DNA transformation system. Unlike many other microorganisms, numerous mutations in genes for biosynthetic pathways are available in *S.cerevisiae* and are conveniently used as selectable growth markers on synthetic media. Being nonpathogenic, yeast can be handled

with little precautions. Large quantities of normal bakers' yeast are commercially available and can provide a cheap source for biochemical studies. The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Genes corresponding to virtually any genetic trait can be identified by complementation of mutants with plasmids from genome libraries. Plasmids can be introduced into yeast cells either as replicating molecules or by integration into the genome. In contrast to most other organisms, integrative recombination of transforming DNA in yeast proceeds exclusively via homologous recombination. Exogenous DNA with at least partial homologous segments can therefore be directed to specific locations in the genome. Also, homologous recombination, coupled with yeasts' high levels of gene conversion, has led to the development of techniques for the direct replacement of genetically engineered DNA sequences into their normal chromosome locations. Thus, normal wild-type genes, even those having no previously known mutations, can be conveniently replaced with altered and disrupted alleles. The phenotypes arising after disruption of yeast genes has contributed significantly toward the understanding of the function of certain proteins *in vivo*. Also unique to yeast, transformation can be carried out directly with synthetic oligonucleotides, permitting the convenient productions of numerous altered forms of proteins. These techniques have been extensively exploited in the analysis of gene regulation, structure-function relationships of proteins, chromosome structure, and other general questions in cell biology. The overriding virtues of yeast are illustrated by the fact that mammalian genes are being introduced into yeast for systematic analyses of the functions of the corresponding gene products. In addition, yeast has proved to be valuable for studies of other organisms genes, by the use of the two-hybrid screening system (for protein-protein interactions), the use of YACs for cloning large fragments of DNA, and the expression systems for the laboratory and commercial preparation of heterologous proteins. During the last two decades, an ever-increasing number of molecular biologists have taken up yeast as their primary research system, resulting in a virtually autocatalytic stimulus for continuing investigations of all aspects of molecular and cell biology. Most significantly, the knowledge of the DNA sequence of the complete genome, which was completed in 1996, has altered the way molecular and cell biologist approach and carry out their studies [1]. In addition, genome sequencing allowed the start-up

of several projects of systematic investigation of genes functions by the in-depth study of strains carrying disrupted genes.

Among the genes sequenced and disrupted in our laboratory, a gene, encoding an atypical Ser/Thr protein kinase (afterwards named Bud32), aroused our interest and was intensively studied. An important trait of this protein was it property of interacting with several proteins, in particular we identified two strong interactors of the protein kinase: the Grx4 glutaredoxin and a putative metallo-endoprotease, that we named Kae1 (kinase-associated endopeptidase 1)

# THE KEOPS/EKC COMPLEX

In 2006, two groups have independently discovered in *Saccharomyces cerevisiae* a protein complex called either KEOPS (for Kinase, Endopeptidase and Other Proteins of Small size) or EKC (for Endopeptidase-like Kinase Chromatin-associated). The first group [2] demonstrated the involvement of the complex in telomere uncapping and elongation, whereas the second one demonstrated its involvement in transcription of essential eukaryotic genes. This complex consists of five subunits: the Ser/Thr protein kinase Bud32, the kinase-associated endopeptidase 1 (Kae1), and the small subunits Pcc1, Pcc2/Gon7 and Cgi121 [2-4].

During a genome-wide screen in *S.cerevisiae,* Downey and colleagues isolated *CGI121*, a component of the KEOPS complex, as a suppressor of *cdc13-1*, an allele of the telomere-capping protein Cdc13.

The telomere is a nucleoproteic structure at the ends of chromosomes, which protects them from destruction [5, 6]. In addition to that, the telomere organizes chromosome-end replication, in part by regulating the recruitment of telomerase, an enzyme consisting of an RNA component and a reverse-transcriptase enzyme [7]. Telomerase catalyzes the addition of tandem G-rich repeats at the 3′ end of linear chromosomes ($TG_{1–3}$ in *S.cerevisiae*). In yeast, telomerase recruitment is mediated by the Cdc13-Ten1-Stn1 complex and the Yku70/80 heterodimer, both of which help to recruit telomerase via direct physical interactions with its components [8, 9]. Cdc13 directly binds TG-rich telomeric single-

stranded (ss) DNA and, together with Yku70/Yku80, also plays a critical role in chromosome end protection. At restrictive temperatures, strains carrying capping-defective alleles of these genes generate large amounts of ssDNA at telomeres and initiate a robust DNA-damage checkpoint response that is dependent on *MEC1* and *RAD9* [10, 11]. In addition to inducing checkpoint arrest and ensuing replicative senescence [12], defects in telomere capping are a potent threat to genome stability [13, 14]. It has been shown that deletion of either genes encoding DNA-damage checkpoint components (*RAD9, MEC1, RAD24*) or the gene encoding the 5′-3′ exonuclease Exo1, suppress the checkpoint arrest imparted by telomere-capping defects [6]. Telomere-capping defects associated with the *cdc13-1* allele can also be partially suppressed by overexpression of the Stn1 protein, which interacts directly with Cdc13 [15].

Using a genome-wide functional genomics screen the authors were able to isolate genetic suppressors of the thermosensitive *cdc13-1* allele; one of these was the *YML036W* gene, successively named *CGI121*.

*CGI121* is highly conserved in eukaryotes, with the exception of *Drosophila*. Human *CGI121* had already been identified in a two-hybrid screen using the human homolog of *BUD32*, the p53-related protein kinase PRPK as bait [16]. In yeast this relationship is conserved and it has been shown that indeed Cgi121 interacts physically with Bud32.

Using tandem affinity purification (TAP) experiments, Bud32 and Cgi121 were found to be part of a complex containing also Gon7 and Kae1. Downey *et al.* proposed that the KEOPS complex acts as a critical regulator of telomere elongation at native telomeres and at double strand breaks (DSBs), and that it can promote telomere uncapping in *cdc13-1* strains. This dual role of the KEOPS complex appears unique among telomeric proteins and suggests that this conserved protein kinase-containing complex links the processes of telomere protection and telomere elongation.

As mentioned before, the same complex was isolated in a parallel study, during a screen to isolate suppressors of a splicing defect which leads to a cold-sensitive phenotype. During the screen, the authors identified a new ORF and they called the gene *PCC1* (for Polarized growth Chromatin-associated Controller 1); this gene encodes a small protein of about 10kDa. *PCC1* is not essential, but *pcc1* null cells grow very slowly and are thermosensitive at 34°C. The construction of a thermosensitive mutant allele indicated that Pcc1 is required for normal cell cycle progression and mating projection formation. In fact Pcc1 is involved

in the expression of some of the genes induced by the mating pheromone α-factor. Mutation of Pcc1 also affects the expression of the *GAL* genes (that are induced by the presence of galactose in the medium) by impairing the recruitment of the SAGA and Mediator co-activators. By the analysis of proteins interacting with Pcc1, the authors isolated the EKC complex, that contains the Bud32 kinase, the putative endopeptidase Kae1 and two additional proteins, Pcc2/Gon7 and Cgi121. Genetic and physical interactions among these proteins strongly suggested that this complex is a functional unit. Chromatin immunoprecipitation experiments and multiple genetic interactions of *PCC1* mutants with mutants of the transcription apparatus and chromatin-modifying enzymes underscore the direct function of the complex in transcription. Sequence analyses and functional complementation experiments [3, 17] have shown that subunits of KEOPS are conserved among species and suggest that the function of the complex in transcription and/or telomere maintenance (and possibly other processes) is also conserved in other species.

## The Kae1 subunit

### A universally conserved protein

The *KAE1* gene of *S.cerevisiae* encodes a 368 aminoacids protein of a prevalent nuclear localization; this gene is essential for yeast cells, as its inactivation is lethal [18]. Kae1 is the most highly conserved member of the KEOPS complex, with sequence identity of roughly 60% between yeast and humans; the human homolog of *KAE1* is called *OSGEP*. The Kae1 protein and its homologues belong to the small set of about 60 universal proteins present in all members of the three domains of life [19]. This protein was placed in 2004 by Galperin and Koonin at the top of their list of 10 ‘known-unknown’ proteins ‘that should be priority targets for experimental study’. Indeed, this putative endopeptidase (of ‘known’ biochemical function; even if until now its function is still not really defined) was the only protein of ‘unknown’ biological role present in all the 70 genomes then available. The endopeptidase activity of Kae1/OSGEP proteins was suggested by the homology with an O-sialoglycoprotein endopeptidase (Gcp) previously purified from *Pasteurella*

*haemolytica* by Mellors and colleagues in 1991; this similarity permitted to include Kae1 in the M22 family of metallo-proteases (MEROPS database,[20]).

**A putative metallo-protease**

In general, metallo-proteases are enzymes that have critical roles in processes like gene expression, regulation of cell cycle, intracellular targeting of proteins and apoptosis. In these enzymes, a divalent cation, usually $Zn^{2+}$ (but also $Co^{2+}$ or $Mn^{2+}$) activates the water molecule. The metal ion is held in place by amino acid ligands, usually three in number. The known metal ligands are His, Glu, Asp or Lys; and at least one other residue, which may play an electrophilic role, is required for catalysis. Of the known metalloproteases, around half contain an HEXXH motif, which has been shown, by crystallographic studies, to form part of the metal-binding.

An HXXXH motif is present also in Kae1: this is essential as the mutagenesis of either of the two histidines (H-141 and H-145) deprives the protein of its functionality (data not published from our laboratory and [3]). It is interesting to notice, however, that in Kae1 and in all its eukaryotic homologues, the residue of Glutamic acid in the second position is replaced by a Cystein (fig. 1). Cystein and Glutamic acid have very different properties and the presence of that residue may create a great steric effect in correspondence of the site of metal binding. This characteristic suggests that Kae1 may act with a different catalytic mechanism with respect to the other metallo-proteases.

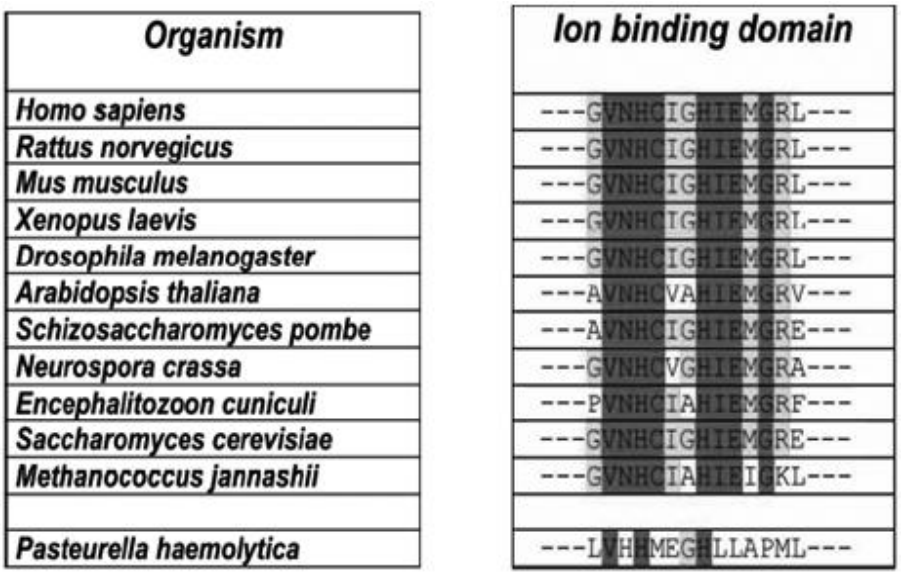



**Fig. 1-Metal ion binding domain in *S. cerevisiae* Kae1 and in its homologs** (from C.Peggion, PhD thesis, 2006)

In 1999, Koonin and co-workers identified an HSP70-actin-like fold (HALF) in Kae1-related proteins and suggested that they were ATP-dependent proteases with chaperone activity [21]. Therefore, it was proposed that yeast Kae1 could modulate the activity of the KEOPS complexes via its proteolytic activity.

In favor of the protease hypothesis, a work of 2006 by Ng *et al.* [22], indirectly demonstrated that a proteasic activity is associated to the mammalian homolog of Kae1, OSGEP. OSGEP is a protein of 335 amino acids, encoded by a gene located on chromosome 14. It lies immediately adjacent to the APEX gene (which encodes the APEX nuclease, a multifunctional DNA repair enzyme) in a 5'-to-5' orientation; transcription of both the *OSGEP* and *APEX* genes is regulated by a bidirectional promoter containing a CCAAT box. Northern blot analysis showed ubiquitous expression of the *OSGEP* gene in several tissues, and similarities in expression patterns between *OSGEP* and *APEX* [22].

Ng *et al*. observed that OSGEP is over-expressed in acute promyelocytic leukemia (APL) cells. In normal cells, accumulation of misfolded nuclear hormone receptor corepressor (N-CoR) as insoluble protein aggregates, induces endoplasmic reticulum stress and activates unfolded protein response (UPR). In contrast, APL cells are resistant to UPR-induced apoptosis. By purification and spectrometric analysis from an APL cell line, it was revealed that the glycoprotease activity of OSGEP is responsible for the resistance of APL cells to the UPR-induced apoptosis, through processing misfolded N-CoR protein. Furthermore, the cleavage of N-CoR in APL cells could be blocked by the broad-spectrum protease inhibitor AEBSF and by RNA interference-mediated down-regulation of OSGEP expression. The protease activity of OSGEP could therefore represent a way exploited by tumor cells to survive the toxic insult of misfolded protein(s) [23].

**A putative endonuclease**

Recently, the structure of the protein YeaZ from *Salmonella typhimurium* (a bacterial homolog of yeast Kae1) has been solved, showing that structurally this protein contains a HALF fold and belongs to the ASKHA (Acetate and Sugar Kinases/Hsp70/Actin) superfamily of phosphotransferases. However, purified YeaZ does not bind ATP and has no glycoprotease activity [24].

In 2007 the analysis of the crystal structure and the biochemical function of the orthologue of Kae1 in the archaeon *Pyrococcus abyssi* (PaKae1, [25]) showed that indeed this protein belongs to the ASKHA protein family. PaKae1 was shown to bind the ATP analogue AMPPNP in a canonical manner. Surprisingly, the nucleotide was found to be directly bound through its phosphate groups to a non-heme iron. Mutation of residues coordinating this iron in the yeast Kae1 (the two histidines of the HXXXH motif) causes lethality, demonstrating the importance of the metal binding for the function of this protein [3]. Although yeast Kae1 was proposed to possess endopeptidase activity, the crystal structure of PaKae1 as well as biochemical activity tests did not support this hypothesis. Rather, it was shown that PaKae1 binds cooperatively to single and double-stranded DNA and induces DNA conformational changes without significant DNA lengthening or shortening. PaKae1 also exhibits a class I apurinic endonuclease activity (AP-lyase), suggesting an important function of this protein in the maintenance of genome integrity [25].

Since the two functions of protease and nuclease are dramatically different, the conserved function of Kae1 remains an open question.

Genome analysis has revealed that homologs of yeast Kae1 and Bud32 are present in all eukaryotes and archaea. The genes encoding the archaeal orthologues of Kae1 and Bud32 are juxtaposed in nearly all archaeal genomes, and they are even fused in several archaea, encoding a single bifunctional protein; this strongly suggests that, even when their genes are separated, Kae1 and Bud32 are physically and functionally linked [26]. To get further insights on the interactions between Kae1 and Bud32, in 2008 the archaeal "fusion" protein MjBud32 of *M. jannaschii* was expressed in *E.coli* and its crystal structure solved [27]. The structure of MJ1130 revealed the strict association of Kae1 and Bud32: the intramolecular interactions identified between the two moieties of MJ1130 suggested that the two yeast proteins might interact similarly. In fact the same interaction between Bud32 and Kae1 has been clearly demonstrated in yeast by mutagenesis of the corresponding residues involved in the surface interaction. The analysis of these mutants demonstrated that they strongly affect the function of the yeast KEOPS complex, as they elicit major phenotypic effects both on transcription and telomere maintenance, thus linking the function of the complex to its structural integrity.

## The Bud32 protein kinase

As mentioned before, the yeast *BUD32* gene was identified and analyzed in our laboratory, after the completion of the *S.cerevisiae* genome sequence [1], in the frame of the EUROFAN project [28]. The deletion of *BUD32* is responsible for different phenotypic effects; among these, the drastic slowdown of cell growth, the inability of homozygous diploid cells to enter sporulation, alterations in the cell wall and random budding [29-32].
*BUD32* encodes a protein of 261 amino acids, initially named piD261, prevalently located in the nucleus. Biochemical data showed that Bud32 is an atypical protein kinase [33-35].

### General characteristics of protein kinases

The phosphorylation event is one of the most frequent and general mechanisms by which nearly all cellular functions are regulated in higher organisms. The enzymes responsible for this kind of reaction are protein-kinases. These enzymes use the γ-phosphate of ATP (or GTP) to generate phosphate monoesters using protein alcohol groups (on Ser and Thr) and/or protein phenolic groups (on Tyr) as phosphate acceptors. Protein-kinases make up a superfamily of homologous proteins in Eukarya, being related by virtue of their catalytic domain. The kinase domain consists of about 250-300 amino acid residues and contains 12 conserved subdomains that fold into a common catalytic core structure. In the α type of cAMP-dependent protein kinase (PKA-Cα), that is considered the paradigm of protein-kinases, this core folds into a two-lobed structure (fig. 2). The smaller, N-terminal lobe, which includes subdomains I-IV, is primarly involved in anchoring and orienting the nucleotide. The larger, C-terminal lobe, which includes subdomains VIA-XI, is largely responsible for binding the peptide substrate and initiating phosphotransfer. Subdomain V residues span the two lobes. The deep cleft between the two lobes is recognized as the site of catalysis.

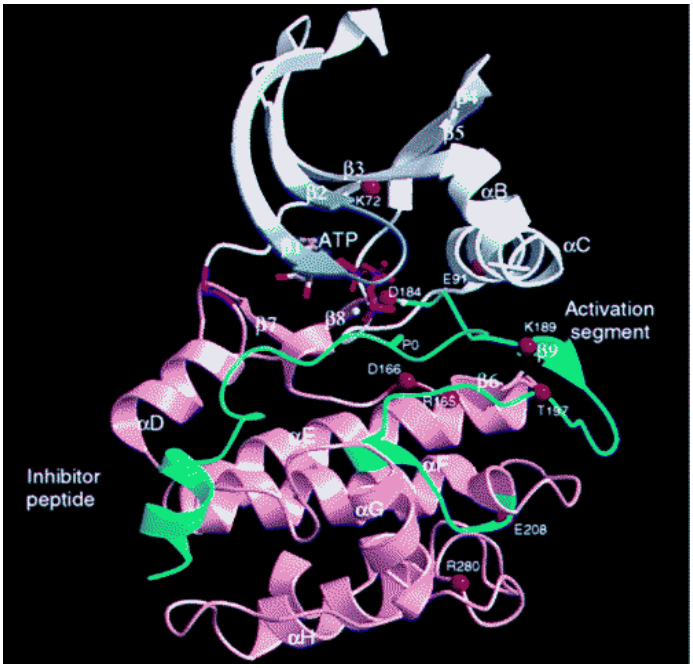



**Fig. 2-The Catalytic Core of PKA.** The N-terminal lobe is colored white, and the C-terminal lobe is pink. The hinge region between the lobes is magenta. An inhibitor peptide is shown in green with the position of the serine that is the target for phosphorylation shown as $P_0$. ATP is in ball and stick representation. The glycine-rich region that is important for localization of the phosphates of ATP is shown colored light purple. α Helices and β strands are labeled. (From Johnson L.N. *et al*., 1996)

Twelve aminoacid residues are invariant or nearly invariant throughout the superfamily, and hence are strongly implicated as playing essential roles in enzymes function. In PKA-Cα, these correspond to Gly50 and Gly52 in subdomain I, Lys52 in subdomain II, Glu91 in subdomain III, Asp 166 and Asn171 in subdomain VIB, Asp184 and Gly 186 in subdomain VII, Glu208 in subdomain VIII, Asp220 and Gly225 in subdomain IX, and Arg280 in subdomain XI.

Subdomain I contains the consensus motif GxGxxGxV ("glycinic loop"), starting with Gly50 in PKA-Cα. It acts as a flexible clamp that covers and anchors the non-transferable phosphates of ATP. Subdomain II contains the invariant Lys (Lys 72 in PKA-Cα), which is essential for maximal enzyme activity: it helps anchor and orient ATP, toghether with the nearly invariant Glu91 (in subdomain III). Subdomain IV contains no invariant residues and does not appear to be directly involved in catalysis or substrate recognition. Subdomain V links the small and large lobes of the catalytic subunit. Subdomain VIA folds into the large hydrophobic α-helix that extends through the large lobe; this part of the molecule appears to act mainly as a support structure. Subdomain VIB contains two invariant residues (Asp 166 and Asn171 in PKA-Cα), that lie within the consensus motif HRDLKxxN, known as the "catalytic loop"; within the loop, Asp166 is the catalytic base. Subdomain VII contains the highly conserved DFG triplet (corresponding to Asp184-Phe185-Gly186 in PKA-Cα), where Asp184 helps to orient the γ-phosphate for transfer.

Subdomain VIII includes the highly conserved APE motif (residues 206-208 in PKA-Cα). This subdomain, named "activation loop", appears to play a major role in recognition of peptide substates; indeed, many protein-kinases are known to be activated by phosphorylation of residues in this domain. In PKA-Cα, maximal kinase activity requires phosphorylation of Thr197, probably occurring through an intermolecular autophosphorylation mechanism. Subdomain IX contains a nearly invariant Asp (Asp220 in PKA-Cα) that acts to stabilize the catalytic loop. Subdomain X is the most poorly conserved subdomain and its function is obscure. Subdomain XI extends to the C-terminal end of the kinase domain; it contains the nearly invariant Arg corresponding to Arg280 in PKA-Cα. [36].

**Characteristics of the Bud32 protein kinase**

Bud32 belongs to the piD261 family of Ser/Thr protein kinases, which has representatives in virtually all eukaryotic and archaeal organisms. Unlike the majority of eukaryotic protein kinases, Bud32 preferentially phosphorylates acidic substrates, like casein and osteopontin [33, 35].

Bud32 is the shortest protein kinase known to date, entirely lacking the C-terminal subdomain XI. If it is considered moreover that the C-terminal 44 residues sequence, including a unique stretch of basic residues, does not display any significant homology with the members of the protein kinase family, it should be concluded that also subdomain X and part of subdomain IX are lacking or deeply altered in Bud32. Bud32 also displays some unique features, such a single glycine left in the glycine loop, proline replaced by leucine in the APE motif and the lysil residue within the DLKPEN sequence, which is diagnostic of Ser/Thr kinases, substituted by a threonyl residue [33]. Despite its small size and low overall similarity with the other members of the protein kinase family, Bud32 displays all the main signatures of a protein kinase catalytic domain, with special reference to the conservation of invariant residues, whose relevance to its catalytic activity has been confirmed by mutational analysis. Starting from the N-terminus, the suspected functional equivalence of Gly-25 with the invariant second glycine of the phosphate anchor motif, GXGXXG (equivalent to PKA Gly-52), was confirmed by mutating it to Val, which gives a fully inactive. Lys-52 must be the functional equivalent of PKA Lys-72, since its

replacement fully suppressed catalytic activity. Another important residue for the kinasic activity is Glu-76, which matches well the invariant glutamyl residue (PKA Glu-91) that defines subdomain III; the mutation of this glutamic acid to alanine fully suppressed catalytic activity. Finally, domain VIB includes the highly conserved catalytic loop, with its invariant motif DXXXXN, whose actual identification with the 161-166 segment of Bud32 (DLTSSN) was validated by showing that the replacement of Asp-161 by Ala nearly abolishes activity. The activation loop of Bud32 is abnormally short; however, it includes two phosphorylatable residues, Ser-187 and Ser-189. Thanks to a mutant in which both Ser-187 and Ser-189 had been replaced by Ala (SS187,189AA) it was seen that those residues do indeed undergo autophosphorylation. All these features suggest that in several respects the properties of Bud32 are unique [34].

The human homolog of *BUD32* is *PRPK*, even if the two proteins share only 30% of identity. This gene was cloned from an interleukin-2 activated cytotoxic T-cell subtraction library and shown to up regulate transcriptional activity of p53 once transfected in COS-7 cells. PRPK binds to and phosphorylates p53 at Ser15 [37], whence the acronym PRPK (p53 related protein kinase). It was shown that also Bud32 is able to interact with human p53 and to phosphorylate its amino-terminal domain, at Ser15 and Ser37; conversely, PRPK partially restores the normal growth phenotype of yeast, when overexpressed in strains where the gene encoding Bud32 has been disrupted. This indicates that the two genes are functional and not only structural homologues.

Several different approaches have shown that Bud32 is able to interact with many yeast proteins [17, 38, 39]. Particularly remarkable is its tight association with Kae1, within the KEOPS complex. Although the kinase activity of Bud32 is relevant for the functions of the KEOPS complex in yeast, it is actually unknown whether Bud32-dependent phosphorylation of other subunits of the complex could directly regulate its activity.

## Atomic structure of the KEOPS complex

In 2008, Mao and colleagues solved the atomic structure of an archaeal-derived KEOPS complex. Recombinant forms of archaeal proteins were expressed in *E.coli* and purified, then the structure and biochemical function of each protein was determined. Using pull-down experiments the authors showed that KEOPS is organized in a linear fashion where Cgi121 binds to Bud32, which binds to Kae1, which in turn binds to Pcc1 (fig. 3). This work highlighted some features of the complex.

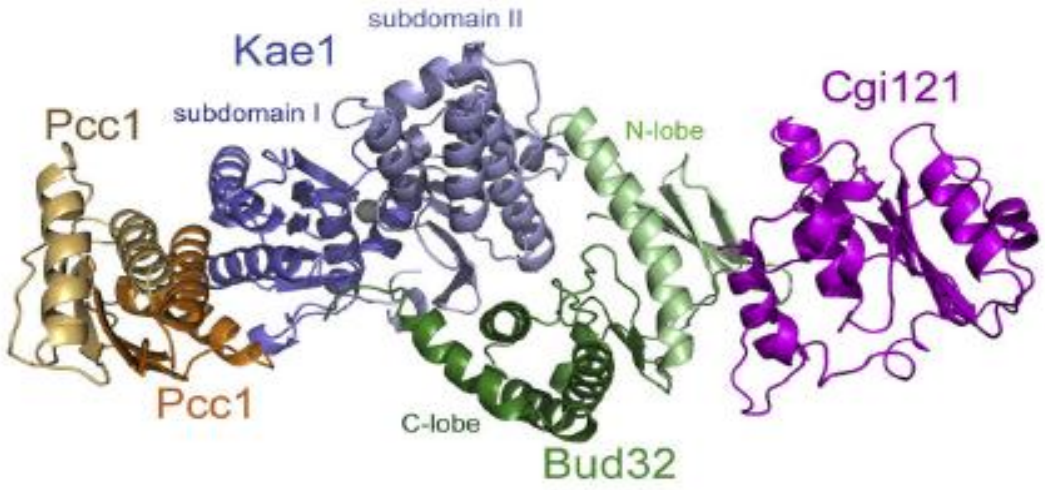



**Fig. 3-Composite model of the KEOPS complex based on the X-ray crystal structures of single subunits, binary complexes, and a ternary complex** (from Mao *et al.* 2008).

Kae1 was shown to bind ATP, but no AP-endonuclease or DNA-binding activity were detected; hence, the exact function of Kae1 remains mysterious. It has been proposed that Bud32 may regulate Kae1 in two ways. First, by phosphorylating Kae1 and second by binding it. Again, mutational analyses on the binding surface between Bud32 and Kae1 indicated that this bonding is critical for KEOPS function. Bud32 engages Kae1 using both its N- and C-terminal lobes; the deletion of the C-terminal tail of Bud32 did not affect its ability to contact Kae1, but had a severe growth defect, suggesting a possible role in regulating Kae1 function. The work also identifies Cgi121 as a positive regulator of Bud32 kinase activity. In fact Cgi121 is a potent activator of Bud32 autophosphorylation *in vitro* and this function was dependent on the ability of Cgi121 to bind Bud32. It was also

.demonstrated that Cgi121 is highly unstable, and probably its binding to Bud32 is important to enhance stability. Finally, Pcc1 was identified as a dimeric protein, structurally homolog to KH (kink in helix) domain-like proteins, that function as a versatile RNA, ssDNA, or RNA hairpin binder. Pcc1 dimer has the ability to bind two Kae1 molecules and this is needed for its function: Pcc1 was proposed to be a dimerization module in the presence of the other subunits, as it dimerizes in solution at all concentrations tested. Since dimerization and multimerization is a common feature of processive enzymes, it has been proposed that KEOPS may be a processive molecular machine [40].

# Bud32 INTERACTS WITH OTHER PROTEINS, BESIDES THOSE OF THE KEOPS COMPLEX

In addition to the components of the KEOPS complex, many other proteins have been identified as Bud32 interactors [17, 38, 39], suggesting that this protein kinase may have additional roles by specifically phosphorylating other substrates. Among these Bud32 interactors, our attention has been drawn to the glutaredoxin Grx4 [17].

## The Grx4 glutaredoxin

Grx4 is a protein of 245 aminoacids, that belongs to the subfamily of yeast monothiolic glutaredoxins, together with Grx3 and Grx5 [41].

Thioredoxins and glutaredoxins are small proteins containing an active site with a redox-active disulfide; they function in electron transfer via the reversible oxidation of two vicinal protein-SH groups to a disulfide bridge. Both can supply ribonucleotide reduction and other reactions with electrons from NADPH. Research on thioredoxins and glutaredoxins in many organisms has revealed that they participate in many cellular

processes, including deoxyribonucleotide synthesis, protein folding and sulphur metabolism. Theyare also very important in regulating the redox state of proteins, hence participating in the protection against oxidative stress [42, 43].

Monothiol glutaredoxins are divided in two classes, those with a single glutaredoxin domain and those with a thioredoxin-like region followed by one or more glutaredoxin domains. In *S.cerevisiae* there are 3 monothiol glutaredoxins: Grx3, Grx4 and Grx5.

Whereas the function of Grx5 in mitochondrial Fe–S cluster assembly has been extensively investigated, the role of the nuclear glutaredoxins Grx3 and Grx4 is less well characterized. The single deletion of either *GRX3* or *GRX4* leads to weak growth defects, but the double deletion strongly affects cellular growth and the response to oxidative stress. As the two proteins display relevant sequence similarity, they might have overlapping or redundant functions. Accordingly, both Grx3 and Grx4 have been identified as modulators of the activity of Aft1, a transcription factor involved in the regulation of the iron regulon genes. The binding of Aft1 to specific promotorial regions induces the expression of this regulon in conditions of iron depletion. It has been demonstrated that the two proteins form a complex with Aft1: this determines the translocation of Aft1 to the cytoplasm in conditions of iron repletion and, as a result, a negative regulation of the iron regulon genes [44, 45].

As mentioned before, results from our laboratory have indicated that Grx4 associates to Bud32 [2, 17]; moreover, Grx4 was shown to be an *in vitro* substrate of the protein kinase, being readily phosphorylated by recombinant, purified Bud32 mainly at Ser134 [17]. This has suggested that Grx4 may be one of the physiological substrates of Bud32 in yeast cells.

In fact, we have recently demonstrated that indeed Grx4 is a physiological substrate of Bud32, and that this relationship is influenced by the phosphorylation state of Bud32 [46].

Bud32, as well as its human homolog PRPK, displays a highly conserved C-terminal sequence, rich in basic amino acids (RxxRxS/THy), that fulfils the consensus recognized by protein kinase B (Akt/PKB) [47]. Interestingly, the activity of PRPK on its known substrate (Ser15-p53) mainly (but not exclusively) depends on the phosphorylation of its Ser250 residue by Akt/PKB [48]. This prompted us to investigate whether the activity of Bud32 in yeast could also be modulated by phosphorylation of its Ser258 residue, possibly mediated by Sch9, which is considered to be a yeast homolog of mammalian Akt/PKB.

## The Sch9 protein kinase

Sch9 is an AGC kinase of 90KDa, initially identified as a high-copy suppressor of the *cdc-25* temperature-sensitive allele and of cAMP-PKA signaling defective mutants. Like cells overexpressing components of the cAMP pathway, cells overexpressing *SCH9* are sensitive to heat shock. *SCH9* is not itself an essential gene, but Δ*SCH9* cells grow slowly and this phenotype is readily suppressed by activation of the cAMP pathway. This suppression may therefore be explained by the functional overlap in the activities of Sch9, PKA and TOR (the Target Of Rapamycin protein) [49].

AGC kinases regulate various signaling events that orchestrate growth and morphogenesis and are readily activated by nutrients availability. Members of the AGC family of Ser/Thr protein kinases share considerable homology in their kinase domains. The catalytic core has a bilobal composition: the smaller N-terminal lobe binds nucleotides whereas the large C-terminal lobe participates in substrate binding and catalysis [50]. This family includes PKA, PKG, PKC and also the phosphoinositide-dependent kinase (PDK), PKB, and the ribosomal protein S6 kinase (S6K).

Sch9 is required for longevity and cell size in budding yeast. Strains that are deficient in *SCH9* form smaller colonies with respect to the wild-type, with a fewer number of cells and cells of smaller cell size (Whi phenotype). They also grow at a slower rate; *sch9* null strains, in fact, are characterized by a prolonged $G_1$ phase of the cell cycle, such that their doubling time is greater than that of wild-type cells [49, 51]. The finding of a role of Sch9 in cell cycle progression is reminiscent of the functions of AGC kinases in other organisms. In *Drosophila*, PKB/Akt controls cell cycle progression and also decreased abundance of PDK or S6K lead to an increase in the proportion of small cells in $G_1$ phase. These similarities between yeast Sch9 and different animal AGC kinases suggest that Sch9 may serve multiple roles that are performed by specific isoforms in higher organisms [52].

Sch9 is also involved in replicative aging, as suggested by the identification of Sch9-regulated genes in a screen for yeast cells with extended replicative life span (defined as the number of daughter cells produced by a single mother cell before its death). It has been shown that *sch9* null strains have also a threefold extension in chronological life span (defined as the time cells survive in stationary phase), compared to wild-type yeast [53]. Longevity in yeast is tightly correlated with multiple stress resistance. Interestingly, cells

lacking *SCH9* showed an increase in the resistance to stress [53, 54]. Recently, Sch9 has been identified as a transcriptional activator that is recruited, only in stress conditions, to the chromatin of genes induced by osmotic stress; moreover, the ability of Sch9 to induce the expression of osmostress genes is directly due to its kinase function. [55].

Analysis of the localization of Sch9 suggests that it has a role as a nutrient sensor. During log phase, Sch9 localizes throughout the cell, but is enriched at the vacuolar membrane; this enrichment disappears in response to carbon starvation. As the vacuole is an important reservoir of amino acids, phosphate and other metabolites, Sch9 may communicate to the cell the status of these internal nutrient pools, thus regulating the initiation of the cell cycle progression (Start). Start is the short interval during late $G_1$ phase after which cells are committed to division; passage through Start requires a critical cell size, nutrient sufficiency, a critical translation rate and absence of mating pheromone. Sch9 is a potent negative regulator of Start and an activator of the ribosomal protein (RP) and ribosome biogenesis (Ribi) regulon, the transcriptional programs that dictate ribosome synthesis rate in accord with environmental and intracellular conditions [51].

Sch9 is regulated by TORC1, the Target Of Rapamycin-Complex1, which phosphorylates six amino acids situated in its C-terminal sequence. TORC1-dependent phosphorylation is required for Sch9 activity and Sch9 is required for TORC1 to properly regulate ribosome biogenesis and translation initiation. Sch9 mediates TORC1 signals to a number of distal readouts: it blocks the induction of genes required for entry into $G_0$ by directly phosphorylating the Ser/Thr kinase Rim15, and thereby antagonizing its nuclear accumulation [57, 58]. Sch9 is critical for TORC1 ability to antagonize eIF2a phosphorylation and thus maintain efficient translation initiation[56]; also, Sch9 plays important roles in the regulated expression of RNA polymerase II (Pol II)-dependent genes required for ribosome biogenesis[51, 56]. Except for Rim15, the substrates of Sch9 involved in these processes are not known. A very recent work took advantage of a mass spectrometry technique to define the TORC1-regulated phosphoproteome. These studies led to the observation that the repressor of RNA Pol III, Maf1, is directly phosphorylated by Sch9, and that Sch9 regulates both Maf1 localization and binding to RNA PolIII. In addition to RNA Pol IIII, Sch9 was found to regulate also the synthesis of RNA Pol I transcripts. Thus, Sch9 appears to play a central role in the regulation of the protein synthesis machinery[59].

## Sch9 is the yeast homolog of mammalian Akt/PKB

The *AKT* gene is the cellular homolog of the v-akt oncogene transduced by AKT8, an acute transforming retrovirus in mice that was originally described in 1977 [60]. Akt is a Ser/Thr protein kinase, composed of an N-terminal pleckstrin homology (PH) domain, followed by a catalytic domain and a short C-terminal tail. The catalytic domain is most similar to cyclic AMP-dependent protein kinase A (PKA; 65% similarity) and to protein kinase C (PKC; 75% similarity); thus, Akt is also frequently referred to as protein kinase B (PKB)[61, 62]. The optimal consensus sequence for phosphorylation by Akt was found to be R-X-R-X-X-S/T [63]; this consensus motif is a common feature of known substrates of Akt, and its presence is useful to predict if a given protein may be phosphorylated by the kinase *in vitro*.

Akt binds to membrane phosphatydilinositol (3,4,5)-trisphosphate ($PIP_3$) thanks to its PH domain. The enzyme responsible for the conversion from phosphatydilinositol (3,4)-bisphosphate ($PIP_2$) to ($PIP_3$) is phosphatydilinositol 3-kinase (PI3K), that is triggered by many growth factors, such as PDGF, EGF or FGF. It has been shown that the interaction of $PIP_3$ with Akt may initiate the activation process, perhaps by recruiting the protein to the plasma membrane [61]. In addition to phosphoinositide binding, Akt needs to be phosphorylated at two key residues to be fully active: Thr308 of the activation loop, by PDK, and Ser473 (in the hydrophobic motif of the C-terminal tail), by mTORC2 (although other molecules can also phosphorylate the latter residue). Phosphorylation by mTORC2 stimulates the subsequent phosphorylation of Akt by PDK1. Activated Akt can then activate or deactivate many substrates via its kinase activity [64].

Consequences of Akt activation include diverse biological responses, ranging from primarily metabolic functions such as glucose transport, glycolysis, glycogen synthesis and the suppression of gluconeogenesis, to protein synthesis and increased cell size. One of the targets of Akt is the protein kinase GSK3: Akt inhibits GSK3 activity, in an insulin-stimulated and PI3K-dependent manner, by direct phosphorylation of an N-terminal regulatory serine residue of GSK3 [62]. Inhibition of GSK3 is thought to contribute to the stimulation of glycogen synthesis and translation of certain mRNAs by insulin [61].

Akt has also important functions in cell-cycle progression and apoptosis suppression. Another target of Akt is in fact the proapoptotic BCL2-antagonist of death (BAD) protein:

once phosphorylated, this protein is retained in the cytosol by the 14-3-3 proteins and its pro-apoptotic activity is neutralized. Moreover, PI3K-dependent Akt activation can be regulated through the tumor suppressor PTEN, which works essentially as the opposite of PI3K: it acts as a phosphatase to dephosphorylate $PIP_3$ back to $PIP_2$. This removes Akt from the membrane and this delocalization significantly decreases the rate of Akt activation. Interestingly, PTEN is often mutated or even silenced in human cancer. Conversely, Akt iperactivation is frequently found in poorly differentiated tumors (and hence considered a negative prognostic marker for disease outcome) (reviewed in [62]).

# THE Sch9-Bud32-Grx4 SIGNALING PATHWAY

As mentioned before, in human cells, phosphorylation at Ser250 of PRPK (Bud32 homolog) by Akt /PKB positively regulates *in vivo* the activity of PRPK on its physiological substrate p53[48]. We then asked if a similar regulation could occur also in yeast cells. We indeed noticed that also Bud32 displays at his C-terminus the consensus sequence for Akt/PKB and that the Bud32 residue, corresponding to the Serine 250 in mammalian PRPK, is the Serine 258. We first looked for a genetic interaction between the two genes and observed that the combination of *SCH9* deletion and Bud32 mutations that alter its catalytic activity affects the growth of yeast cells more severely than each of the two single mutations. We then looked at the phosphorylation status of Bud32: using an antibody anti-pSer258, which recognizes the phosphorylated target site for Akt at the C-terminus of Bud32, we could see that Serine 258 is phosphorylated *in vivo*. Consequently, the recognition is virtually absent when the Ser258 is replaced with an Alanine. A similar result could be seen when we analyzed a mutant strain in which *SCH9* was deleted, supporting the idea that Sch9 is implicated in the phosphorylation of Bud32 at Ser258, *in vivo*.

It is known that the abundance of Sch9, that is a nutrient-sensitive kinase, is modulated by the presence of nutrients in the medium. We then wanted to verify if the amount of Sch9 had any effect on the phosphorylation of Bud32. The amount of Sch9 in cells grown in glucose was indeed higher than in cells grown in glycerol; interestingly, we observed that

also the phosphorylation of Bud32 at Ser258 was higher in cells grown in glucose than in cells grown in glycerol. These results suggest that Bud32 might be a physiological target of Sch9, representing one of the effectors of this protein kinase known to be involved in multiple cellular processes. We then demonstrated that Sch9 and Bud32 are able to interact and, thank to an *in vitro* reaction using the immunoprecipitated Sch9 and recombinant Bud32 as a substrate, that Sch9 phosphorylates Bud32 at Ser258.

In order to check if this regulation could have some effects on Bud32 catalytic activity, we compared the two forms of Bud32 (phosphorylated, or not, at Ser258) for their ability to phosphorylate recombinant, purified Grx4. We could see that when Bud32 was not Ser258-phosphorylated, i.e. in the *ΔSCH9* strain, or when it carried the S258A mutation, phosphorylation of Grx4 was reduced by up to 40% of the wild-type activity, similarly to what was seen with the catalytic (*K52A* and *D161A*) mutants. Nevertheless, the catalytic activity of Bud32 was unaffected in the *BUD32*$^{S258A}$ strain, as results from its ability to still autophosphorylate and phosphorylate casein. These results may therefore indicate that Ser258 modification could modulate the ability of Bud32 to recognize the Grx4 substrate; in support of this hypothesis, we noticed that wild type Bud32 associated with Grx4, while the mutant Bud32 $^{S258A}$ did not.

We could therefore describe a novel *S.cerevisiae* signaling pathway that implicates Bud32 and Sch9 in modulating the phosphorylation state of Grx4 in yeast cells, with possible implications for the regulation of its activity. Analysis of the growth of the *S258A* mutant revealed that cells were almost unaffected when compared to catalytically inactive or null mutants of Bud32. Our hypothesis is that the S258 phosphorylation could affect growth only in specific environmental conditions. [46].The search for a physiological role of this newly described signaling pathway was a matter of my research PhD project.

# AIM OF THE THESIS

During the first year of my PhD I collaborated in the description of a novel *S. cerevisiae* signaling pathway that implicates the protein kinase Bud32, Sch9 (the yeast homolog of mammalian Akt), and the glutaredoxin Grx4. We demonstrated that Grx4 is not only phosphorylated *in vitro* by Bud32, but that it is also a physiological substrate of the kinase, and that this relationship is influenced by the phosphorylation status of Bud32. In fact Bud32 is phosphorylated at Ser258 by the Sch9 kinase and this has the effect of upregulating the ability of Bud32 to interact with Grx4 and to phosphorylate it [46].

The aim of my subsequent work was to study the physiological significance of this new phosphorylation cascade. To do this, I analyzed the phenotype of mutant cells in which Grx4 was not phosphorylatable by Bud32, and evaluated a series of parameters, first of all cell growth. I then asked if this cascade could be able to modulate the activity of the whole KEOPS complex, *via* the Bud32 subunit. I thus analyzed both the levels of *GAL1* transcription (one of the main transcriptional targets of the complex) and telomere length in strains in which the cascade was impaired.

As described in the introduction, Grx4 is important in the maintenance of the redox state of proteins, and thus in the protection against oxidative stress. Moreover Grx4, together with Grx3, participates in iron homeostasis, through the control of the cellular localization of the Aft1 transcription factor [44, 45]. Therefore I evaluated if these known functions of Grx4 are affected when the Sch9-Bud32-Grx4 cascade is impaired. The hypothesis was that Bud32 might be indirectly involved in iron homeostasis, through its capacity of phosphorylating the glutaredoxin. This modification, in fact, might activate or inhibit the ability of Grx4 to mediate the cytoplasmic translocation of Aft1, and consequently the transcription of the iron regulon. To verify this hypothesis, I looked at the cellular localization of Aft1 in strains in which Bud32 was wild-type or mutagenized in the key residue D161A and also measured the levels of transcription of two iron regulon genes in different conditions (iron-repletion or depletion) and strains (wild-type, $BUD^{D161A}$, $BUD^{S258A}$).

I then devoted my study to the KEOPS subunit Kae1. Kae1 has been classified as a metalloprotease, due to its similarity with a glycoprotein of *P. haemolytica*. Indeed, Kae1

contains the conserved HXXXH metal binding motif, typical of metalloproteases: the substitution of one or both histidines of the motif (H-141 and H-145) deprives the Kae1 protein of its functionality and cells cannot survive. Interestingly, in Kae1 and in all its eukaryotic homologues, the residue of glutamic acid in the second position of the motif is replaced with a cystein, while a glutamic acid is present straight after at position +2. In order to understand the role of Kae1 I used two mutants already available in my laboratory, in which the residues of Cys142 and Glu147 were mutagenized. Both mutant strains in fact show a slow growth phenotype, but are still viable, allowing the study of Kae1 function within cells. I first asked if these mutations could interfere with the functions of the KEOPS complex. As done before for Bud32 mutants, I thus analyzed both telomere length and transcription activation; I also studied the cellular localization of wild-type and mutant Kae1 proteins.

In a recent work that describes the atomic structure of an archaeal-derived KEOPS complex, the authors speculate that Bud32 could be a regulator of Kae1, by binding and phosphorylating it [40]. I then decided to verify if the same was true for the yeast proteins, by setting up a series of phosphorylation assays using the recombinant yeast Bud32 and Kae1 purified from *E. coli*.

As a final part of my PhD, I tried to verify if the yeast Kae1 protein could have a nuclease activity. In fact, in a work of 2007, made on the *P. abyssi* Kae1, the authors have indicated that this protein was able to bind cooperatively to single and double-stranded DNA and exhibited a class I apurinic endonuclease activity (AP-lyase) [25].

# Results and discussion

## PHOSPHORYLATION OF THE *S. cerevisiae* Grx4 GLUTAREDOXIN BY THE Bud32 KINASE UNVEILS A NOVEL SIGNALING PATHWAY INVOLVING Sch9, A YEAST MEMBER OF THE Akt/PKB SUBFAMILY

### Phosphorylation at Ser134 of Grx4 by Bud32 contributes to the functionality of the glutaredoxin in yeast cells

As described in the introduction, the yeast KEOPS/EKC complex is conserved throughout evolution and is involved in transcription regulation and telomere maintenance [2, 3]. The complex is composed of five proteins: the hypothetical endonuclease Kae1, the protein kinase Bud32, the still uncharacterized Cgi121, and two other proteins of small size, Pcc1 and Pcc2/Gon7. For many years, our attention has been focused on the Bud32 protein. It came to light that this atypical protein kinase interacts with many other proteins, in addition to the components of the EKC/KEOPS complex [17], suggesting that it may have additional roles by specifically phosphorylating other substrates. Among these Bud32 interactors, our attention has been drawn to the glutaredoxin Grx4, which is an *in vitro* substrate of the protein kinase, being readily phosphorylated by recombinant, purified Bud32 at Ser134 [17]. Several data collected in our laboratory and described by C. Peggion in her PhD thesis (2006), confirmed that Grx4 is able to interact with Bud32 in yeast cells, by co-Immunoprecipitation data, and that it is also an *in vivo* substrate of the kinase. We also noticed that this relationship is influenced by the phosphorylation status of Bud32 itself [46]. In fact, Bud32, which displays a highly conserved C-terminal sequence that fulfils the consensus recognized by the mammalian Akt/PKB protein kinase, is phosphorylated at its Ser258 residue by Sch9, the yeast homologue of Akt/PKB. We proved that phosphorylation of Grx4 by Bud32 is also activated by Sch9 [46]. We thus identified a novel phosphorylation cascade, implicating Sch9, Bud32 and Grx4.

Starting from these data, my PhD project focused on the *in vivo* effects of the phosphorylation of Grx4 by Bud32 and on the possible interactions between this new phosphorylation cascade and the functions of the KEOPS complex. As indicated by the *in vitro* data, phosphorylation of Grx4 by recombinant Bud32 would occur mainly at the Ser134 residue and, more weakly, at Ser133, two residues embedded in a highly acidic stretch of the protein [17]. This sequence is situated in the linker region between the thioredoxin-like and the glutaredoxin domains of Grx4 [41], and its modification would be likely to influence, directly or indirectly, the activity of the enzyme. To evaluate the contribution of the phosphorylation at these residues to the biological competence of Grx4 in yeast cells, I constructed a series of yeast plasmids expressing the unphosphorylatable mutants of Grx4 S134A, S133A, and SS133-134AA, as well as the phospho-mimic mutant S134D, in order to test their capacity to complement the *GRX4* deletion, in comparison with that of the wild-type *GRX4* sequence.

*S. cerevisiae*, however, possesses another nuclear monothiolic glutaredoxin, Grx3, which is very similar to Grx4; the two proteins cooperate and show interchangeable roles, *e.g*. in the transcriptional regulation of iron-dependent genes [44, 45]. Therefore, to specifically investigate *in vivo* the effect of mutating Grx4, we created the double null strain *ΔGRX3/ΔGRX4* (for more details see *Material and methods*). Surprisingly, we noticed that, unlike what was observed with other commonly used yeast strains (such as BY4742 and CML128), cells containing the double mutation are nonviable in the W303 genetic background (Fig. 4), indicating that in the W303 strain the functions of nuclear monothiolic glutaredoxins are essential. This may reflect the subtle differences existing between yeast laboratory strains, in particular with regard to the responses to environmental changes or stresses involving these oxidoreductases.

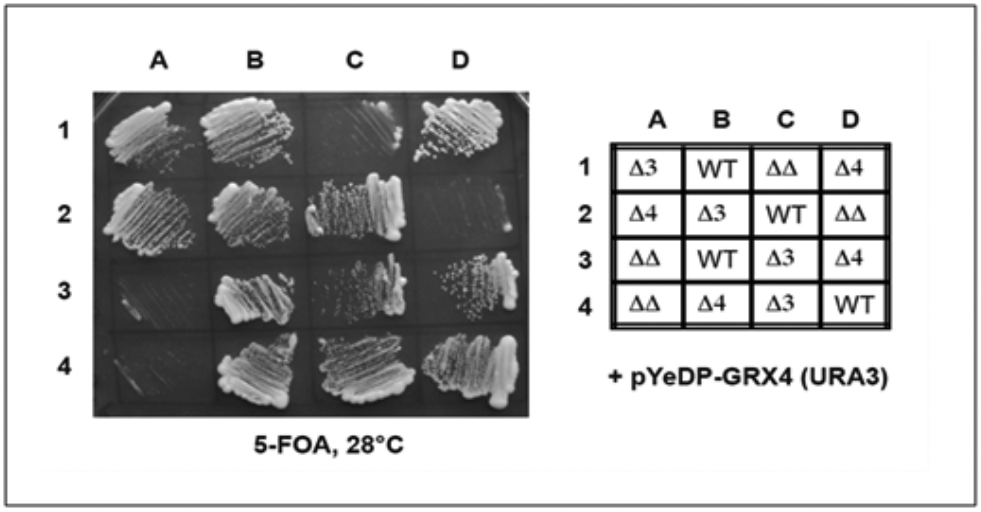



**Fig 4-Activity of the Grx3/Grx4 nuclear monothiolic glutaredoxins is essential in the W303 yeast strain.** One copy of both *GRX3* and *GRX4* unessential genes has been specifically deleted in the wild type diploid W303 yeast strain. Heterozygotic cells have been transformed with the pYeDP-GRX4 plasmid, carrying the wild-type *GRX4* sequence and the *URA3* marker (counter selectable on 5'-FOA containing medium). After tetrad dissection, haploid spores have been recovered and genotypes determined. Yeast cells containing the plasmidic *URA3* marker and coming from complete tetrads have been plated on 5-FOA, wich allows only growth of cells without plasmid. Only cells containing the double deletion *ΔGRX3/ΔGRX4* cannot lose the plasmid and therefore do not grow on 5'-FOA plates, indicating that the presence of at least one nuclear glutaredoxin is required for viability.

The wild type *GRX4* coding sequence inserted in the pYeDP vector, that contains a galactose-inducible promoter, was already available in our laboratory. I then constructed the other mutants using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene); the correct substitution of each residue was confirmed by sequence analysis.

The pYeDP vectors containing the wild-type or the mutagenized *GRX4* sequence were then used to transform heterozygous diploid Δ*GRX4::Kan*/Δ*GRX3::HIS3* cells. After sporulation and tetrad dissection, I isolated a complete set of haploid Δ*GRX4*/Δ*GRX3* strains containing wild-type or mutant *GRX4* plasmids.

I then compared their respective growth rates in glucose medium, where the plasmidic alleles are weakly expressed, and observed that, in these conditions, wild-type *GRX4* was able to fully restore yeast growth, similarly to the *bona fide* positive control (wild-type W303 cells carrying the empty plasmid). The in vivo functionality of Grx4 was not affected by the substitution of Ser133 by Ala (S133A), while the mutation of Ser134 (S134A) slightly impaired its function, as the mutant was not able to fully restore growth.

Consequently, growth was completely restored by the expression of Grx4 carrying the phospho-mimic substitution of Ser134 by Asp (S134D). Accordingly, the double mutation of Ser133 and Ser134 (SS-AA) showed the same effect as the single S134A mutation, confirming that, also *in vivo*, phosphorylation of Ser134 has a major role with respect to that of Ser133. We then checked the effects of Grx4 overexpression, by growing the yeast strains in galactose medium, in which the expression of plasmid-carried genes is strongly induced. We observed that overexpression of wild-type Grx4 was toxic to yeast cells, whereas overexpression of either the single S134A mutant or the double S133A-S134A mutant was less detrimental, indicating that these substitutions somehow impaired the activity of the glutaredoxin, rendering its excess less toxic to the cell. Remarkably, the phospho-mimic substitution S134D is as toxic to yeast cells as the wild-type, further supporting the relevance of Ser134 phosphorylation to the biological properties of Grx4 (fig. 5). Taken together, these data indicate that Ser134 affects mildly Grx4 functionality, under normal growth conditions, but it could be relevant in the regulation of specific pathway(s) upon environmental changes, allowing yeast cells to respond appropriately to these stimuli.

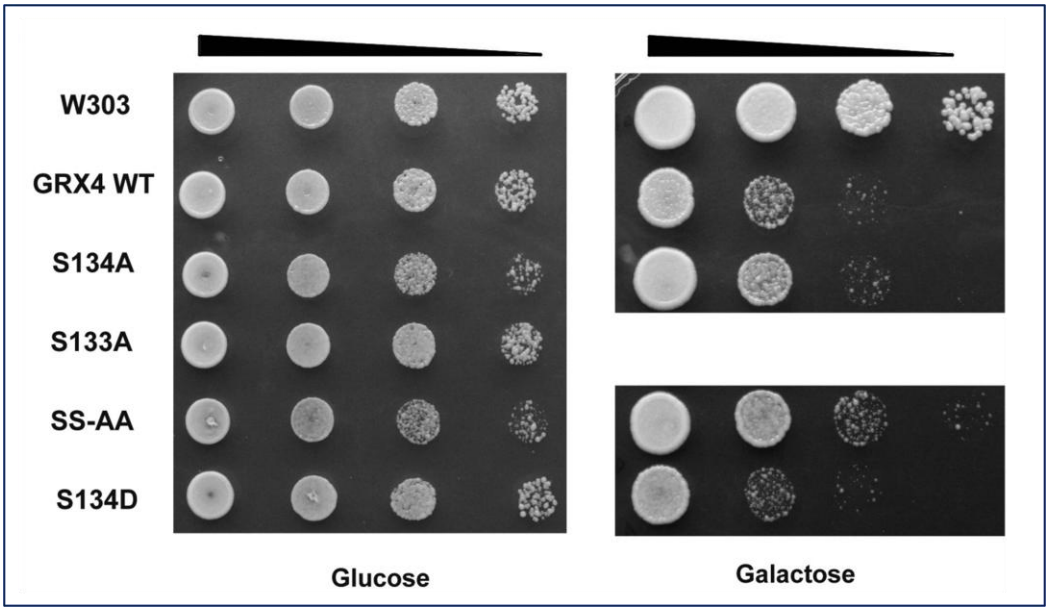



**Fig. 5-Ser134 phosphorylation of Grx4 by Bud32 contributes to its functionality in vivo.** The wild-type W303 strain carrying the empty plasmid and the mutant strain *ΔGRX3/ΔGRX4*, carrying the plasmids coding for either wild-type or mutant Grx4 (S134A, S133A, SS-AA, S134D) were grown until stationary phase in SD selective medium and diluted at 3 $10^7$ cells/ml. Tenfold serial dilutions were spotted either onto solid SD (Glucose) or SG (Galactose) plates. Growth was observed after 3 days at 28 °C.

Finally, to evaluate the specific contribution of Bud32 to Grx4 phosphorylation at Ser134 and Ser133, I constructed a mutant version of recombinant, His-tagged Grx4 (S133A-S134A), and, after expression in *E.coli*, used it as substrate for an *in vitro* phosphorylation reaction by native Bud32 immunoprecitated from yeast cells. Despite several purification attempts, the recovery of wild-type and mutant Grx4 was low; however, as shown in fig. 6, the results demonstrated that Bud32 was able to phosphorylate the low amount of wild-type Grx4 present in the reaction (left, upper panel), whereas the mutant Grx4 was not phosphorylated at all (right, upper panel), despite the presence of a higher amount of recombinant protein, as revealed by the western blot (lower panels).

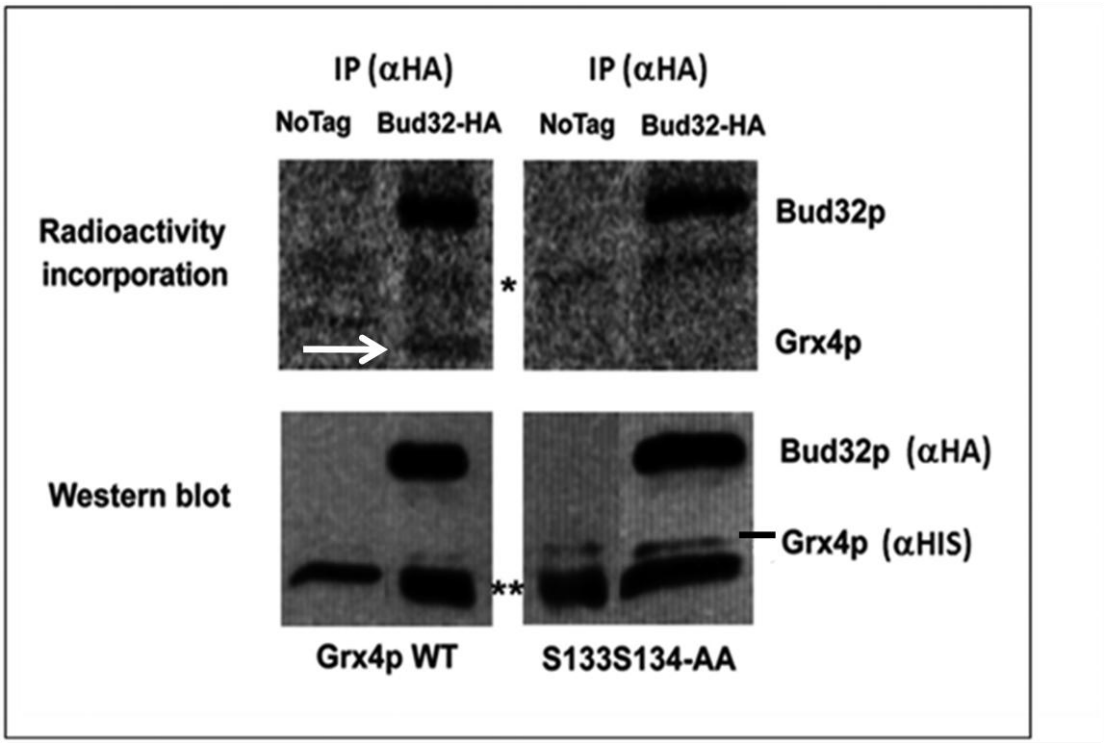



**Fig. 6-Total protein lysates (500 μg) of yeast cells expressing wild-type, HA-tagged, Bud32 (Bud32–HA) were used to immunoprecipitate Bud32.** Immune complexes were subjected to an in vitro phosphorylation reaction in the presence of [γ-$^{33}$P]ATP and 25 ng of recombinant wild-type Grx4p (WT) or 50 ng of the Grx4p double mutant S133A/S134A (SS-AA). After SDS/PAGE and blotting, filters were autoradiographed (upper panels), and then visualized with antibodies against the HA-tag (for Bud32) or the HIS-tag (for Grx4) (lower panels). The marked radiolabeled bands (*) are produced by an unidentified contaminant of the recombinant Grx4p proteins. The strong signals in western blots (**) correspond to the IgG light chains released by the resin used for Bud32–HA precipitation. As a negative control, the immunoprecipitation with anti-HA antibodies was performed also from a wild-type strain (lanes NoTag).

## Phosphorylation of Bud32 at Ser258 is unrelated to its functions within the KEOPS complex

The observation that phosphorylation of Bud32 at Ser258 has an effect on Grx4, suggested that it might also influence the activity of the whole KEOPS complex, of which Bud32 is a crucial component. Notably, Bud32-Ser258 modification might have an impact on the two functions (transcription control and telomere homeostasis) in which the KEOPS complex is involved [2, 3]. We therefore investigated whether phosphorylation of Bud32-Ser258 is linked to these processes, first by analyzing telomere length in several wild-type and mutant strains. As shown in fig. 7, catalytically inactive or null *BUD32* mutations (K52A; D161A; *ΔBUD32*) led to shorter telomeres in comparison to the wild-type, whereas the telomere length of the S258A mutant was unaffected, being almost identical to that of the wild-type (W303 or *BUD32-HA*). Remarkably, deletion of either *SCH9* or *GRX4* did not impair telomere elongation.

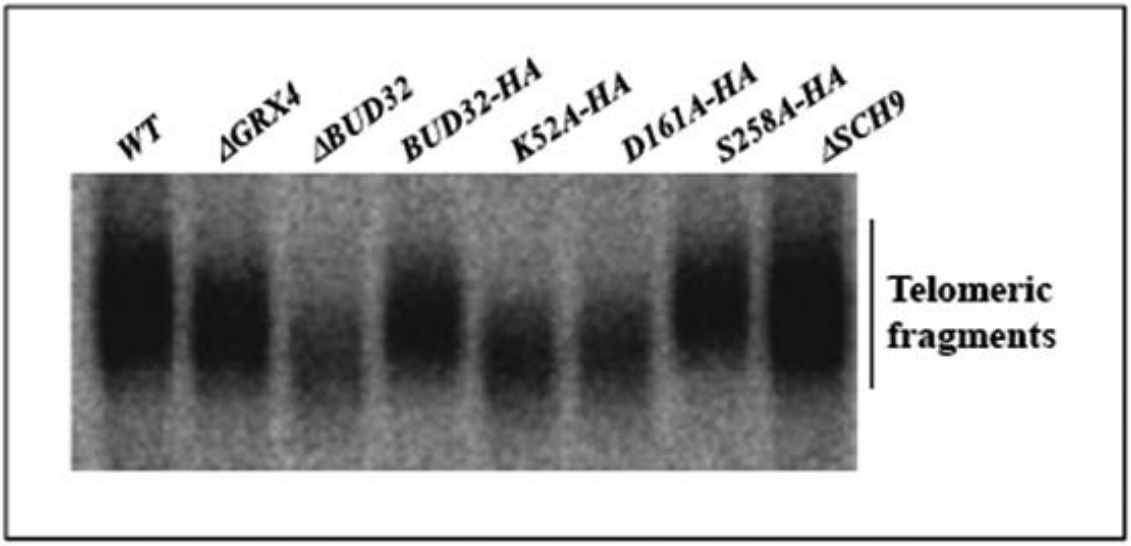



**Fig. 7-Telomere length is unaffected by Ser258 mutation.** Telomere length analysis in wild-type and in mutant strains harboring defects in the Sch9–Bud32–Grx4 cascade. Genomic DNA of the indicated strains (grown in rich medium until exponential phase) was purified and digested with XhoI, producing telomeric terminal DNA fragments of about 1 kb, which were separated on 1.2% agarose, tranferred onto a nitrocellulose membrane, and checked with a $^{33}$P-labeled probe specific for telomeric $TG_{1–3}$ repeats.

We have then examined the effects of Ser258 phosphorylation of Bud32 on the transcriptional activity of the KEOPS complex: this was done by analyzing the activation

rate of the galactose-inducible gene *GAL1*, known to be regulated by the complex. By using real-time RT-PCR and northern blot analyses, we compared the levels of *GAL1* mRNA in wild-type and *BUD32* mutant strains upon transcription induction (a representative northern blot is shown in fig. 8).

When cells are grown in raffinose, the signal corresponding to the *GAL1* gene is absent, as expected. In a medium containing raffinose, that is a trisaccharide composed of galactose, fructose and glucose, the transcription of the *GAL1* gene is inactivated, even though not completely inhibited, as happens in a glucose-containing medium. Therefore, switching from a raffinose- to a galactose-containing medium avoids the necessity to encompass the glucose-induced inhibition, and promptly activates *GAL 1* transcription.

After galactose induction we could observe (as expected) a reduction of *GAL1* mRNA levels in kinase-dead or null mutants, but no differences between the wild-type and the S258A mutant strain, in accordance with the effects observed on telomere elongation.

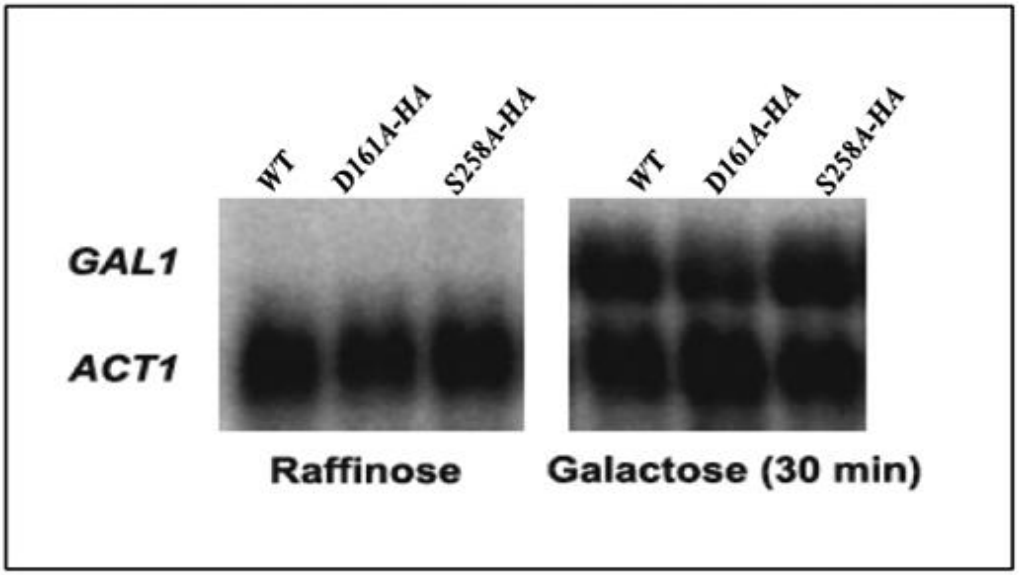



**Fig. 8-Transcriptional activation of GAL1 is unaffected by Ser258 mutation.** Yeast strains were grown in non-inducing raffinose medium until exponential phase, and then incubated for 30 min in galactose medium to activate the *GAL* regulon. Total mRNAs were subjected to standard northern blot analysis. *GAL1* mRNA, and *ACT1* mRNA (considered as a loading control), were detected by the use of specific radiolabeled probes (see Experimental procedures).

The results presented here indicate that the phosphorylation cascade involving Sch9, Bud32 and Grx4 is apparently not relevant to the telomeric or the transcriptional function

of the Bud32-containing KEOPS complex. Accordingly, Grx4 has never been isolated as a component of the KEOPS complex, indicating that the phosphorylation of Grx4 by Bud32 is independent from the known activities of the complex and must therefore be involved in different functions and possibly unrelated pathways. Furthermore, we noticed that growth of *BUD32* mutants lacking the highly conserved C-terminal tail was not affected, similarly to what has been observed in the case of the single S258A substitution, indicating that these mutations do not impair the main biological properties of the kinase. Moreover, recent data from the 3D structure of an archeal Bud32 homolog (Mj1130p) [27] indicate that the Bud32 C-terminal tail is located far from the catalytic site, suggesting that its alteration should not be detrimental to the overall structure. Finally, our data are consistent with a cellular role of Bud32 (via Grx4 phosphorylation) which would be unrelated to the functions of Bud32 as a component of KEOPS, but could be involved in the response to environmental stimuli or endogenous stresses. Whether Bud32 could achieve the two tasks while being simultaneously associated with the KEOPS complex and the Grx4 substrate or, alternatively, whether only a cellular fraction of Bud32, not included in KEOPS, could associate with the glutaredoxin, will be a matter for future investigation.

## Does the Sch9-Bud32-Grx4 phosphorylation cascade affect any function of Grx4?

As briefly explained in the Introduction, Grx4, together with Grx3, participates in iron homeostasis, through the control of the cellular localization of the Aft1 transcription factor. We hypothesized that Bud32 might be indirectly involved in this pathway, by phosphorylating the glutaredoxin. This kind of modification, in fact, might be able to activate or inhibit the ability of Grx4 to mediate the cytoplasmic translocation of Aft1. If this is the case, the activity of Bud32 would influence the transcription of the iron regulon.
To verify this hypothesis, I first looked at the cellular localization of Aft1 in strains in which Bud32 was wild-type or mutagenized in the key residue D161A. I thus created two strains, espressing Aft1 $^{3}$HA-tagged, combined to wild-type or mutagenized Bud32 (*AFT1*$^{HA}$*/BUD*$^{WT}$, *AFT1*$^{HA}$*/BUD*$^{D161A}$) and then observed the signal at the fluorescence microscope (see *Materials and Methods* for details). Fig. 9 shows that the cellular

distribution of Aft1, under iron-depleted conditions (6 hours of growth in the presence of 0.1 mM BPS, an iron chelant), appears to be the same in both the *AFT1*$^{HA}$*/BUD*$^{WT}$ and in the *AFT1*$^{HA}$*/BUD*$^{D161A}$ strains.

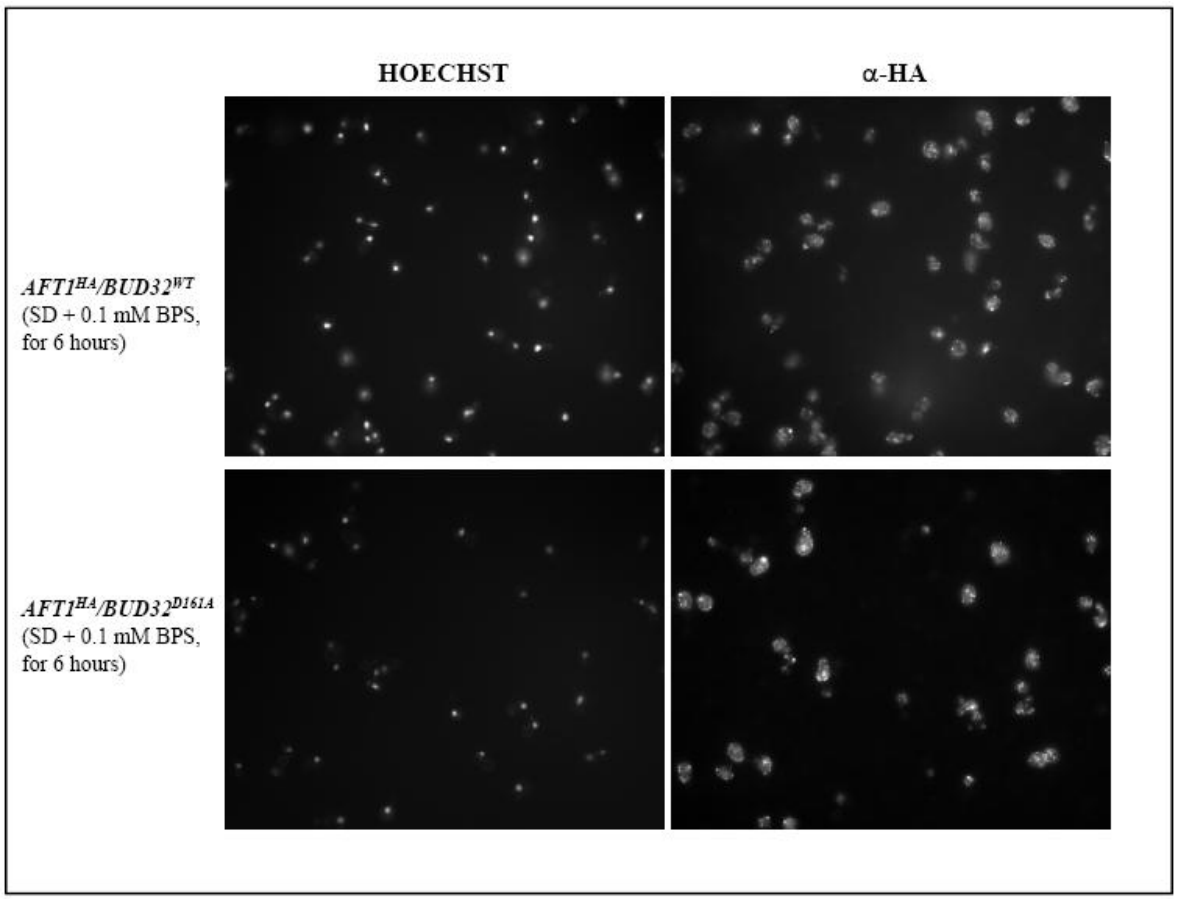



**Fig. 9-Cellular localization of Aft1 transcription factor is unaffected in Bud32 mutants.** Cells were observed after treatment with 0.1 mM BPS for 6 hours. BPS sequestrates iron, and as a consequence Aft1 enters the nucleus to activate the transcription of the iron regulon. There is no evident difference in Aft1 localization between a wild-type strain and a strain in which Bud32 is catalytically inactive (*AFT1*$^{HA}$*/BUD32*$^{D161A}$).

To be sure of the significance of this result, I also measured the levels of transcription of two genes belonging to the iron regulon in different conditions (iron-repletion or depletion) and strains (wild-type, *BUD*$^{D161A}$, *BUD*$^{S258A}$). The genes under examination were *FIT3* and *FET3*; the former encodes a mannoprotein involved in the retention of siderophore iron in the cell wall and the latter is an iron-$O_2$-oxidoreductase required for high affinity iron uptake and is located in the plasma membrane. I extracted total RNAs from all these strains

in two different conditions, iron repletion or depletion, and controlled them both by Northern Blot and Real-Time PCR.

In figure 10 a representative Northern blot is shown; experiments were performed several times and I could never observe a difference between the strains as regards *FIT3* and *FET3* transcription rate. I must therefore conclude that the phosphorylation cascade involving Sch9, Bud32 and Grx4 does not impair Grx4 function in the regulation of the iron regulon transcription. Another possible explanation is that the pathway is somehow bypassed by other proteins that have redundant functions.

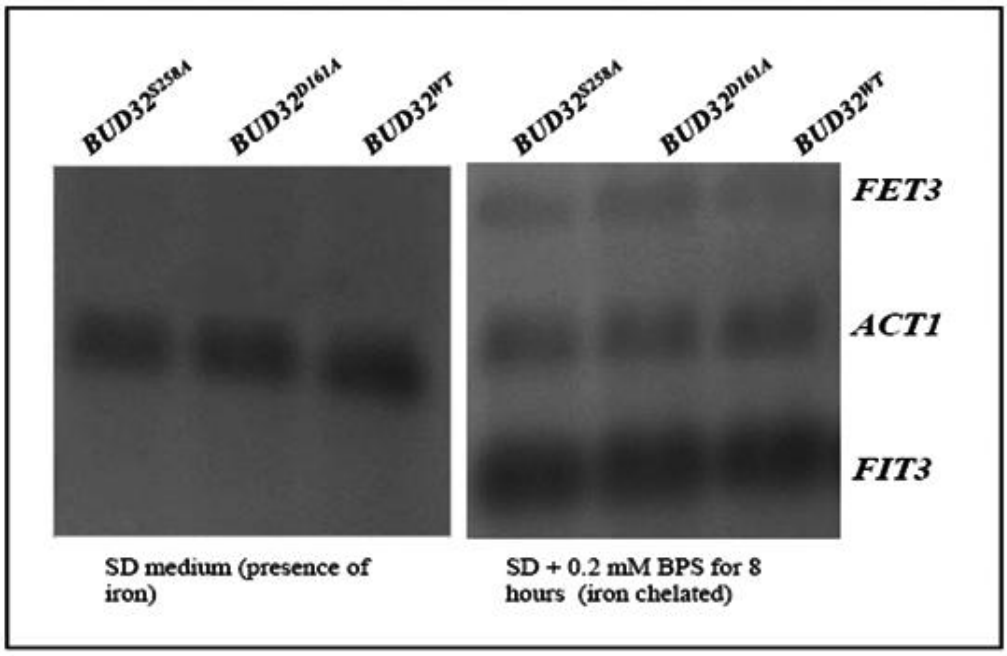



**Fig. 10-Transcriptional activation of the iron regulon is unaffected by Ser258 mutation.** In the presence of iron, no transcription of the *FET3* and *FIT3* genes occurs (left panel). After incubation with BPS for 8 hours, cellular iron is completely chelated, and an activation of the iron regulon occurs. This activation is not affected by either catalytic mutations in Bud32 (*BUD32*$^{D161A}$) or by the S258A mutation.

Beside the role in the control of iron homeostasis, Grx4 acts in the antioxidant defence by reducing the redox state of proteins. We thus controlled if there were any alterations in the response to oxidative stress when *GRX4* was mutagenized at Ser134, compared to the wild-type strain. Exposure of cells for 30 min to $H_2O_2$ (2.5 mM) or Menadione (10 and 20 mM), another commonly used compound that causes oxidative stress, led to an important

decrease in cell viability, as expected. However, no statistically significant differences existed between the wild-type and the mutants as regarded cell survival (data not shown).
The results here described do not enable us to define a role for the Sch9-Bud32-Grx4 phosphorylation cascade within yeast cells. The possibility that the phosphorylation at the Ser134 residue of Grx4 alters the interaction between the glutaredoxin and its partners (numerous, as judged by a two-hybrid screening) will be matter of further investigation.

# ANALYSIS OF THE PHYSIOLOGICAL ROLE OF Kae1

## Analysis of the phenotype of *KAE1* mutants

The study of the Kae1 protein was forbidden by the unavailability of viable mutants. In fact, the mutagenesis of one or both histidines of the conserved HXXXH motif (H-141 and H-145), typical of metalloproteases, deprives the Kae1 protein of its functionality and cells cannot survive (data not published from our laboratory; [3]). In order to obtain a viable mutant strain with a clear phenotype, we observed that in Kae1 and in its eukaryotic homologues, the residue of Glutamic Acid in the second position of the motif is replaced by a Cystein. We therefore decided to substitute the residue of Cystein with a Glutamic Acid (C142E) and to mutagenize the Glutamic Acid present straight after the motif at position +2 in an Alanine (E147A). We hypothesized that this residue (E147) could function as the Glutamic Acid situated inside the HXXXH motif in the majority of metalloproteases (C.Peggion, PhD thesis, 2006). As it can be seen in figure 11, at the optimal temperature of 28 °C, both mutant strains show a slow growth phenotype, in comparision to the wild-type strain. The most affected is the $KAE1^{E147A}$ mutant; its phenotype is exacerbated when cells are grown at 37 °C, indicating a thermosensitive mutant. Instead, the $KAE1^{C142E}$ mutant can be defined as crio-sensitive, as its phenotype is more evident at the temperature of 22°C.

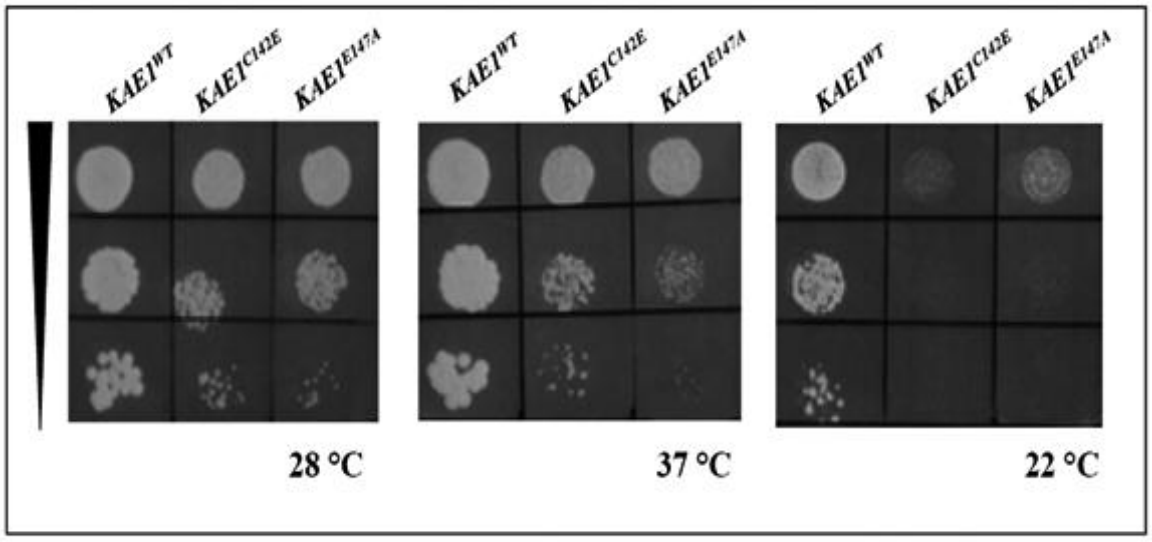



**Fig. 11-Phenotype of *KAE1* mutants.** $KAE1^{WT}$, $KAE1^{C142E}$ and $KAE1^{E147A}$ were grown in liquid YPD at 28°C overnight, until stationary phase. Cultures were then increasingly diluted (1:10, 1:100, 1:1000) and spotted on solid YPD medium; plates were incubated for two days at the indicated temperature

Also, a preliminary co-immunoprecipitation analysis performed in my laboratory indicated that these Kae1 mutants show a decreased ability to interact with Bud32. It was therefore interesting to understand if these mutations altered the functions of the KEOPS complex.

## The $KAE^{E147A}$ mutant shows a strong defect in the regulation of *GAL1* transcription

As reported before, one of the functions of the KEOPS complex is the regulation of transcription. I therefore analyzed, by Northern blot, the *GAL1* mRNA levels in the two *KAE1* mutant strains just described, in comparison with a wild-type one (see *Material and Methods* for more details). After the shift of cells to the galactose-containing medium, the signal of the *GAL1* gene increased in the wild-type and, to a lesser extent, in the $KAE1^{C142E}$ strain, while transcription of the *GAL1* gene was very low in the $KAE1^{E147A}$ mutant (fig. 12).

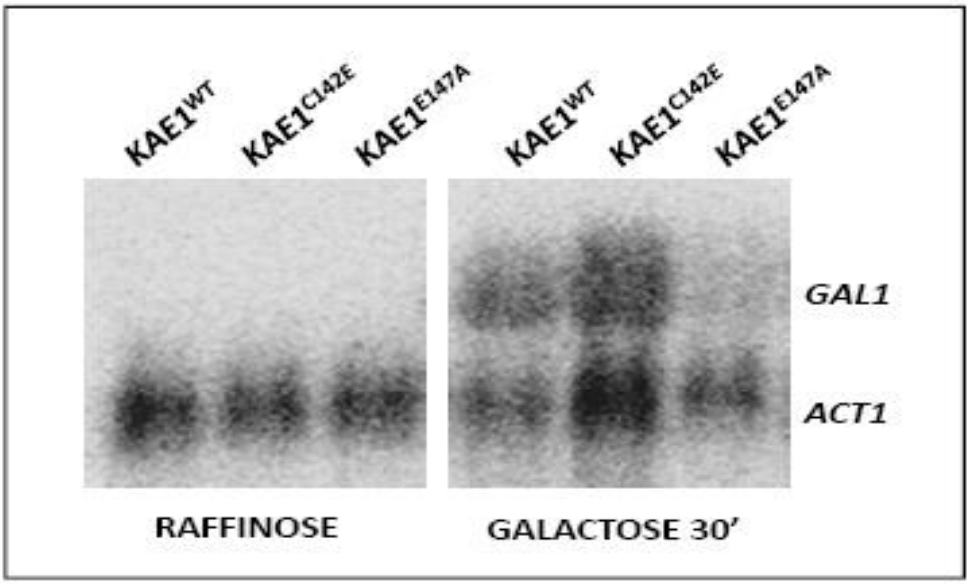



**Fig. 12-Northern blot showing the levels of *GAL1* transcription in $KAE1^{WT}$, $KAE1^{C142E}$ and $KAE1^{E147A}$ strains.** Cells were grown in raffinose-containing medium until $OD_{600}$=0.4 and then were shifted in galactose-containing medium for 30 min, to activate the transcription of the *GAL1* gene. The transcription of the *ACT1* gene serves as an internal control to assure that the basal level of transcription is the same in all strains.

This result suggests that the biochemical activity of the Kae1 protein is important for the functions of the entire complex, as the substitution of one residue probably important for the metallic-ion binding impairs the regulation of transcription.

## The *KAE1* mutations impair telomere homeostasis

After the analysis of transcription regulation, I observed (by Southern blot) telomere lengths in $KAE1^{C142E}$ and in $KAE1^{E147A}$ mutant cells. The probe used corresponded to the Y' region, that is present in almost half of yeast telomeres. As we can see in figure 13, the wild type strain telomeres are longer than those of the two mutant strains. Moreover, telomeres of $KAE1^{C142E}$ are even shorter than telomeres of the Δ*BUD32* strain.

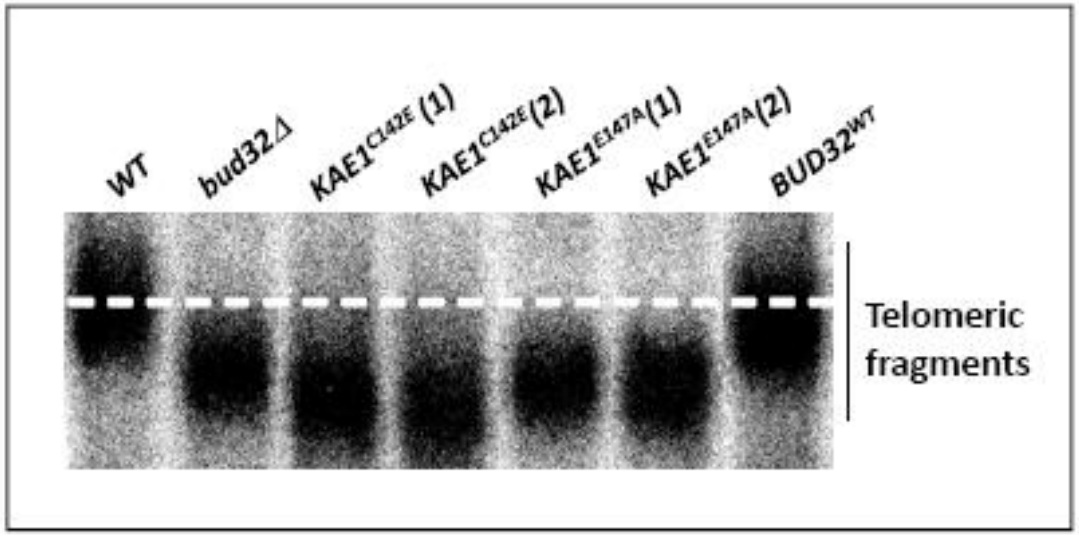



**Fig. 13-Southern blot. Telomere length analysis in wild-type and in *KAE1* mutant strains.** The *ΔBUD32* strain is used as a control of shortened telomeres. Genomic DNA of the indicated strains (grown in rich medium until exponential phase) was purified and digested with XhoI, producing telomeric terminal DNA fragments of about 1 kb, which were separated on 1.2% agarose, tranferred onto a nitrocellulose membrane, and checked with a 33P-labeled probe specific for telomeric Y'regions.

This result is consistent with that obtained on transcription analysis, further attesting a biochemical importance to the two residues of Cystein 142 and Glutamic Acid 147, within the metal ion-binding domain.

## Mutations of Kae1 alter its cellular localization

As described in [17], the Kae1 and Bud32 proteins have a nuclear localization. I then checked if the *KAE1* mutants showed the same behavior.

First of all, I inserted an HA-tag at the 3'-terminus of both the wild-type and mutant *KAE1* sequence, using a PCR-based technique (see Material and methods). The Kae1 protein could therefore be recognized by an anti-HA antibody, and the localization signal was visible using a fluorescent probe-labeled secondary antibody (the probe emits at 570 nm if excited with UV light). In wild-type cells, the signal of Kae1 corresponds to that of the nuclei, confirming the nuclear localization of the protein (fig. 14). As regards the $KAE^{C142E}$ and $KAE^{E147A}$ mutants, the signal is not only nuclear, but is scattered across the cell. It is possible that the mutagenized proteins acquire a different conformation, that alone impairs

its binding with the transporter protein(s), or that the observed diminished affinity with Bud32 is involved in this alteration of the transport.

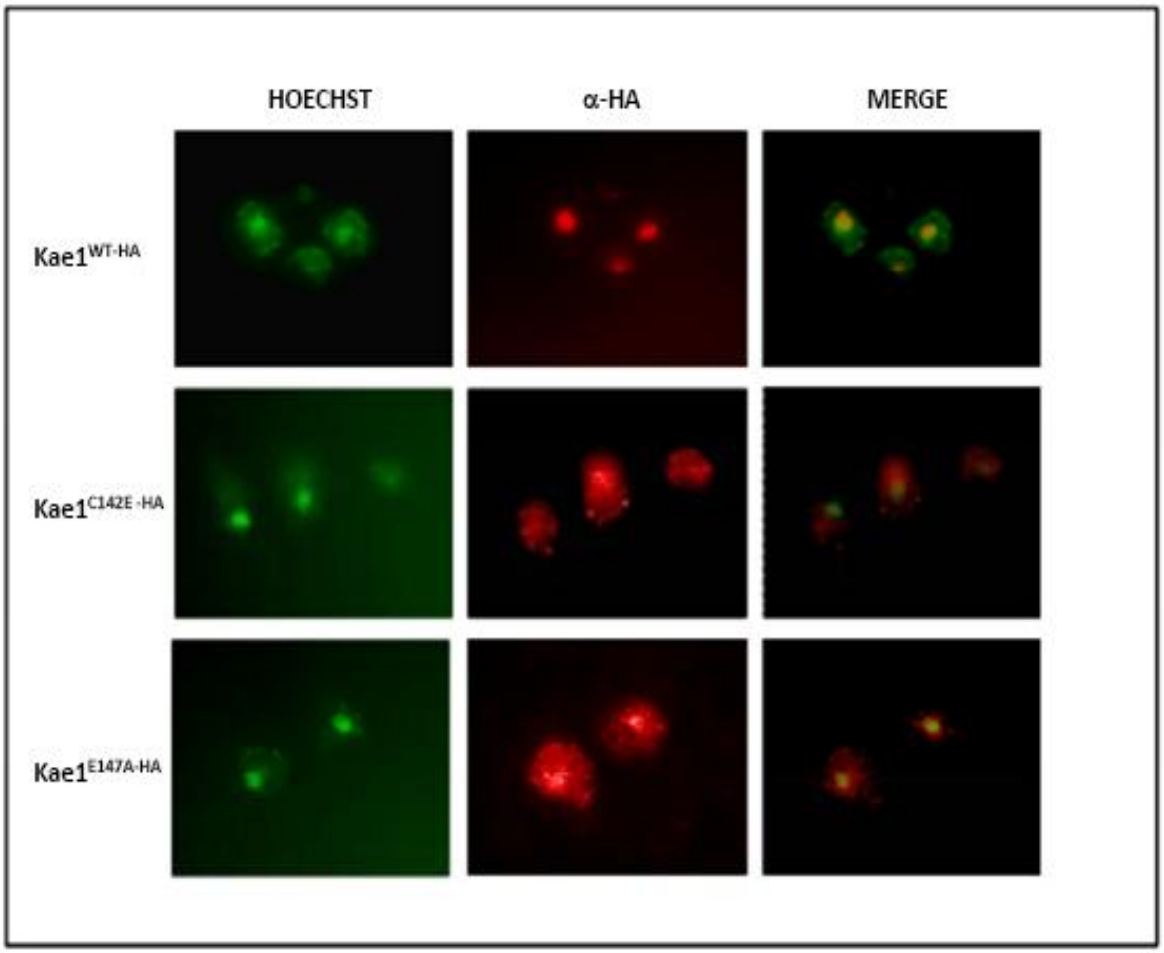



**Fig. 14-Cellular localization of Kae1.** Cells were grown in liquid YPD until exponential phase ($OD_{600}$ 0.8), then fixed on a poly-L-lysine coated slide. After the primary α-HA antibody, the exposition to a fluorescent probe-conjugated secondary antibody, allows the recognition of the protein by a fluorescence microscope.

This result well matches the alterations in transcription regulation and telomere homeostasis exhibited by the two mutants.

It is interesting to notice that while the mutant Kae1 proteins change in cellular localization, this behavior is not showed by Bud32 mutant proteins (data not shown).

## Is yeast Kae1 endowed with nuclease activity?

In 2007, thanks to a work of Hecker et al. [25], it was shown that the orthologue of Kae1 in the archaeon *Pyrococcus abyssi* (PaKae1) is able to bind cooperatively to single and double-stranded DNA and exhibits a class I apurinic endonuclease activity (AP-lyase).
In order to verify if this function could be assigned also to yeast Kae1, I made up a series of nuclease assays and chose telomere-like probes as substrates, on the basis that Kae1 functions in the regulation of telomere homeostasis. Since yeast telomeres are characterized by $TG_{1–3}$ repeats, I used radiolabeled 42-bp $(TGTGGG)_7$ (TG-P) or the complementary $(ACACCC)_7$ (AC-P) oligonucleotides as probes for the nuclease assay. A first result is shown in the beneath figure (fig. 15): I incubated the same amount of Kae/Bud for increasing times, with both the TG-P or the AC-P probe. The signal correspondent to the probes (∗) seemed to decrease when I added the protein, and another signal, corresponding to a lower molecular weight (∗∗), seemed to appear. This did not happen when I added EDTA to the reaction.

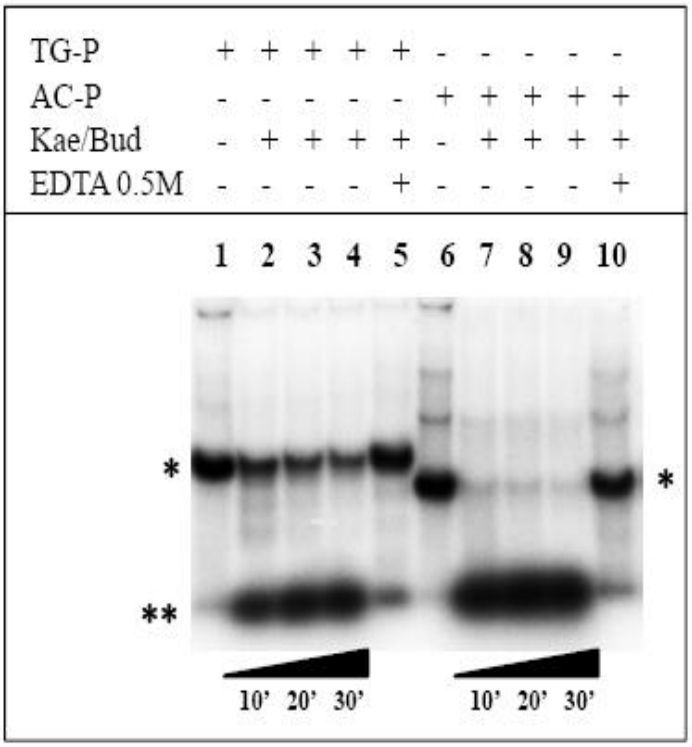



**Fig. 15-Nuclease assay on radiolabeled TG- and AC- probes.** The upper bands (∗), which correspond to the radiolabeled probes (alone, in lanes 1 and 6), progressively diminishes or disappears, and a band of a lower molecular weight appears (∗∗), when the Kae/Bud proteins are added (lanes 2-5 and 7-9). This is more evident for the AC-probe (lanes 7-9). Increasing times of reaction were checked. When EDTA (final concentration of 15 mM) is added to the reaction mix, we cannot see any diminution of the band (lanes 5 and 10).

I then tried to characterize the reaction and to verify if this activity was specifically due to the presence of the Kae1 protein.

I first checked if the addition of $MgCl_2$ in the reaction buffer was essential for the observed activity, and performed the reaction using various divalent ions. The figure below shows that in a buffer containing $CaCl_2$ or no ions, the reaction could not occur, similarly to what occurred when I used $MgCl_2$ plus EDTA. The same could be seen for the other ions tested (data not shown). Note that in this case I used a random-48 bp oligonucleotide probe: this means that the cleavage is not specific for a telomere-like probe, but could occur also elsewhere (fig. 16).

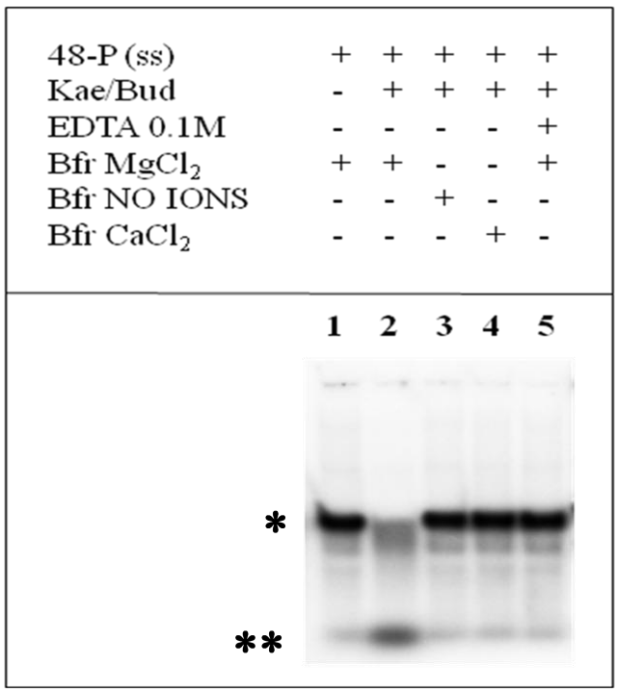



**Fig. 16-Nuclease assay on random 48bp-probe.** In lane 1 the probe alone and in lanes 2-5 the probe incubated with the Kae/Bud proteins, in different conditions. Lane 2: presence of MgCl2 in the reaction buffer; lane 3: the reaction buffer does not contain any ion; lane 4: presence of CaCl2 in the reaction buffer; lane 5: presence of MgCl2 in the reaction buffer supplemented with EDTA. Only in lane 2 the upper band corresponding to the probe alone (∗) is sensibly reduced and a lower band appears (∗∗).

I repeated the same experiment using a double-stranded TG-P/AC-P probe, but I could not observe any cleavage: this suggested that the observed nucleolytic activity is directed only to single-stranded DNA. Moreover, in this case a band of lower molecular weight, present in the lane containing the probe alone (lane 1), disappeared after the addition of the protein. This however has to be considered an aspecific signal, since it disappeared also with the different buffers tested and even in the presence of EDTA (fig. 17).

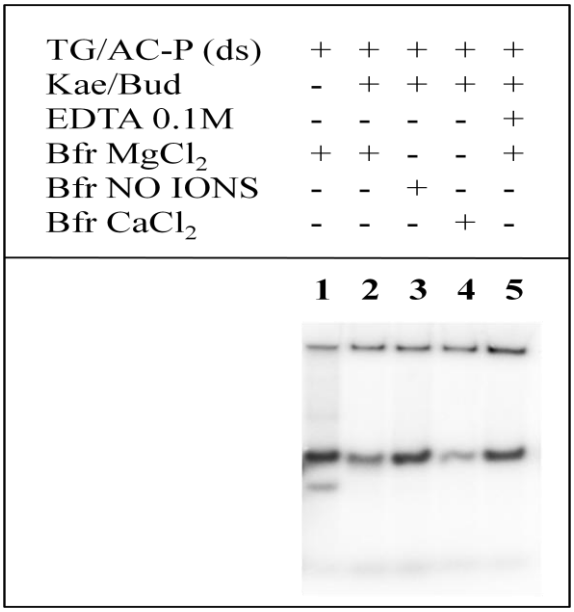



**Fig. 17-Nuclease assay on a double stranded probe, obtained by mixing the two single-stranded (radiolabeled) TG- and AC-probes.**
Even if in the presence of $MgCl_2$ and $CaCl_2$ (lanes 2 and 4) the probe seems partially degraded, it is not possible to detect the presence of the lower band as in previous experiments.

To further evaluate the specificity of this biochemical reaction, I purified a mutagenized version of the Kae1/Bud coexpressed proteins, in which the histidines 141 and 145 of Kae1, responsible for the coordination of the metal ion, and thus of the ATP moiety, were substituted with Alanine residues ($Kae1^{HH141\text{-}145AA}$/Bud32). The integrity of those histidines is essential in yeast cells and so I expected to observe no activity when these are substituted with alanine.

In order to work in the same conditions for the wild-type and the mutagenized protein, I purified again the $Kae1^{WT}$/Bud32, obtaining a cleaner enzyme as that used so far (compare fig. 22, page 48-49 and fig. 18, below).

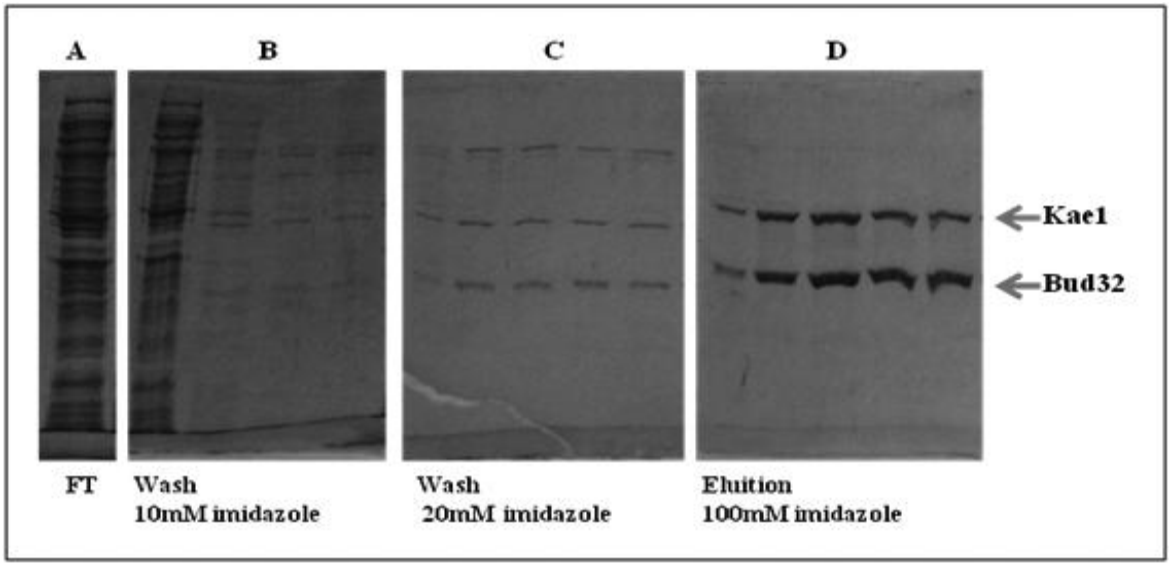


**Fig. 18-New purification of coexpressed Kae/Bud.** Panel A: the flow-through of the resin-containing column. Panel B: four fractions of the first column-washing (Tris/HCl pH 7.5, 0.3 M NaCl, 10 mM imidazole). Panel C: five fractions of the second column-washing, containing 20 mM imidazole. Panel D: five fractions of the eluition of the protein from the column with a buffer containing 100 mM imidazole.

Below in figure 19, a nuclease test using both the wild-type and the mutated protein is shown. The result is not very clear although the disappearance of the upper band seems to be quantitatively more important in the presence of the wild-type protein, compared to the mutated one. However, a lot of radioactive signal is retained in the wells of the gel, especially when the Kae1$^{WT}$/Bud32 protein is used, and that signal may be part of the cleaved or the uncleaved moiety.

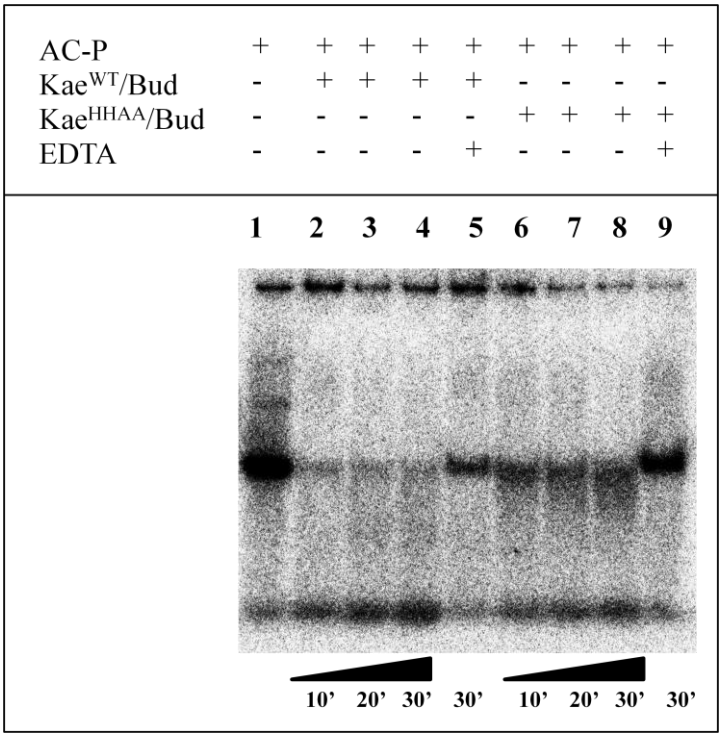



**Fig. 19-Nuclease assay on the AC-probe, in the presence of the wild-type (Kae1WT/Bud32) or the mutagenized (Kae1HHAA/Bud32) protein.** After addition of the wt protein (lanes 2-4), the upper band, corresponding to the protein alone, nearly disappears, even if a lot of radioactivity is retained in the gel wells, thus making the result difficult to interpret. When the mutated protein is added (lanes 6-8), the cleavage seems to be less efficient. In lanes 5 and 9 EDTA is added to the raction.

As the result was not clear, I made other attempts, testing different reaction conditions. Unfortunately, in the following experiments I was not able to see any cleavage, as it can be highlighted in the two figures below (and in many other experiments not shown).

In figure 20, the band corresponding to the probe disappears in the presence of both the wild-type and the mutated proteins. However it cannot be explained why a part of signal seems to disappear after the addition of the protein; in fact, we cannot see a corresponding

increasing of the lower band (but a lot of radioactivity still remains in the gel wells). Unexpectedly, when Bud32 alone was used (lane 10), the upper signal corresponding to the probe is nearly completely vanished. As this could not be observed in the subsequent experiments (see for example fig. 21), we conclude that in our hands these experiments are not enough reproducible and that probably also the results previously obtained were not specifically due to an activity of the Kae1 protein alone. What I had observed might be due to the presence of some contaminants in the protein eluate.

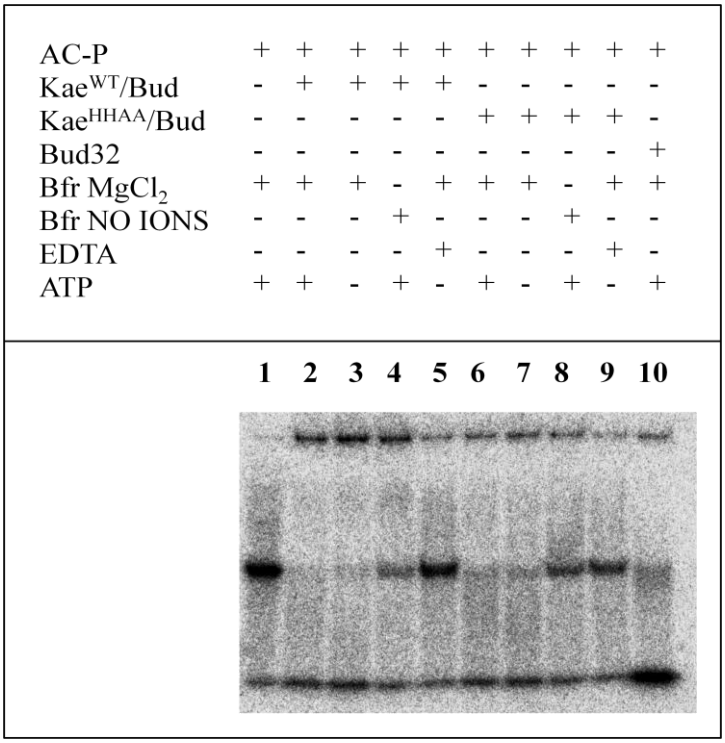



**Fig. 20-Nuclease assay on the AC-probe, in the presence of the wild-type (Kae1WT/Bud32) or the mutagenized (Kae1HHAA/Bud32) protein.** Unfortunately, both the wt and the mutated protein seem to cleave the probe (lanes 2-3 and 6-7), while the reaction is abolished by EDTA (lanes 5 and 9) or the absence of divalent ions (lanes 4 and 8). Surprisingly, also Bud32 alone seem to be able to completely cleave the probe (lane 10). Notice that no differences occur if ATP is added to the reaction (lanes 2-3 and 6-7).

As a last experiment (fig.21), I compared the activity of the previous, less clean, purification of Kae/Bud ($Kae1^{WT}$/Bud OLD, see figure 15) with those of the newly purified wild-type and mutated proteins (see fig. 18).

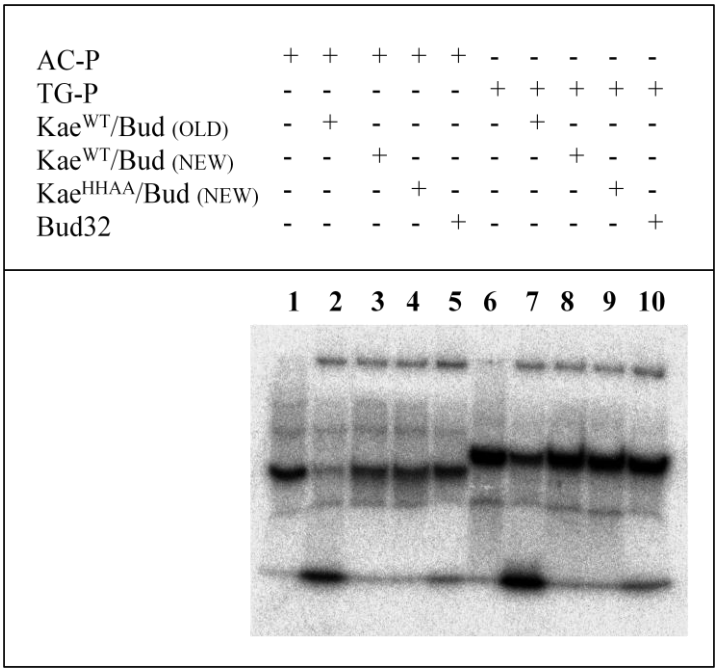



**Fig. 21-Nuclease assay comparing the activities of the first purification of Kae/Bud (Kae1WT/Bud OLD) with those of the newly purified wild-type and mutated proteins (NEW).** In this experiment, the activity of the newly purified proteins (lanes 3-4 and 8-9) is null, compared to the old purification (lanes 2 and 7); in this case Bud32 does not cleave at all (lanes 5 and 10).

It can be clearly seen that the activity of the cleaner proteins is null, comparing to the old purification, thus confirming the non reproducibility of the results obtained and the hypothesis that the activity previously observed was due to the presence of a contaminant, bacterial nuclease. Furthermore, in this case Bud32 does not cleave at all. I must therefore conclude that no nuclease activity for the yeast Kae1 can be clearly assessed.

# Kae1 IS AN *IN VITRO* SUBSTRATE OF Bud32

In a very recent work that describes the atomic structure of the KEOPS complex, the authors speculate that Bud32 could be a regulator of Kae1, by binding and phosphorylating it [40]. The results they obtained, however, were on the archaeal *M.jannaschii* proteins. Accordingly, a previous immunoprecipitation of Bud32 from wild-type yeast cells, performed in our laboratory, followed by a phosphorylation test *in vitro*, had indicated that Kae1 was phosphorylated. However, the result was not very clear. In order to better understand this interesting relationship, I tried to perform the same experiments on the yeast Bud32 and Kae1 proteins, expressed from *E.coli*.

Unluckily, despite several attempts, I was not able to purify the Kae1 protein alone, as it precipitates almost completely in the bacterial inclusion bodies.

I then used a polycistronic pET28 vector (kindly given by P. Forterre's lab) apt to coexpress Kae1 and Bud32 (both genes carrying their own ribosome binding sites). Bud32 has an histidine tag at its C-terminus that allows its purification through a nickel affinity resin (NiNTA, SIGMA). When we checked the eluate for the presence of Bud32, we noticed that it contained also Kae1, indicating that the two proteins co-purify. This evidence is an interesting confirmation of the binding between Bud32 and Kae1, that is maintained also for the recombinant proteins (fig. 22).

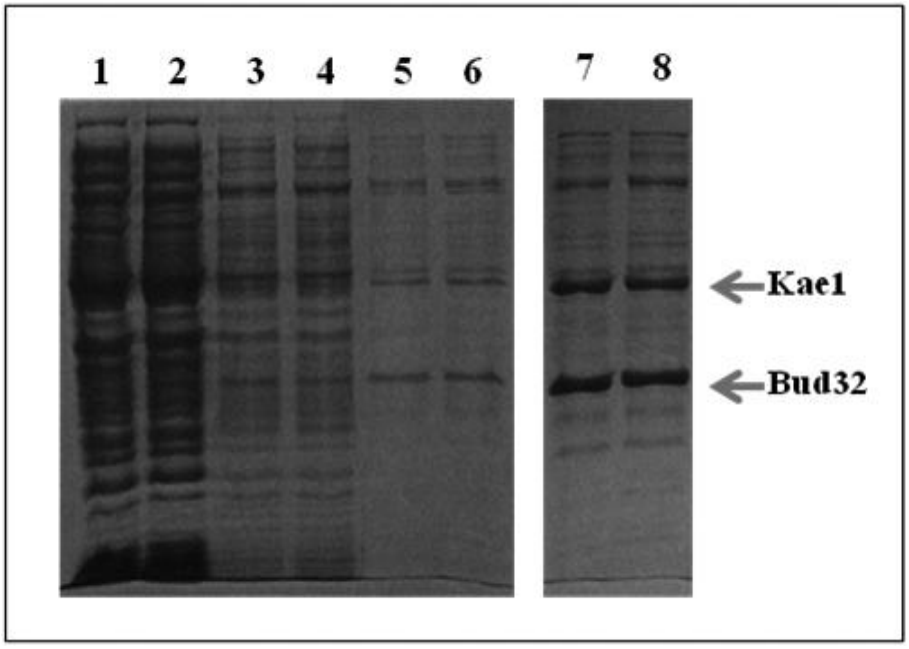


**Fig. 22-Batch purification of coexpressed Bud32 and Kae1 from the pET28 polycistronic vector.** Bud32 has an histidine tag to its C-terminus that allows its purification through a nickel affinity resin (NiNTA, SIGMA); when Bud32 is purified, Kae1 co-elutes with it. Lane 1: flow-through KaeWT/Bud, lane 2: flow-through KaeHHAA/Bud (MUT), lane 3-4: first wash of the resin with 10mM imidazole (3, WT; 4, MUT); lanes 5-6: second wash of the resin with 20mM imidazole (5, WT; 6, MUT); lanes 7-8: eluition with 100mM imidazole (7, WT; 8, MUT).

I then performed a series of phosphotransferase assays using the coexpressed proteins.

*P. abyssi* Kae1 has been proposed to bind ATP through coordination of a metal ion, held in place by two histidines (His107 and His110) and one tyrosine residue (Tyr127) [25]. Therefore, to exclude that Kae1 alone could retain the ATP mojety, I also used a mutagenized version of the coexpressed proteins, in which the two corresponding histidines of yeast Kae1 (His141 and His145) were replaced with Alanine residues.

In figure 23 the autoradiography panel shows that a phosphorylated band that corresponds to Kae1 can be seen both in the case of wild-type Kae1 and of the $Kae1^{HH141-145AA}/Bud32_{6HIS}$ mutant. This indicates that the phosphorylation signal is not a simple retention of ATP by Kae1.

It is also interesting to notice that the presence of Kae1 strongly affects the autophosphorylation of the kinase (observe the difference between the first and the other two lanes), indicating that Kae1 is able to inhibit the catalytic activity of Bud32. This evidence is a confirmation of what observed by Hecker *et al.*[27].

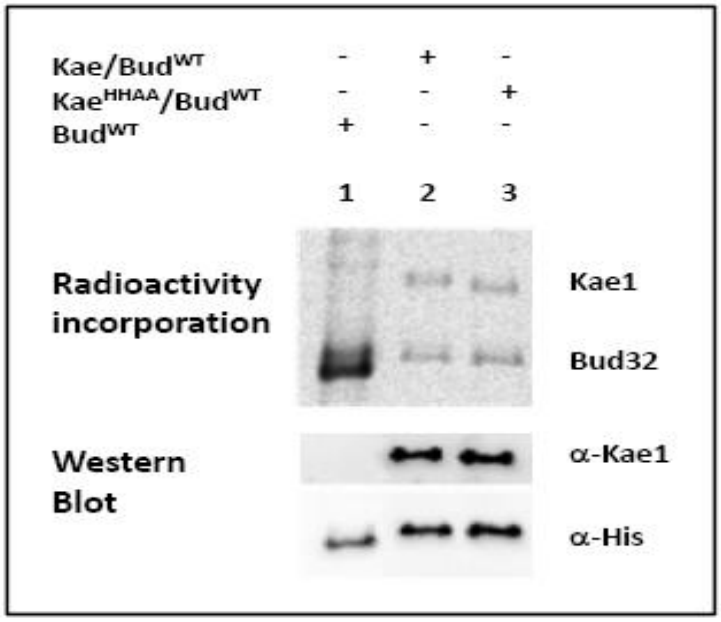



**Fig. 23-Kae1 is phosphorylated by Bud32.** Lane 1: Bud32 alone; lane 2: KaeWT/Bud326HIS ; Lane 3: KaeHH141-145AA/Bud326HIS. Reaction conditions: incubation with [γ-33P]ATP at 37 °C, for 15'

Then, in order to exclude that Kae1 could autophosphorylate or be phosphorylated by some contaminants present in the purification eluate, I made another assay using mutagenized versions of Bud32, in which two key residues for the catalytic activity were substituted: the Lysine 52 (K52A) and the Aspartic acid 161 (D161A) (see the *Introduction* for more details). As evident from figure 24, when Kae1/Bud32$^{K52A}$ was used (lane 4), the phosphorylation signal of Kae1 completely disappeared. However the Kae/Bud$^{D161A}$ still maintained some catalytic activity in agreement with previous observations on the recombinant Bud32 [34]. This preliminary result indicates that the phosphorylation of Kae1 is dependent on Bud32.

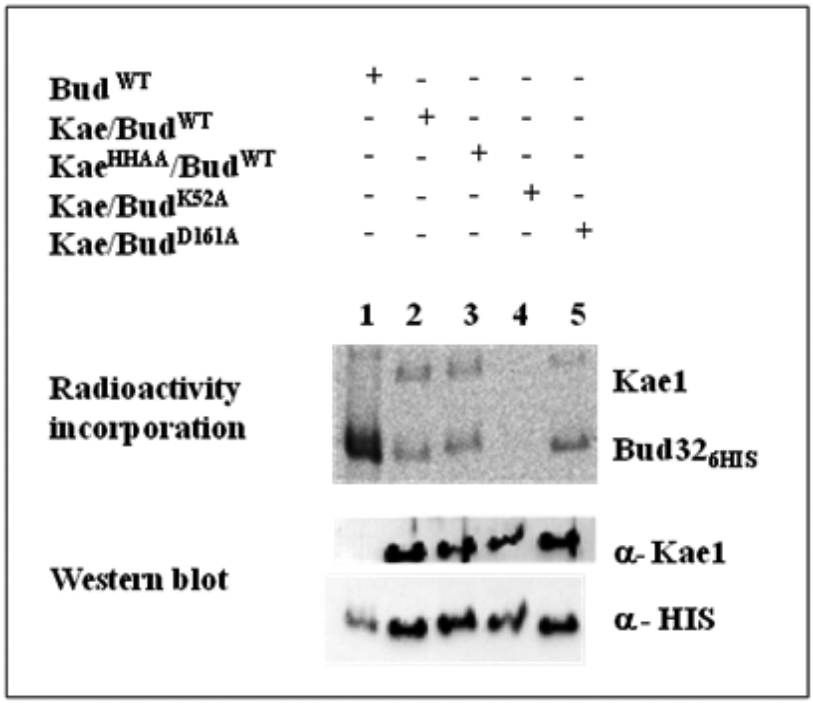



**Fig. 24**-Lane 1: Autophosphorylation of Bud32. Lane 2: KaeWT/Bud326HIS; Lane 3: KaeHH141-145AA/Bud326HIS; Lane 4: KaeWT/BudK52A; Lane 5: KaeWT/BudD161A. Reaction conditions: incubation with [γ-33P]ATP at 37 °C, for 15'.

To get a further insight into this relationship, I should have worked with different amounts of the substrate, which was impossible. So I only added an additional amount of Bud32$_{6HIS}$ alone to the Kae1/Bud32 incubation mixtures. I performed a kinase assay using as substrate the wild-type Kae1/Bud32$^{WT}$ or the mutant Kae1/Bud32$^{K52A}$, and then I added the kinase Bud32$_{6HIS}$ alone. I used either the wild-type Bud32$^{WT}$ or a double mutant Bud32$^{K52A+D161A}$; in the latter, both residues important for the catalytic activity are replaced by Alanine residues, leading to an almost fully inactive protein kinase. The result can be seen in figure 25.

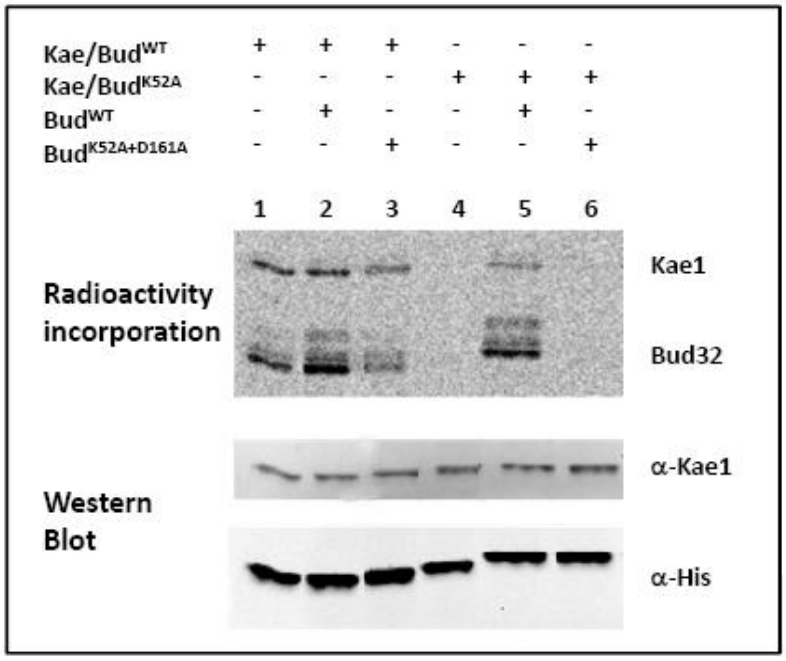



**Fig. 25. Incubation of the wild-type or mutagenized Kae/Bud32 proteins with either the wt or the catalytically inactive double mutant Bud32.** Lane 1: Kae1/Bud32$^{WT}$ alone, the same plus Bud32$^{WT}$ (lane 2) or Bud32$^{K+D}$ (lane 3). Lane 4: Kae1/Bud32$^{K52A}$ alone, the same plus Bud32$^{WT}$ (lane 5) or Bud32$^{K+D}$ (lane 6).

The phosphorylation signal on Kae1 was immediately evident when the wild-type Kae1/Bud32 coexpressed proteins were used and did not increase when wild-type Bud32 was added; only an increase of the autophosphorylation of Bud32 can be noticed (lane 2) and this further confirms that Kae1 exerts a quantitative inhibition on Bud32 activity. When the double mutant Bud32$^{K52A+D161A}$ was added (lane 3), both signals (phosphorylation of Kae1 and autophosphorylation of Bud32) seem to slightly diminish, but this result is not sufficient to give rise to speculations.

More interesting, when I used the mutant Kae1/Bud32$^{K52A}$ proteins, the basal signal of Kae1 phosphorylation was not visible in autoradiography (lane 4) and so we could appreciate the appearance of a phosphorylation band when the wild-type Bud32 was added (lane 5). Instead, when the double mutant Bud32$^{K52A+D161A}$ protein was added, no signal of phosphorylation could be detected (lane 6). This result is a further confirmation of the relevance of Bud32 activity for Kae1 phosphorylation.

As mentioned before, I could not obtain a soluble purified Kae1 protein. I then tried to purify Kae1 in denaturing conditions (with 8M urea) and to carry out a refolding of the protein directly on the Ni-NTA resin; immobilization of the protein on the matrix, in fact, should theoretically circumvent protein-protein aggregation due to the spatial separation of the bound proteins. However the attempt failed because all the protein was retained by the resin. I then used this resin-immobilized protein as a substrate for another

phosphotransferase assay. Increasing amounts of wild-type Bud32 or double mutant Bud32$^{K52A+D161A}$ where added to the same amount of Kae1 bound to the resin. The result is shown in figure 26: a not well defined, although visible, band is present when a sufficient amount of the wild-type Bud32 is added to the resin (lanes 1-5). The signal raised with the quantity of protein kinase until saturation. Conversely, this could not be seen when the mutagenized Bud32$^{K52A+D161A}$ was added (lanes 6-9).

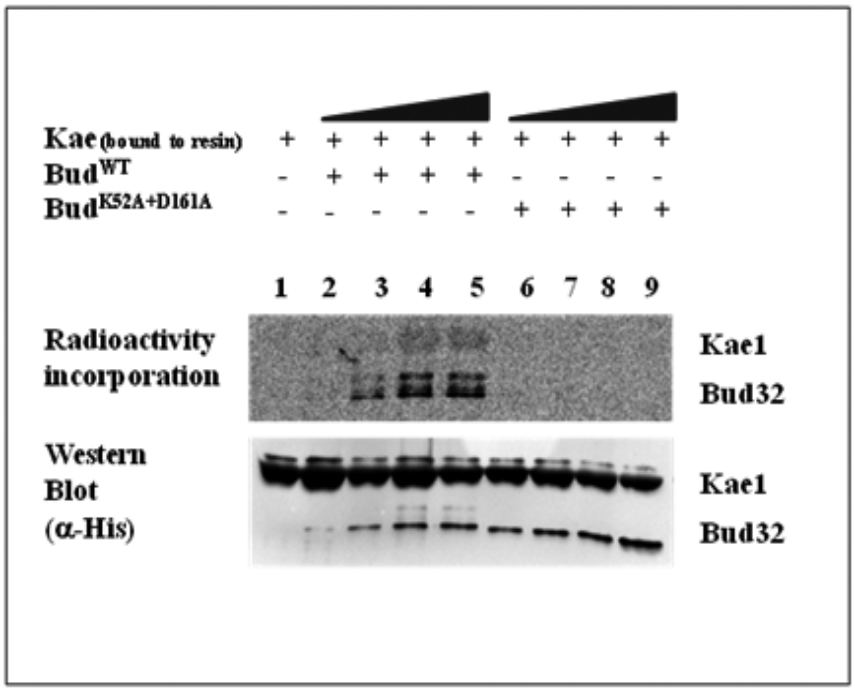



**Fig. 26-Phosphorylation assay on the resin-immobilized Kae1.** Lane 1: resin-bound Kae1 alone. Lane 2-5: increasing amounts of Bud32$^{WT}$ are added to the resin suspension(100, 200, 300, 400 ng). Lane 6-9: increasing amounts of Bud32$^{K+D}$ are added to the resin suspension(100, 200, 300, 400 ng).

Altogether these results indicate that, at least *in vitro*, a regulatory relationship exists between Bud32 and Kae1; in fact, Bud32 phosphorylates Kae1, which conversely inhibits the catalytic activity of the kinase. This is very interesting as the two proteins are liable to carefully modulate the functions of the entire KEOPS complex.

## MS analysis of phosphorylated Kae1

I then tried to identify the specific residue(s) of Kae1 phosphorylated by Bud32. First of all I measured the level of phosphorylated Kae1, in an *in vitro* assay on Kae1/Bu32$^{WT}$, and found that 2.6 % of the total protein is phosphorylated. Thanks to Dott. G. Arrigoni

(University of Padova), we made a MS analysis on recombinant Kae1, after incubation with additional Bud32 for 30 minutes, in the presence of 62.5 μM ATP (see *Materials and Methods* for more details). The analysis revealed that the Kae1 peptide DFS*ETVVTQK is phosphorylated at the Serine indicated; other three peptides are phosphorylated, but they have not yet been sequenced: IPNEPSPGYNIEQLAK, DYYIALGLEGSANK, AQNPVVLYVSGGNTQVIAYSEK (fig. 27)

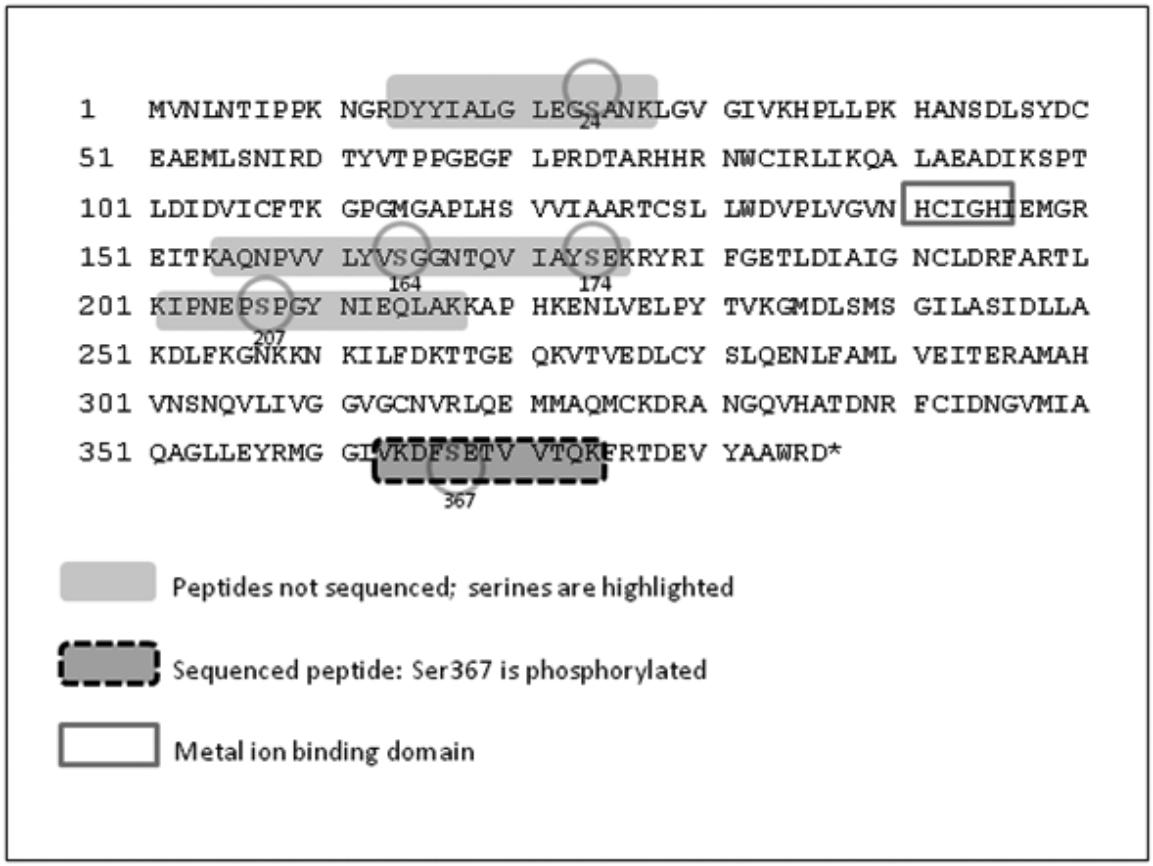



**Fig. 27-Aminoacid sequence of the Kae1 protein,** with the sequenced peptide and the site of phosphorylation, and the three other peptides that are supposed to be phosphorylated.

These new data encourage to perform further experiments in order to confirm the biochemical relationship between Bud32 and Kae1. A first step will be the mutagenesis of Ser367 of recombinant Kae1 protein to an Alanine residue. If this is the main residue phosphorylated by Bud32, the mutant Kae1 will show a decreased, or even a null signal, in an *in vitro* kinase assay. However, also the three other peptides may be substrates of Bud32. If this was confirmed, we will mutagenize other residues, to locate the main targets of the kinase.

The other important step will be the identification of a physiological role of this phosphorylation of Kae1 by Bud32, through the analysis of the phenotype of Kae1 mutants, including the analysis of transcription regulation and telomere homeostasis, in order to know if this phosphorylation is involved in the functions of the KEOPS complex.

It is interesting to notice that, in the structure described by Mao *et al.*, [40], the Ser367 residue lies within insert 2, a very conserved sequence, specific of the Kae1 family, that is exposed in the protein structure and hence accessible to the active site of Bud32. In this work, Mao and colleagues write that "partial (373-end) or full deletion (365-end) of the C-terminal tail of yeast Kae1 caused a severe growth defect". In particular, the authors identified a residue within this region, T369, which leads to the same growth defect if mutagenized to Alanine. They thus assumed that T369 might be the phosphoregulatory site. However, our identification of Ser367, that is only two residues from Thr369 allows the hypothesis that the T369A phenotype may be due to the close proximity of S367, either because the T369A substitution alters the conformation of the local sequence, and thus the accessibility of Bud32, or because the previous phosphorylation of T369 is essential to S367 phosphorylation.

# Conclusions

## ROLE OF Bud32 IN A NEW SIGNALING PATHWAY IN YEAST

In the first part of my PhD thesis, I have described my contributions to the unveiling of a novel signaling pathway in *S.cerevisiae*, that implicates Sch9, the yeast homolog of mammalian Akt, Bud32 and the glutaredoxin Grx4. We showed that Grx4, previously described as an *in vitro* substrate of Bud32 [17], is also a physiological target of the kinase, that phosphorylates it in the Ser 133 and, mainly, Ser134 residues. This phosphorylation contributes to the functionality of the glutaredoxin in yeast cells; in fact the growth of mutant cells in which Grx4 is unphosphorylatable by Bud32 is affected, even if in a mild way.

Moreover, this relationship is influenced by the phosphorylation status of Bud32. Bud32 is phosphorylated at the Ser258 residue by the Sch9 kinase, and this phosphorylation upregulates the ability of Bud32 to bind and phosphorylate Grx4 [46].

When searching for a physiological role for this new phosphorylation cascade, however, we noticed that phosphorylation of Bud32 at Ser258 is unrelated to the role of KEOPS complex in telomere homeostasis and transcription regulation, suggesting that Bud32 partecipates in multiple pathways in yeast cells.

Also, the cascade does not seem to affect some known functions of Grx4, like iron homeostasis and oxidative stress protection. We may then hypothesize that it plays a role in different and still uncharacterized functions of Grx4.

It is important to highlight that this regulatory system is evolutionarily conserved. In fact, it has been demonstrated that in human cells Akt (Sch9 homolog) phosphorylates PRPK (Bud32 homolog) at the Ser250 residue, and that this phosphorylation positively regulates *in vivo* the activity of PRPK on its physiological substrate, p53 [48].

# ANALYSIS OF Kae1 ACTIVITY

A part of my PhD study was devoted to the subunit of the KEOPS complex named Kae1. As described in the introduction, Kae1 was previously classified as a protease, and indeed it contains a HXXXH motif, highly conserved in metalloproteases. The motif is essential in Kae1, as the mutagenesis of one or both histidines (H-141 and H-145) deprives the protein of its functionality and is lethal to the cell. Interestingly, in Kae1 and in all its eukaryotic homologues, the residue of Glutamic Acid in the second position (HEXXH), normally present in metallo proteases, is replaced with a Cystein, while a Glutamic Acid is present after the second histidine, in position + 2 (HCGIHIE). These characteristics suggest that Kae1 may act with a different catalytic mechanism compared with the other metallo-proteases. In order to understand the role of Kae1, I used two mutants already available in my laboratory, in which these two residues were substituted ($KAE^{C142E}$ and $KAE^{E147A}$). Both mutants, in fact, showed a slow growth phenotype, but were still viable. I first asked if those mutations could interfere with the functions of KEOPS. Indeed, I observed that both transcription regulation and telomere homeostasis were affected in these mutant cells. I thus concluded that the biochemical activity of the Kae1 protein must be important for the functions of the entire complex, as the mutation in two residues within the metal ion binding domain impairs both telomere homeostasis and transcription regulation

Then I verified if the mutations of Kae1 could alter the cellular localization of the protein: in fact I could observe that, in contrast with the wild-type, the mutant proteins are not exclusively nuclear, but are scattered across the whole cell. This result well matches with the alterations in the KEOPS functions exhibited by the two mutants, as both telomere homeostasis and transcription regulation functions are performed within the nucleus. As the two mutations are not part of a localization sequence, we may hypothesize that they could somehow interfere with the binding to other proteins, like cellular transporters. It is also interesting to notice that the catalytic mutants of Bud32 do not show alterations in Bud32 cell localization with respect to the wild-type, *i.e.* the protein is always nuclear.

In order to attribute a biochemical activity to Kae1, I wanted to verify if the yeast Kae1 protein could be a nuclease. In fact, in a work of 2007 it was shown that the orthologue of Kae1 in the archaeon *Pyrococcus abyssi* (PaKae1;) is able to bind cooperatively to single and double-stranded DNA and exhibits a class I apurinic endonuclease activity (AP-lyase)

[25]. Moreover, a nuclease function is hypothesized to be essential for telomerase, as the latter needs 3'ssDNA overhangs to add new telomeres at the level of double-strand breaks (DSBs). The identification of a nuclease activity for Kae1 would then be very important to clarify the molecular mechanism by which KEOPS exerts its function in telomere maintenance. Hovewer, from our experiments, no nuclease activity for the yeast Kae1 can be clearly assessed. This is in agreement with what observed by Mao *et al*. in 2008, when the authors assessed that the archaeal MjKae1 has an ATPase, but no AP-endonuclease or DNA-binding activity [40].

## BIOCHEMICAL RELATIONSHIP BETWEEN Bud32 AND Kae1

In the work describing the atomic structure of an archaeal-derived KEOPS complex [40], Mao and colleagues suggested that MjBud32 phosphorylates MjKae1, and that this phosphorylation has a regulatory function. We then decided to verify if the same was true for the yeast proteins. As, despite several attempts, I was not able to purify the Kae1 protein alone, due to its precipitation in the bacterial inclusion bodies, I used a polycistronic vector, allowing the coexpression of yeast Kae1 and Bud32. I then performed a series of kinase assays using the coexpressed proteins and demonstrated that, at least *in vit*ro, a regulatory relationship exists between Bud32 and Kae1. In fact, I showed that Bud32 is able to phosphorylate Kae1 and that Kae1 inhibits the catalytic activity of the kinase. These results confirm those obtained with the archaeal proteins, suggesting a conserved mechanism within the complex. An ulterior important confirmation comes from the MS analysis of phosphorylated Kae1, that identified Ser 367 as a target of Bud32. However this might not be the only phosphorylated residue.

Therefore the relationship between Bud32 and Kae1 is liable to carefully modulate the functions of the entire KEOPS complex.

# Materials and methods

## STRAINS

### *E. coli* strains

| STRAIN | GENOTYPE |
|---|---|
| InvαF' | endA1, recA1, hsdR17($r^{-k}$, $m^{+k}$) supE44, $\lambda^-$, thi-1, gyrA, relA1, φ80lacZαΔM15Δ (lacZYA-argF),deoR$^+$, F |
| BL21 | F-, *omp*T, *hsd*Sβ(rβ-mβ-), *dcm*, *gal*, (DE3) tonA |

### *S. cerevisiae* strains

| STRAIN | GENOTYPE |
|---|---|
| W303-1B | Mat a/α ; ade2-1 ; hys3-11,15 ; leu2-3,112 ; trp1-1 ; ura3-1 ; can$^r$ |
| W303-1B 2n a/α | ade2-1/ade2-1; hys3-11,15/hys3-11,15; leu2-3,112/leu2-3,112; trp1-1/trp1-1; ura3-1/ura3-1; can1-100/can1-100 |
| W303-1B/*BUD32$^{WT}$-HA$_3$* | |
| W303-1B/*BUD32$^{D161A}$-HA$_3$* | |
| W303-1B/*BUD32$^{S258A}$-HA$_3$* | |
| **STRAINS CONSTRUCTED IN THIS WORK:** | |
| W303-1B 2n/*GRX4::KanMX4/GRX3::HIS3* | |
| W303-1B /*GRX4::KanMX4/GRX3::HIS3*+pYeDP *GRX4 WT* | |
| W303-1B /*GRX4::KanMX4/GRX3::HIS3*+pYeDP *GRX4 SS133-134AA* | |
| W303-1B /*GRX4::KanMX4/GRX3::HIS3*+pYeDP *GRX4 S134A* | |
| W303-1B /*GRX4::KanMX4/GRX3::HIS3*+pYeDP *GRX4 S133A* | |
| W303-1B /*GRX4::KanMX4/GRX3::HIS3*+pYeDP *GRX4 S134D* | |
| W303-1B /*KAE1$^{WT}$-HA$_3$* | |
| W303-1B /*KAE1$^{E147A}$-HA$_3$* | |
| W303-1B /*KAE1$^{C142E}$-HA$_3$* | |

# MEDIA

| **MEDIA FOR *E. coli*** | |
|---|---|
| **LB:** | **SOC:** |
| • 1% Bacto Triptone (Difco);<br>• 0.5% Yeast Extract (Difco);<br>• 0.5% NaCl;<br>• 2% Agar (only for solid media). | • 1% Bacto Triptone (Difco);<br>• 0.5% Yeast Extract (Difco);<br>• 0.5% NaCl;<br>• 2.5 mM KCl;<br>• 10 mM $MgCl_2$;<br>• 10 mM $MgSO_4$;<br>• 20 mM dextrose. |
| When necessary, Ampicillin (100 μg/ml final concentration) or Kanamycin (20 μg/ml final concentration) were added to the medium. | |

| **MEDIA FOR *S. cerevisiae*** | |
|---|---|
| **YPD (rich medium):** | **SD (minimal medium):** |
| • % Bacto Peptone (Difco);<br>• 1% Yeast Extract (Difco);<br>• 2% Dextrose;<br>• 2% Agar (only for solid media).<br><br>When necessary, geneticin (G-418, GIBCO BRL; 200 μg/ml) was added to this medium. | • 0.17% Yeast Nitrogen Base (Difco);<br>• 2% Dextrose;<br>• 0.5% Ammonium Sulfate;<br>• 2.3% Agar (only for solid media).<br><br>When necessary, aminoacids were added to this medium: Adenine, 40 mg/ml; $_L$-Leucine, 60 mg/ml; $_L$-Triptophane, 20 mg/ml; $_L$-Histidine, 10 mg/ml; Uracil, 50 mg/ml (SIGMA) |

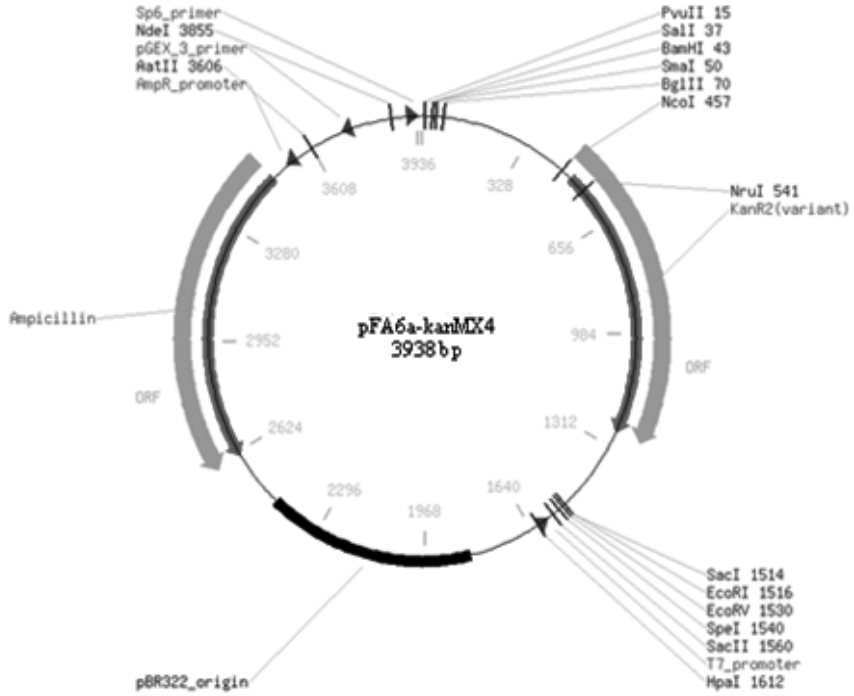
| **SPORULATION MEDIUM:** | **FOA:** |
|---|---|
| • 1% KAc;<br>• 2% Agar. | • 0.17% Yeast Nitrogen Base (Difco);<br>• 2% Dextrose;<br>• 0.5% Ammonium Sulfate;<br>• 2% Agar.<br>• Aminoacids (Ade, 40 mg/ml; Leu, 60 mg/ml; Trp, 20 mg/ml; His, 10 mg/ml; Ura, 50 mg/ml) and the 5'-Fluoroorotic acid (1 g/l) are filtered-sterilized and added after the medium has been autoclaved |

# VECTORS

## pFA6a-kanMX4

(Achim W. et al., 1994)

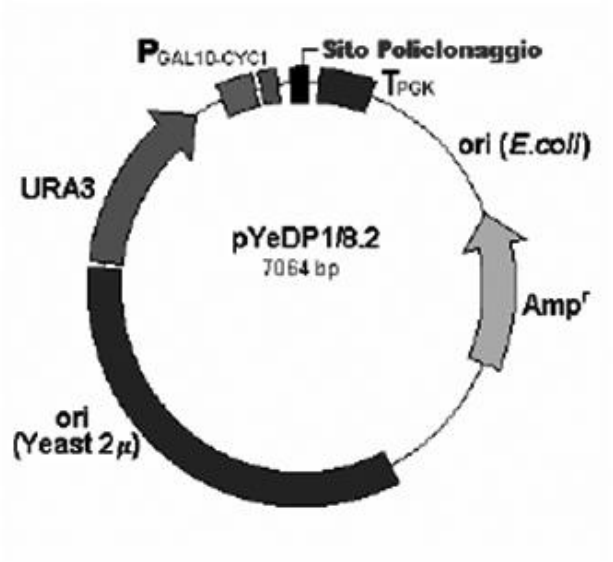
This vector of 3938 bp carries a selectable marker for both *E.coli* and yeast cells. It is used as a template for PCR with oligonucleotides that contain regions complementary to the target gene to be deleted (*GRX4* in our case). PCR products are then used for high efficiency transformation of yeast cells. We also used a modified vector in which the KAN cassette was substituted with an HIS3 cassette, cloned in the BglII site. This latter plasmid was used for the deletion of the *GRX3* gene.




## pYeDP1/8.2

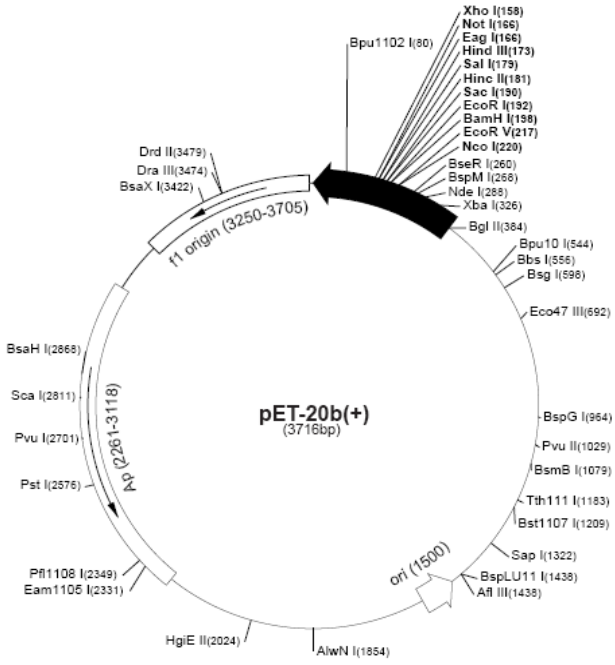
(Cullin C and Pompon D., 1988)

It is a vector for gene expression in yeast cells. The $P_{GAL10\text{-}CYC1}$ promoter is formed by the fusion of the CYC1 promoter (that regulate the transcription of Cytocrome C, inhibited by glucose) and GAL10 promoter (that promotes the transcription of the *GAL10* gene, in the presence of galactose in the medium). We used this plasmid to overexpress wild type and mutagenized alleles of the *GRX4* gene in a *ΔGRX4/ΔGRX3* contest, simply by growing cells in galactose medium. The vector can be replicated both in *E.coli* and in yeast and carries a selectable marker for both organisms.



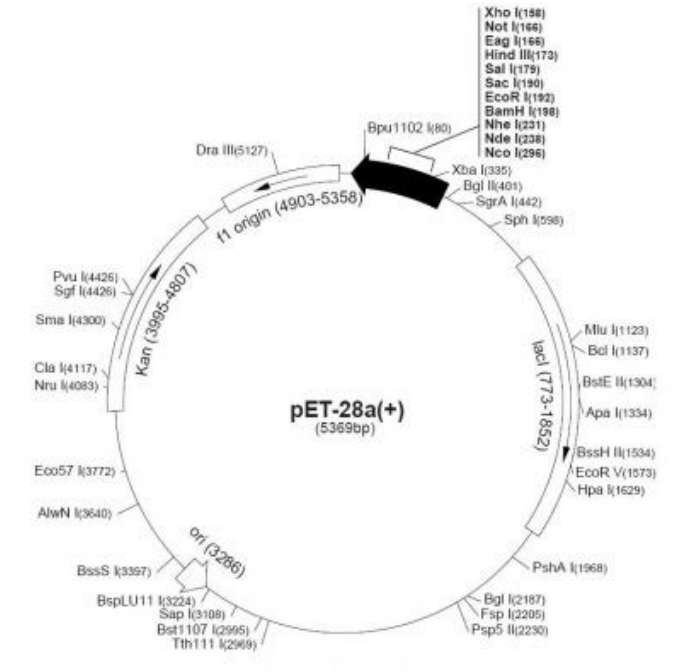

## pET-20 (Novagen)

The pET-20b(+) vector was used to express recombinant Bud32, Cgi121 (both cloned in the XbaI-HindIII sites; a rbs (ribosome binding sequence) was placed before the XbaI cloning site) and Grx4 (cloned in NdeI-XhoI sites) proteins, as it carries a C-terminal His•Tag® sequence, that allows purification by affinity chromatography.



## pET-28 (Novagen)

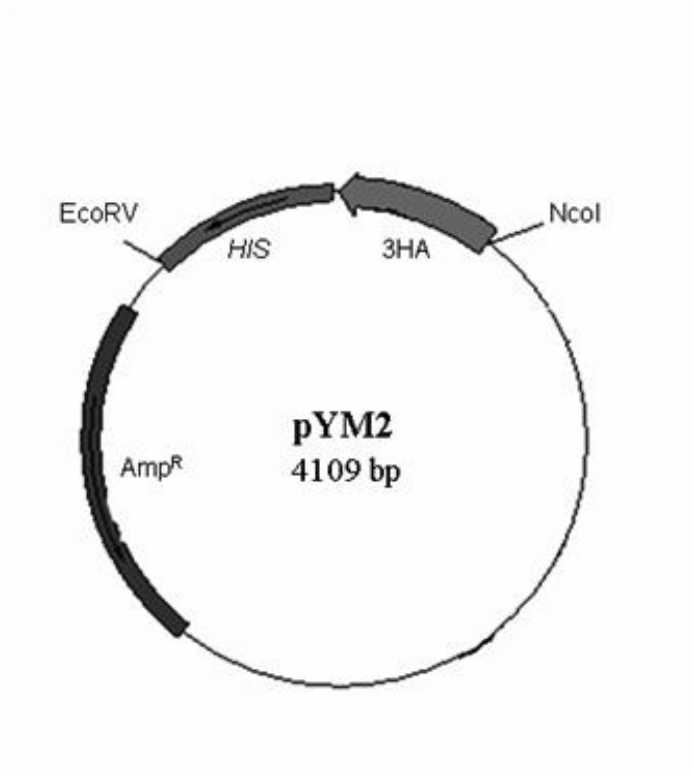
The pET-28a(+) vector was used to co-express Kae1 and Bud32 as described in Hecker et al., 2008. The *KAE1* gene was amplified by PCR and ligated at the NotI restriction site followed by the *BUD32*-his gene which has a 6His-tag encoding sequence at the 3' end. *E. coli* expression with this polycistronic vector leads to single transcripts bearing the two genes that both have their own rbs [27].




## pYM2

(M.Knop *et al*., 1999)

This vector carries a selectable marker for both *E.coli* and yeast cells. It is used as a template for PCR with oligonucleotides that contain regions complementary to the target gene to be tagged (*KAE1* and *AFT1* in our case). PCR products are then used for high efficiency transformation of yeast cells. Transformants thus express proteins that contains a $HA_3$-tag at their C-terminus and this allows for their recognition through a specific monoclonal antibody.




## ONE-STEP TRANSFORMATION OF YEAST

(Chen et al., Curr. Genet. 21:83, 1992)

Inoculate 2 ml medium in a 13-ml sterile tube with a single yeast colony and grow overnight at 30°C.

Transfer culture to a 2-ml microcentrifuge tube and pellet cells. Discard supernatant and add 5 μl carrier DNA (10 mg/ml), 0.5-1 μg of the desired plasmid DNA, and 100 μl of transformation solution (40% PEG 3350, LiAc 0.2M pH 7.5, DTT 0.1M).

Vortex vigorously and incubate 30 min at 45 °C. Transfer in ice 2 min.

Centrifuge 3 min at top speed, discard supernatant. Resuspend pellet in 600 μl of the desired medium or sterile $H_2O$ and spread suspended cells onto selective plates.

# HIGH EFFICIENCY LIAC TRANSFORMATION OF YEAST

(Gietz et al., YEAST 11:355-360, 1995)

Inoculate 2-5 ml of liquid YPD and incubate with shaking overnight at 30°C.

Count o/n culture and inoculate 50 ml of warm YPD to an $OD_{600}$=0.2.

Incubate the culture at 30°C on a shaker at 200 rpm until the absorbance reaches $OD_{600}$=0.7-0.8. This will take 3 to 5 hours. This culture will give sufficient cells for 10 transformations.

Harvest the culture in a sterile 50 ml centrifuge tube at 1000 x g for 5 min.

Pour off the medium, resuspend the cells in 25 ml of sterile water and centrifuge again.

Pour off the water, resuspend the cells in 1 ml 100 mM LiAc and transfer the suspension to a 1.5 ml microfuge tube.

Pellet the cells at top speed for 15 sec and remove the LiAc with a micropipette.

Resuspend the cells to a final volume of 500 µl in 100 mM LiAc.

Boil SS-DNA for 5 min. and quickly chill in ice water.

Vortex the cell suspension and pipette 50 µl samples into microfuge tubes. Pellet the cells and remove the LiAc with a micropipette.

Add the "transformation mix": 240 µl PEG (50% w/v), 36 µl 1.0 M LiAc, 5 µl SS-DNA (10 mg/ml), X µl DNA*, 70-X µl Sterile $ddH_2O$. Carefully add these ingredients in the order listed. The PEG should go in first, which shields the cells from the detrimental effects of the high concentration of LiAc.

Vortex each tube vigorously until the cell pellet has been completely mixed. Usually takes about 1 min.

Incubate (w/o agitation) at 30 °C for 30 min.

Heat shock in a water bath at 42°C for 20 min.

Microfuge at 6-8000 rpm for 15 sec and remove the transformation mix with a micropipette.**

Pipette 600 µl of sterile water into each tube and resuspend the pellet by pipetting it up and down gently. Spread suspended cells onto selective plates.

*For gene disruption via PCR, you can use an entire reaction of 50 µl without any further purification.

**When you are attempting to disrupt a gene with a PCR-amplified KAN::MX4 cassette, you have to resuspend yeast cells in YPD (about 20 ml), after step 15, and let them grow 2 to 3 hours before plating on YPD+G-418.

# PREPARATION OF YEAST DNA

Inoculate 2 ml medium in a 13-ml sterile tube with a single yeast colony and grow overnight.
Transfer culture to a 2-ml microcentrifuge tube (proper for the MagNA Lyser Instrument, Roche) and pellet cells. Discard supernatant and add an equal amount of glass beads, 200 μl TE buffer, 0.1 M NaCl, 2% TRITON X-100, 1% SDS, 100 μl basic phenol and 100 μl chloroform:isoamyl alcool (24:1).
Vortex vigorously in the MagNA Lyser three time for 30 sec at 6000 rpm, with 1 min in ice, and add 200 μl TE. Centrifuge at 13000 rpm for 10 min, 4°C.
Transfer aqueous (top) phase to a clean 1.5-ml microcentrifuge tube, add 2.5 volumes of ice-cold 100% ethanol and precipitate 30 min (or overnight), -80°C. Microcentrifuge 15 min at 13000 rpm, 4°C.
Dry the pellet and resuspend in 50 μl sterile $H_2O$ and add 1 μl RNAse A; let 15 min at 37 °C. Determine the concentration spectrophotometrically and run electrophoresis gel.

# POLYMERASE CHAIN REACTION (PCR)

(Sambrook K.J. et al., 1989; Ausubel F.M. et al., 1989)

The polymerase chain reaction (PCR) is a technique used to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences

complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus.*

Reactions were performed in these conditions:

- 5-10 ng DNA template (that contains the DNA region (target) to be amplified);
- 200 μM deoxynucleoside triphosphates (dNTPs);
- 1 μM of each primer (that are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target);
- 1X buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase and containing a monovalent cation (KCl) (Finnzymes);
- 1.5 mM $MgCl_2$;
- 1U Phusion or DyNAzyme™ II DNA polymerase (Finnzymes);
- $H_2O$ to reach volume.

Common volumes are 20–50 μl and reactions were carried out in a GeneAmp® System 9700 (PE Applied Biosystem) thermal cycle.

Reaction conditions varies depending on the type of DNA polymerase used, but are usually the following:

- Initialization step: 5 min at 94 °C (for DyNAzyme™ II DNA polymerase ), or 30 sec at 98 °C (for Phusion).

Then 25-35 cycles of:

- Denaturation step: 1 min at 94 °C (for DyNAzyme™ II DNA polymerase ), or 10 sec at 98 °C (for Phusion).for 20–30 seconds.
- Annealing step: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 °C below the $T_m$ of the primers used.

- Extension/elongation step: The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. We used a temperature of 72 C for 1min/Kb DNA target (for DyNAzyme™ II DNA polymerase ), or 15-30 sec/Kb DNA (for Phusion).
- Then a final elongation step at 72 °C for 5–10 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4 °C for an indefinite time may be employed for short-term storage of the reaction.

## MUTAGENESIS OF *GRX4, BUD32* AND *KAE1*

Mutagenesis of *GRX4, BUD32* and *KAE1* coding sequences, inserted in different vectors, were performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). In the table below are listed the oligonucleotides used, designed according to the instruction manual. With small letters are indicated the substituted bases. Controls of the correct mutagenesis were performed both by PCR, when allele-specific primers were available (see below), and by sequencing (BMR genomics service).

**OLIGOs FOR *GRX4***

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
|---|---|---|
| GRX4-S133-134A-S | GAAAGCAGCGGGgCTgCCGATGATGAAGAG | Mutagenesis of both residues of Grx4 (Ser-134 and Ser-133) that are phosphorylated by Bud32, in pYeDP and pET20 vectors |
| GRX4-S133-134A-AS | CTCTTCATCATCGGcAGcCCCGCTGCTTTC | |
| GRX4-S134A-S | AGCAGCGGGTCTaCCGATGATGAAGAGGAC | Substitution of the main serine phosphorylated by Bud32 with an unphosphorylatable residue, in the pYeDP vector |
| GRX4-S134A-AS | GTCCTCTTCATCATCGGtAGACCCGCTGCT | |

| | | |
|---|---|---|
| GRX4-S133A-S | GAAAGCAGCGGGaCTTCCGATGATGAAGAG | |
| GRX4-S133A-AS | CTCTTCATCATCGGAAGtCCCGCTGCTTTC | |
| GRX4-S134D-S | AGCAGCGGGTCTgaCGATGATGAAGAGGAC | Substitution of the Ser-134 with a residue that mimic a constitutively phosphorylated condition, in the pYeDP vector |
| GRX4-S134D-AS | GTGCTCTTCATCATCGtcAGACCCGCTGCT | |

**OLIGOs FOR *BUD32***

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
|---|---|---|
| K52A-S | GTATATTATCgcATATAGGCCACC | Mutagenesis of two residues important for the catalytic activity of Bud32 (see introduction), in the pET-28 vector, containing *KAE1/BUD32* coding sequences and in the pET-20 vector, containing only the *BUD32* coding sequence. |
| K52A-AS | GATAATATACgcTTGATGAGAATCC | |
| D161A-S | GTCATGGTGcgTTGACAAGTTC | |
| D161A-AS | GAACTTGTCAAcgCACCATGAC | |
| **OLIGO USED FOR THE CONTROL OF THE MUTAGENESIS:** | | |
| BUD K52 ASP | CATCAAAAGTATATTATCgc | Control of K52 mutation: it anneals with the mutagenized sequence |
| BUD wt ASP | CATCAAAAGTATATTATCaa | Control of K52 mutation: it anneals with the wt sequence |
| BUD32 D161 ASP | ATGACTACTGTCATGGTGcg | Control of D161 mutation: it anneals with the mutagenized sequence |
| BUD32 D161 ASP WT | ATGACTACTGTCATGGTGat | Control of D161 mutation: it anneals with |

| | | |
|---|---|---|
| | | the wt sequence |
| BUD32 S258A-AS | TCCTAGCATAGCTCTCTTACGACC | Antisense oligo used with the other 5 on top |

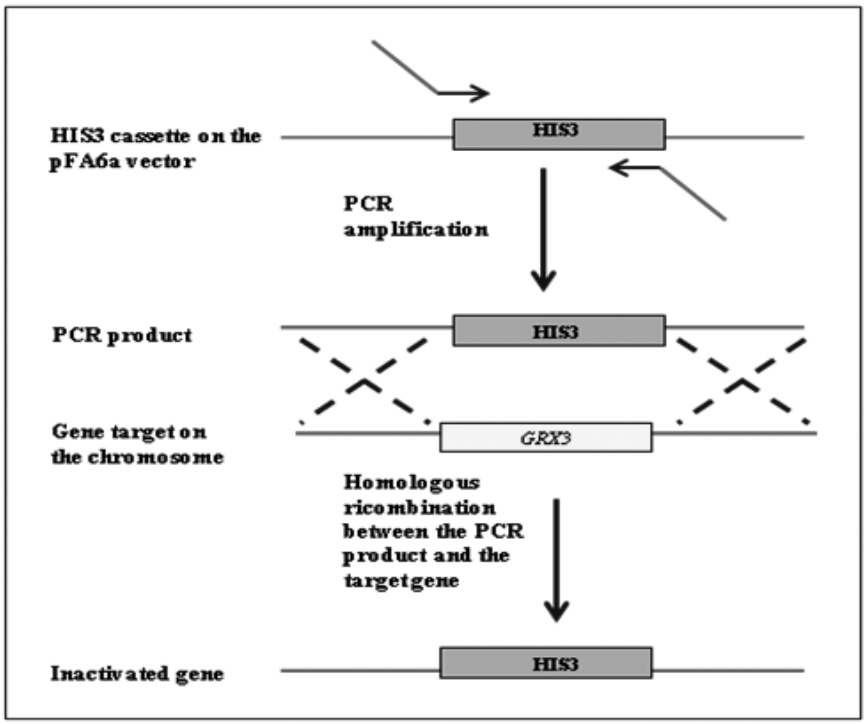
**OLIGOs FOR *KAE1***

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
|---|---|---|
| HHKAE AA-S | GGAGTAAACgcCTGCATTGGTgcCATCGAAATGG | Mutagenesis of the two histidines of Kae1 (H141 and H145), important for the metal ion binding (see introduction), in the pET-28 vector, containing *KAE1/BUD32* coding sequences. |
| HHKAE AA-AS | CCATTTCGATGgcACCAATGCAGgcGTTTACTCC | |

# CREATION OF THE STRAINS USED FOR THE PHENOTYPIC ANALYSIS OF THE EFFECT OF THE PHOSPHORYLATION ON Grx4 BY Bud32

For this analysis, we deleted both the *GRX4* and the *GRX3* coding sequences on yeast genome, as the two genes share overlapping functions. We then transformed the heterozygous diploid strain with galactose-inducible pYeDP plasmids, carrying a wild-type or a mutagenized version of *GRX4* (see on top).

Both genes were deleted in a PCR-mediated fashion, by amplification of either the *KAN::MX4* cassette (for the *GRX4* gene) or the *HIS3* cassette (for the *GRX3* gene) with specific primers that contained a sequence complementary to the plasmid (pFA6A, in which the cassette is inserted), and two protruding sequences complementary to the region in which the cassette has to be inserted in the genome (see explanatory figure for the *GRX3* gene, but the same occurred for the *GRX4* gene).




Primers used for the inactivation are listed in the table below. The PCR products were used for a high efficiency transformation of yeast diploid cells (see the method). We inactivated the *GRX3* gene in an already available heterozygous diploid $2n^{+/-}$ Δ*GRX4* strain, so cells were sprung in SD-HIS, after transformation. Transformants were then controlled by PCR, as the *GRX3* and the *HIS3* sequences have different lengths.

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
|---|---|---|
| GRX3-ALA 3 | TTAAGATTGGAGAGCATGCTGCA AAAAATCAGGGTCTTCCTCCAAGC ATAGGCACTAGTGGATCTG | Primers used for the inactivation of the *GRX3* gene |
| GRX3-ALA 5 | ATGTGTTCTTTTCAGGTTCCATCT GCATTTTCTTTTAACTACACCCAG CTGAAGCTTCGTACGC | |
| GRX3-Prom-S | ACTGGTTACTTCCTAAGACTG | Primers used for the control of the inactivation |
| GRX3-Prom-AS | TTTCTCCGGGCAATGGTTGTT | |

A one-step transformation (see the method) with the pYeDP vector was carried out on that clones that had both the genes inactivated and cells were sprung on SD-URA. Heterozygous transformed diploids were then subjected to tetrad dissection.

# TETRAD DISSECTION

Heterozygous diploid strains were allowed to sporulate on 1% potassium acetate solid medium for 4-6 days at 28 °C. After the addiction of zymoliase, about 10 tetrads/diploid strains were dissected and the spores allowed to germinate on YPD medium at 28 °C. For each spore, the presence of a determined cassette/phenotype was controlled by growth on selective media.
The deletion of *GRX4* gene was thus controlled by growth on YPD+ G-418, the deletion of *GRX3* gene was controlled by growth on SD-HIS and the presence of the pYeDP vector was controlled by growth on SD-URA.

# EPITOPE-TAGGING OF *KAE1* AND *AFT1*

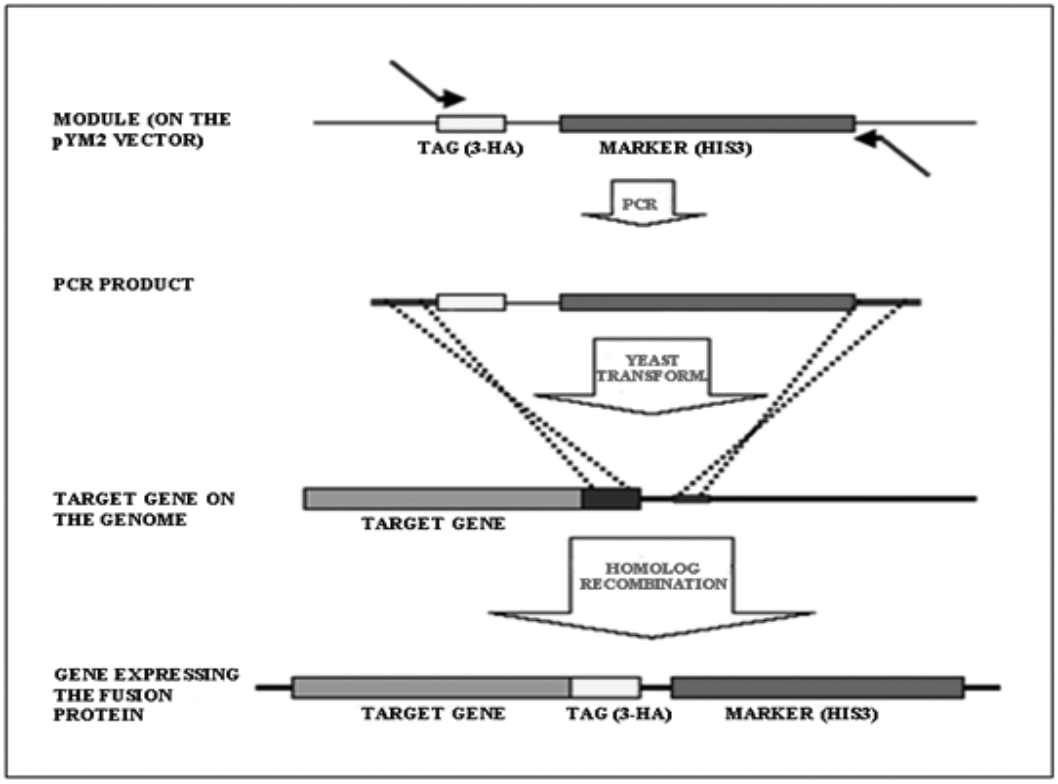
(Knop et al., Yeast, 1999)

To add a C-terminal tag on these two genes, we used a protocol described in Knop et al. (1999). Amplification of the tag from the pYM2 vector is carried out using specific primers that contain a sequence complementary to the plasmid (and that is the same for all pYM vectors, allowing the insertion of different tags at one locus, using a unique pair of primers), and two protruding sequences complementary to the region in which the tag has to be inserted in the genome. The oligonucleotides used for *KAE1* and *AFT1* are listed in the table below: bases that are corresponding to a sequence on the vector are underlined.

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
|---|---|---|
| YKR9MYC-S | ACGCAGAAATTCAGAACCGATGAA GTATACGCAGCCTGGCGTGGCGTG ATCGTACGCTGCAGGTCGAC | Primers used for insertion of the HA epitope on the *KAE1* gene |
| YKR9MYC-AS | TATATATTCGTAGTGTGAACATTAG GCTAACTGTAACAAAAATCGATCG | |

| | | |
|---|---|---|
| | ATGAATTCGAGCTCG | |
| AFT1-pYM12-S | ATTAAAATGGTGAACGGCGAGTTG AAGTATGTGAAGCCAGAAGATCGT ACGCTGCAGGTCGAC | Primers used for insertion of the HA epitope on the *AFT1* gene |
| AFT1-pYM12-AS | TGAAAATGGACGAGAGATACGTCT AAGTTTGATTTCATCTATATGATCG ATGAATTCGAGCTCG | |

The PCR products were used for high efficiency transformations of yeast diploid cells. In the figure below the method is explained: the PCR products contains flanking homologous sequences that allows their targeted integration into the genome of yeast cells.




Transformants were then controlled for the presence of the epitope by PCR, using the primers listed below.

| **OLIGO USED FOR THE CONTROL OF THE EPITOPE-TAGGING:** | | |
|---|---|---|
| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
| KAN&HISPRIMER | TGGGCCTCCATGTCGCTGG | PRIMER REV USED FOR BOTH *KAE1* AND *AFT1*; IT ANNEALS AT +427 BP FROM THE 5' POSITION OF INTEGRATION |
| YKR PROM-S | GGATCCGATAATGGTTGATTCTT ATTTAGAC | Primer fwd for the control of the integration in *KAE1*. It anneals at 366 bp upstream of ATG |
| AFT1-1814-S | TGTTGACCATCGTCATCTCA | Primer fwd for the control of the integration in *AFT1*. It anneals at 1841 position in the ORF of *AFT1* |

# PREPARATION OF YEAST RNA BY EXTRACTION WITH HOT ACID PHENOL

(John Wiley & Sons, Current Protocols in Molecular Biology, 1995)

Grow yeast cells in 10 ml of desired medium to mid-exponential phase ($OD_{600}$=1.0).
Transfer culture to 50-ml centrifuge tube and centrifuge cells 3 min at 7000 RPM, 4°C.
Discard supernatant, resuspend pellet in 1 ml ice-cold water. Transfer to a clean 1.5-ml microcentrifuge tube. Microcentrifuge 10 sec at 4°C, and remove supernatant. Proceed to step 4 or if desired immediately freeze pellet by placing tube in dry ice. The frozen pellet can be stored for months at -80°C.
Resuspend cell pellet in 400 µl TES solution (10 mM Tris/HCl, pH 7.5; 10 mM EDTA pH 8; 0.5% SDS). Add 400 µl acid phenol (pH 5) and vortex vigorously 10 sec. Incubate 20 to 60 min at 65 °C with occasional, brief vortexing.
Place on ice 5 min. Microcentrifuge 10 min at 13000 rpm, 4°C.

Transfer aqueous (top) phase to a clean 1.5-ml microcentrifuge tube, add 400 µl acid phenol and vortex vigorously. Incubate 15 min at 65 °C with occasional, brief vortexing. Repeat step 5.

Transfer aqueous phase to a clean 1.5-ml microcentrifuge tube and add 400 µl chloroform:isoamyl alcool (24:1). Vortex vigorously and microcentrifuge 15 min at 13000 rpm, 4°C.

Transfer aqueous phase to a new tube, add 40 µl of 3M sodium acetate, pH 5.3, and 1 ml of ice-cold 100% ethanol and precipitate 1 hr (or overnight), -80°C. Microcentrifuge 15 min at 13000 rpm, 4°C. Wash RNA pellet by vortexing briefly in ice-cold 70% ethanol. Microcentrifuge as before to pellet RNA.

Dry the pellet and resuspend in 25-40 µl DEPC-treated $H_2O$. Determine the concentration spectrophotometrically by measuring the $A_{260}$ and $A_{280}$. Store at -80 °C, or at -20 °C if it is to be used within 1 year.

## ANALYSIS OF *GAL1* TRANSCRIPTION

Yeast cells were grown to $OD_{600}$ 0.4 in Raffinose-containing medium and then shifted to galactose-containing medium to activate the *GAL* regulon. Samples were taken after 30 min of induction and RNAs were prepared as previously described and subjected to Northern Blotting.

## ANALYSIS OF *FET3/FIT3* TRANSCRIPTION

Yeast cells were grown in SD medium until $OD_{600}$ 0.6 and then Bathophenanthroline disulfonic acid (BPS, Sigma) was added, at the final concentration of 0.2 mM or 0.1 mM, for 30 minutes to 1 hour. BPS is a chelating reagent, that forms complexes with various metal ions, particularly, Fe(II). As a result, levels of iron within cell decrease and starts the activation of iron-sensing genes.

# REAL-TIME PCR

Reverse transcription (RT) followed by the polymerase chain reaction (PCR) is the technique of choice to analyze mRNA expression derived from various sources. Real-time RT–PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression. Moreover, it is easy to perform, provides the necessary accuracy and produces reliable as well as rapid quantification results. The simplest detection technique for newly synthesized PCR products in real-time PCR uses SYBR Green I fluorescence dye that binds specifically to the minor groove double-stranded DNA. The quantification method of choice depends on the target sequence, the expected range of mRNA amount present in the tissue, the degree of accuracy required and whether quantification needs to be relative or absolute. Two different methods of analyzing data from real-time, quantitative PCR exist: absolute quantification and relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study. Furthermore, a normalization of the target gene with an endogenous standard (mainly non-regulated reference genes or housekeeping genes like G3PDH or GAPDH, albumin, actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA) is recommended. In our study, we used a mathematical method for relative quantification, as described from Pfaffl in 2001 [65]. It is necessary to determine the cycle threshold (Ct) for each transcript, that is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative expression ratio (*R*) of a target gene is calculated based on *E* and the Ct deviation of an unknown sample versus a control, and expressed in comparison to a reference gene:

$$Ratio = \frac{\left(E_{target}\right)^{\Delta Ct(Control\ -sample\ )}}{\left(E_{reference}\right)^{\Delta Ct(Control\ -sample\ )}}$$

Where $E_{\text{target}}$ is the real-time PCR efficiency of target gene transcript; $E_{\text{reference}}$ is the real-time PCR efficiency of a reference gene transcript; $\Delta Ct_{\text{target}}$ is the Ct deviation of control –

sample of the target gene transcript; $\Delta Ct_{ref}$ = Ct deviation of control – sample of reference gene transcript.

As a reference gene we used actin.

For mRNA-based PCR, the RNA sample is first reverse transcribed to cDNA with reverse transcriptase. To do retro-transcription, we use a commercial kit (SuperScript™ III First-Strand Synthesis System for RT-PCR, Invitrogen) and oligo-dT to amplify total mRNAs.

For the real-time reaction, we used a Corbett Research Rotor-Gene™ instrument and the following parameters: 95°C for 2 min, then 40 cycles of: 95°C for 15 sec and 60°C for 30 sec.

Every reaction contains:

- 25 μl Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen)
- 1 μl Forward primer 10 μM
- 1 μl Reverse primer 10 μM
- ≤ 10 μl Template (cDNA generated from 10 pg to 1 μg of total RNA)
- DEPC-treated $H_2O$ up to 50 μl

# NORTHERN BLOT

The northern blot is a technique used to study gene expression by detection of RNA (or isolated mRNA) in a sample.

Total RNA extracted from yeast cells are separated by gel electrophoresis. The gel contains 1% agarose, 1X MOPS buffer (0.2 M MOPS, pH 7; 80 mM NaAc; 10 mM EDTA, pH 8) and 6% formaldehyde as a denaturing agent for the RNA to limit secondary structure. Sample are prepared as following:

- RNA (10 μg) and DEPC-treated $H_2O$ to 5 μl
- 15 μl MIX (50% formamide, that lowers the annealing temperature of the probe-RNA interaction preventing RNA degradation by high temperatures; 6% formaldehyde; 1X MOPS)

Samples are then denatured at 65 °C for 10 min and held in ice for 2 min. Before loading gel, 2 μl of 10X loading gel solution are added. Gel electrophoresis is carried out overnight at 20 V.

The gel can be stained with ethidium bromide (EtBr) and viewed under UV light to observe the quality and quantity of RNA before blotting. Since the large ribosomal subunit is 28S (approximately 5kb) and the small ribosomal subunit is 18S (approximately 2kb) two prominent bands will appear on the gel, the larger at close to twice the intensity of the smaller. After that, the gel is washed twice in sterile $H_2O$, for 10 min, to eliminate formaldehyde.

The RNA samples, now separated by size, are transferred to a nylon membrane through a capillary blotting system. We use a nylon membrane with a positive charge (Hybond™-$N^+$, Amersham), and SSC 20X (3 M NaCl, 0.3 M Sodium citrate, pH 7) as blotting buffer. The capillary transfer takes about 20 h (overnight).

Once the RNA has been transferred to the membrane it is immobilized through covalent linkage to the membrane by UV light, for 5 min.

At this point the membrane can be hybridized with the probe or stored at 4 °C.

## DETECTION USING RADIOLABELED PROBES:

The membrane is first pre-hybridated at 42 °C for 30 min in the ULTRAhyb hybridization buffer (Ambion). The labeled probe is then denatured at 100 °C for 3 min and then added to the tube and let overnight (14-24 h) in hybridization at 42 °C.

After that, the hybridization solution can then be recovered (if reused within few days) and the membrane is washed twice for 5 min in SSC 2X, SDS 0.1% and twice for 15 min in SSC 0.1X, SDS 0.1%. The membrane is left to dry and exposed to autoradiography for about 1 h, then scanned using the Cyclone apparatus (Packard).

## DETECTION USING NON-RADIOACTIVE PROBES:

When we used non-radioactive probes, the transfer of the gel was performed on nitrocellulose membrane.

The membrane is first pre-hybridated at 50 °C for 30 min in 30 ml of pre-hybridation solution (5X SSC, 0.5% SDS, 1 mM EDTA pH 8). Discard the pre-hybridation solution and add 10 ml (or more) of hybridation solution (0.75 M NaCl, 0.15 M Tris/HCl pH8, 10 mM EDTA, 0.1 M phosphate buffer pH 6.8, 0.1% Sodium pyrophosphate, 0.1% SDS, 0.2% PVP, 0.2% Ficoll 400, 0.2% BSA) plus 50 µl of boiling-denatured ss-DNA (10 mg/ml) and incubate at 65 °C for at least 2 hours. At that time, denature the probe for 10 min at 100 °C and add it to the membrane. Let it incubate overnight at 65 °C.

After that, wash the membrane twice for 5 min in washing buffer A (SSC 2X, SDS 0.1%) and twice for 5 min in washing buffer B (SSC 0.5X, SDS 0.1%, at 65 °C). A final wash with washing buffer C (100 mM maleic acid, 150 mM NaCl, 0.3% tween 20) for 5 min at room temperature. Saturate the membrane is with saturation buffer (100 mM maleic acid pH 7.5, 150 mM NaCl, 3% BSA) for 30 min, and after that add the anti-digoxigenin antibody (1:10000) and let 30 min or more. Then wash twice for 5 min in washing buffer C. At this point, equilibrate the membrane for 2 min with the alkaline phosphatase buffer (AP) (0.1 M Tris/HCl pH9.5, 0.1 M NaCl, 5 mM $MgCl_2$) and incubate in the dark without shaking with the revelation solution: 10 ml of AP buffer with 1 tablet of NBT/BCIP (Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate, Roche) until some signal appears. BCIP is the AP substrate, which after dephosphorylation is oxidized by NBT to yield a dark-blue indigo precipitating dye. NBT is thereby reduced to a dark-blue precipitating dye and serves to intensify the color reaction making the detection more sensitive.

# PREPARATION OF RADIOLABELED PROBES FOR NORTHERN AND SOUTHERN BLOTTING

As a detecting system, we use radiolabeled probes, made of PCR-amplified target DNA. The probe is prepared accordingly to the DECAprime™ II *Random Primed DNA Labeling Kit* (Ambion). The reaction is performed using random decamer oligonucleotide primers and exonuclease-free klenow enzyme.

The probe is then purified using NucAway™ Spin Columns (Ambion), that are useful to recover the labeled DNA while removing salts and unincorporated nucleotides.

In the table below are listed the oligonucleotides used to amplify the target DNA, from its chromosomal locus.

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | **DESCRIPTION** |
|---|---|---|
| GAL1-3 S | CTGCAAATGTTTTAGCTGCCACGTA | Primers used to amplify the *GAL1* gene |
| GAL1-3 AS | CATCTTTGTTAACCGTTCGATGCC | |
| ACT1-cDNA-S | ACCATGTTCCCAGGTATTGCC | Primers used to amplify the *ACT1* gene |
| ACT1-cDNA-AS | ACTTGTGGTGAACGATAGATG | |

# PREPARATION OF NON-RADIOACTIVE PROBES FOR NORTHERN BLOTTING

The analysis of the iron regulon transcription by northern blotting were performed using random-priming labeled probes. The Klenow enzyme copies DNA template in the presence of hexameric primers and alkali-labile Digoxigenin-11-2'-deoxy-uridine-5'triphosphate. On average, the enzyme inserts one DIG moiety in every stretch of 20-25 nucleotides. The resulting labeled product is a homogeneously labeled, sensitive hybridization probe.

*FIT3*, *FET3* and *ACT1* genes were first PCR-amplified with oligonucleotides listed in the previous and following tables.

| | | |
|---|---|---|
| FIT3-cDNA-S | TGTCTGGACTGGTGAAGGCAG | Primers used to amplify the *FIT3* gene |
| FIT3-cDNA-AS | ATCAAACCAGTACCAGCGGTG | |
| FET3-cDNA-S | ATGACATTCTCCTGCTTTGCC | Primers used to amplify the *FET3* gene |
| FET3-cDNA-AS | TCAGTGGAATGACGATCTTCG | |

About 1 μg of template DNA (in sterile water, for a final volume of 15 μl) was boiled for 2 min and then put in ice for 2 min. Then we added:

2 μl of Hexanucleotide Mix 10 X(Roche), 2 μl of DIG DNA Labeling Mix 10 X(Roche), and 1 μl of DNA polymerase I large (Klenow) fragment; then the reaction was left for 2 hr/overnight at 37 °C. To stop the reaction, the day after we added 2 μl EDTA 0.5 M pH 8 and 2 μl LiAc 4 M.

The DNA was then precipitated, by adding 70 μl 100% Ethanol and left at -80 °C for about 30 min. We then centrifuged for 20 min at 13000 rpm, at 4 °C, discarded flow-through and repeated with70% ethanol. The DNA pellet was then left to dry and resuspended with 50 μl of sterile $H_2O$.

## SOUTHERN BLOT ANALYSIS OF TELOMERE LENGTH

Yeast strains were grown in YPDA to exponential phase ($OD_{600}$ 0.8-1.0) and cells were collected. Genomic DNAs have been purified from yeast cells as previously described and approximately 50 μg were digested with XhoI and separated on 1.2% agarose gel. After transfer onto Hybond™-$N^+$ membrane (Amersham), filters have been hybridized with a specific radiolabeled probe for telomeric Y'-$TG_{1-3}$ repeats.

# PREPARATION OF THE PROBE FOR NUCLEASE ASSAY

To detect the possible nuclease activity of Kae1, we use radiolabeled probes. The probes are 40-50 bp oligonucleotides (listed below) that are labeled at their 5'terminus by polynucleotide kinase (PNK). The reaction volume was 10 μl and contained:

- 2 μl of 10 μM oligonucleotides
- 1 μl PNK (BioLabs)
- 1 μl of 10X PNK buffer
- 1 μl of fresh [$^{32}$P] ATP

The reaction was incubated at 37 °C for 1 hr and then PNK was heat-inactivated for 20 min at 65 °C. 40 μl of sterile $H_2O$ were added to the mix and then the probe was purified using NucAway™ Spin Columns (Ambion). A further purification with phenol/chloroform was performed. After ethanol precipitation, the probe was finally resuspended in 50 μl of sterile $H_2O$.

| **OLIGONUCLEOTIDE** | **5'-3' SEQUENCE** | DESCRIPTION |
|---|---|---|
| Tel-probe1-S | TGTGGGTGTGGGTGTGGGTGTGGGTGTG GGTGTGGGTGTGGG | Primer that binds to the $TG_{1-3}$ repeats |
| Tel-probe1-AS | CCCACACCCACACCCACACCCACACCCA CACCCACACCCACA | Primer that binds to the complementary AC repeats |

# NUCLEASE ASSAY

The possible nuclease activity of Kae1 was tested incubating the coexpressed Kae1/Bud32$_{6HIS}$ proteins (increasing amounts: 0.2-2 μg) at 37 °C for different times (10 to 30 min) in a 1X buffer (20mM Tris/HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT), with a dilution of the radiolabeled probe (prepared as described before). The

reaction contained also 10 mM cold ATP. The presence or not of cold ATP, different divalent cations ($MgCl_2$, $ZnSO_4$, $FeSO_4$, $CaCl_2$ or no ions) and EDTA (to chelate them) was tested.

## PURIFICATION OF PROTEINS FROM *E. coli* CELLS

To overexpress wild-type and mutant forms of Bud32, Grx4, Kae1/Bud32 we used the pET-20 or the pET-28 vectors, described before. BL21 *E.coli* strains were transformed with the plasmid that contained the desired sequence and clones were grown in LBA medium until cellular density reached an $OD_{600}$ of about 0.5. At that point, the expression of the protein was induced by adding isopropil β-D-1-tiogalattopiranoside (IPTG) to the medium, at the final concentration of 0.5 mM, and cells were left to grow overnight at 16 °C.

We usually do a trial scale purification, on 1 ml of culture, that is left in IPTG at 37 °C for about 3 hr before attempting a large-scale purification, to determine if the standard operating conditions will work for the recombinant protein of interest.

Bacteria were then harvested and resuspended in 5 ml/g of pellet of purification buffer (50 mM Tris/HCl pH 7.5, 0.3 M NaCl, 10% glycerol, 0.2 mM PMSF, 5 mM β-mercaptoethanol, 0.1% TRITON X-100 and 2 mM imidazole). Purification was performed according to the protocol of the manufacturer by an affinity column containing HIS-Select™ Nickel Affinity Gel (SIGMA). The proteins were eluted with 100 mM imidazole.

All the step of purification are controlled in SDS-PAGE: 16 μl of each sample are mixed with 4 μl of 5X *Laemmli Buffer* (that reaches the 1X concentration: 60 mM Tris/HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol or 100 mM DTT, 10% glycerol, bromophenol blue), boiled for 5 min and loaded on 12% SDS-PAGE. The gel is then stained with coomassie blue or blotted to a nitrocellulose membrane.

All protein concentrations were determined both by coomassie-gel staining and by the Bradford method using BSA as a standard.

# SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE is a technique to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). The solution of proteins to be analyzed is mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Heating the samples to at least 60 degrees C shakes up the molecules, helping SDS to bind.

A tracking dye may be added to the protein solution (of a size smaller than protein) to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

The gels generally consist of acrylamide, bisacrylamide, SDS, and a Tris-Cl buffer with adjusted pH. Ammonium persulfate (an initiator for gel formation) and TEMED (that catalyze the polymerization of acrylamide) are added when the gel is ready to be polymerized. Gels are formed by 2 parts: the separating or resolving gel and the stacking or loadinggel. The former is usually more basic (is prepared with Tris/HCl at pH 8.8) and has a higher polyacrylamide content than the loading gel. The stacking gel is a large pore and is prepared with Tris/HCl buffer pH 6.8 of about 2 pH units lower than that of electrophoresis buffer (Tris/Glycine).

The gel is actually formed because the acrylamide solution contains a small amount, generally about 1 part in 35 of bisacrylamide, which can form cross-links between two polyacrylamide molecules. The ratio of acrylamide to bisacrylamide can be varied for special purposes. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins.

Gels are polymerized in a gel caster. First the separating gel is poured and allowed to polymerize. Next a thin layer of isopropanol or 0.1% SDS is added. Next the loading gel is poured and a comb is placed to create the wells. After the loading gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

The denatured sample proteins are added to the wells one end of the gel with a syringe or pipette. Finally, the apparatus is hooked up to a power source under appropriate running conditions to separate the protein bands.

An electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the anode. Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty (they encounter more resistance). After a set amount of time (usually 1 hour at 120 V), the proteins will have differentially migrated based on their size; smaller proteins will have traveled farther down the gel, while larger ones will have remained closer to the point of origin. Following electrophoresis, the gel may be stained with Coomassie Brilliant Blue R-250, allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different proteins will appear as distinct bands within the gel.

For coomassie brilliant blue R-250 staining, proteins are simultaneously fixed with methanol:glacial acetic acid (45% $H_2O$, 45% methanol and 10% glacial acetic acid) and stained with coomassie brilliant blue R-250 (0.25 g for 100 ml solution). The gel is immersed for several hours in the dye, and excess dye is then allowed to diffuse from the gel during a prolonged period of destaining (in methanol:glacial acetic acid solution, without the dye).

## WESTERN BLOT

In order to make the proteins accessible to antibody detection, after SDS-PAGE, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The blotting buffer is composed of 25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS. Proteins are transferred through electroblotting, that uses an electric current to pull proteins from the gel into the nitrocellulose membrane. Blotting takes about 1 hr at the constant current of 350 mA.

The proteins move while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection.

Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.

Since the membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein (since the antibody is a protein itself). Blocking of non-specific binding is achieved by placing the membrane for 1 hr in a solution of 3% Bovine serum albumin (BSA) in Tris-buffered saline (TBS, 1X: 50 mM Tris/HCl pH 7.5, 150 mM NaCl) with a minute percentage of detergent such as Tween 20, 0.1%. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached.

After blocking, a dilute solution of primary antibody (in TBST/BSA 1%) is incubated with the membrane under gentle agitation, for 1-2 hr at room temperature, or overnight at 4 °C.

The membrane is then washed three times for 10 min with TBS+Tween 0.1% and then exposed to another antibody, directed at a species-specific portion of the primary antibody (secondary antibody). Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. After the secondary antibody, the membrane is washed three more times, as described before and then revealed using the Kodak (NEN) instrument, after treatment with the development solution that contains $H_2O_2$. Exposition time varies according to the substrate, from 30 sec to 5 min about.

# PHOSPHOTRANSFERASE ASSAY

The protein kinase activity of Bud32 was assayed by incubating the recombinant His-tagged protein (about 100 ng) at 37 °C for 15 min in 20 µl of medium containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 62.5 µM "cold" ATP and 25 µM [$\gamma$-$^{33}$P]ATP (Amersham Biosciences; specific radioactivity, 2000–3000 c.p.m./pmol) and the Grx4 (about 400 ng) or the Kae1/Bud32 coexpressed proteins as phosphorylatable substrates. The reaction was stopped by adding 5 µl of the gel electrophoresis loading buffer 5X (*Laemmli buffer*, Tris-HCl 50 mM pH 6.8, 2% SDS, 1% β-mercaptoethanol and coomassie blue staining) and

samples were subjected to SDS/PAGE (12% gels). Gel was then blotted and the membrane scanned using the Cyclone apparatus (Packard), after an overnight exposition. The scanned membranes were further analyzed by revelation with specific antibodies to verify presence and amount of the proteins.

To measure the level of Kae1 phosphorylation, I performed the kinase assay incubating the Kae1/Bud32 coexpressed protein with an additional amount of the kinase, for 30 minutes (that proved to be the optimal condition for Kae1 phosphorylation). Samples were then subjected to SDS/PAGE and the gel stained with Coomassie blue. The band corresponding to Kae1 was cut out and radioactivity measured using the Liquid Scintillation Analyzer (Packard). Then, for MS analysis, I repeated the same procedure, using only cold ATP. In order to have a sufficient amount of Kae1 protein, 10 gel lanes band were cut. MS analysis was performed by Dott.G.Arrigoni (Univ. of Padua).

# PROTEIN EXTRACTION FROM YEAST

Inoculate 2-5 ml of liquid YPD and incubate with shaking overnight at 30°C.
Count o/n culture and inoculate 100 ml of warm YPD to an $OD_{600}$=0.2.
Incubate the culture at 30°C on a shaker at 200 rpm until the absorbance reaches $OD_{600}$=0.7-0.8. Pellet cells at 4000 rpm for 3min, at 4 ºC. Dump supernatant.
Resuspend in 1ml sterile $H_2O$. Transfer to a 2-ml microcentrifuge tube (proper for the MagNA Lyser Instrument, Roche) and pellet cells; dump supernatant.
Add an equal amount of glass beads and 1 ml of ice-cold buffer: 50 mM Tris/HCl, pH 8, 150 mM NaCl, 1 mM PMSF, 0.2 mM NaOV, 10 mM NaF, 0.1% Nonidet. Shake each tube in the MagNA Lyser for 30 sec at 6000 rpm and repeat 4 time, with 1 min in ice.
Centrifuge at 13000 rpm for 15 min at 4 ºC. Save the supernatant and centrifuge again.
Do protein concentration assay (Bradford; Bradford M.M., 1976).

# PROTEIN IMMUNOPRECIPITATION

For the immunoprecipitation (IP) use about 500 ng of total proteins and incubated them for 2 hr in rotation at 4 °C with 10 µl of a resin, covalently linked to the anti-HA antibody (HA.11 Monoclonal Antibody, Affinity Matrix, COVANCE).

Centrifuge for 3 min at 2000 rpm at 4 °C, discard supernatant and wash twice with 900 µl of 50 mM Tris/HCl pH 7.5, 500 mM NaCl. Centrifuge again, discard supernatant and wash with 900 µl of 50 mM Tris/HCl pH 7.5 (without salt).

You can use the IP directly to do an *in vitro* phosphorylation assay (see method before). In this case, reaction volume is higher (40 µl, as the resin itself is 10 µl), and we used $MgCl_2$, instead of Mn $Cl_2$.

# ANTI-HA IMMUNOCYTOLOGY

Cells are grown in 5 ml of YPD to an $O.D._{600}$ of 0.75 to 1.

To fix, 1/10 volume of 37% formaldehyde is added to the culture, which is shaken for a further 40 minutes at room temperature. Cells are then recovered by centrifugation at 1400 X g for 2 minutes, and washed (i.e. gently resuspended then recovered and the supernatant discarded) twice with Solution A (1.2 M sorbitol, 50 mM $KPO_4$, pH 7).

To spheroplast, cells are resuspended in 500 ul of Solution A containing 0.1% β-mercaptoethanol and 10 µg/ml zymolyase. The suspension is incubated at 37°C without shaking and checked periodically. As soon as the settled cell pellet loses its creamy, yellow colour and becomes translucent (30 to 45 min), cells should be recovered and washed twice with Solution A.

Cells are resuspended in 200 ul of solution A. A drop is placed on a poly-L-lysine coated slide and allowed to sit for 10 minutes. Excess solution is aspirated. The adhered cells are covered with PBS (150 mM NaCl, 50 mM $NaPO_4$, pH 7.4) plus 0.1% BSA. The solution is allowed to sit for 5 minutes before aspiration. This wash is repeated twice with PBS/0.1% BSA containing 0.1% NP40.

The cells are covered with PBS/0.1% BSA containing the primary antibody (anti-HA-mouse monoclonal Sigma, 1:1000). The slide is set on wet paper towels in a sealed chamber and incubated at 4$^{o}$C overnight.

Excess solution is aspirated. The cells are covered with PBS/0.1% BSA, which is allowed to sit for 5 minutes before aspiration. This wash is repeated with PBS/0.1% BSA containing 0.1% NP40, then again with PBS/0.1% BSA.

The cells are covered with PBS/0.1% BSA containing the secondary antibody (Cy3-conjugated anti-mouse IgG, Sigma). The slide is set on wet paper towels in a sealed chamber and incubated at room temperature for 2 hours.

Excess solution is aspirated. The cells are covered with PBS/0.1% BSA, which is let sit for 5 minutes before aspiration. This wash is repeated with PBS/0.1% BSA containing 0.1% NP40, then again with PBS/0.1% BSA.

Mount solution (70% glycerol containing 2% n-propyl gallate and 0.25 ug/ml Hoechst) is placed on the cells. A coverslip is placed and sealed with nail polish. Slides are stored at -20$^{o}$C until examination.

Samples are visualized with a fluorescence microscope (Leica) and images are processed with Adobe Photoshop.

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