Molecular genetic and biochemical interaction studies of the transcriptional activator Gal4 and its repressor Gal80 in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

D i s s e r t a t i o n

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

vorgelegt der

Naturwissenschaftlichen Fakultät I
Biowissenschaften
der Martin-Luther-Universität Halle-Wittenberg

von

Frau Constance Zugowski

geb. am 03.07.1982 in Lichtenstein (Sachsen)

Gutachter /in

1. Prof. Dr. K. Breunig, Institut für Biologie, MLU Halle-Wittenberg
2. PD Dr. H. Lilie, Institut für Biochemie und Biotechnologie, MLU Halle-Wittenberg
3. PD Dr. K. Melcher, Van Andel Institute, Grand Rapids (Michigan), USA

Tag der öffentlichen Verteidigung: 24.01.2013

Contents

List of Figures

List of Tables

Abbreviations

AA	amino acid
A_{280}	extinction at 280 nm
AD	activation domain
ATP	adenosine triphosphate
bp	basepair
BSA	Bovine serum albumine
ChIP	chromatin immunoprecipitation
C-terminal	at the Carboxy-terminus of a protein
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GSH	reduced glutathione
GST	glutathione S-transferase
His_6-tag	hexahistidine-tag
kb	kilobases
kD	kilodalton
MW	molecular weight
NADP	nicotinamide adenine dinucleotide phosphate
Ni-NTA	nickel-nitrilotriacetic acid
N-terminal	at the Amino-terminus of a protein
OD_{600}	optical density at 600 nm
o/n	over night
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PCR	polmerase chain reaction
PTM	posttranslational modification
rpm	revolutions per minute
sc-medium	synthetic complete medium
U	Units (enzyme activity)
UAS	upstream activation sequence
v/v	volume per volume
w/v	weight per volume

1 Introduction

1.1 Regulation of *GAL/LAC* gene expression in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

The mechanisms underlying glucose and galactose utilization in the baker's yeast *Saccharomyces cerevisiae* and the milk yeast *Kluyveromyces lactis* are subject of extensive research in molecular biology. Today the so called "*GAL/LAC* switch", which describes the regulation of the genes required for galactose respectively lactose metabolism, is one of the most intensely studied transcriptional switches. The findings gained from analysis of the *GAL/LAC*-regulon are fundamental for the understanding of gene regulation by activators and repressors, and although genetic and structural data are accumulating, there are still open questions.

1.1.1 Metabolism of galactose – the enzymes of the Leloir pathway

The central carbohydrate in cell metabolism is the monosaccharide glucose, and although glucose and galactose have a very similar structure, the enzymes involved in glucose utilization are so specific for their substrate, that galactose has to be converted into glucose-6-phosphate before it can enter the glycolytic pathway. Therefore several enzymatic steps (referred to as the Leloir pathway, reviewed by Frey 1996) are necessary. The enzymes of the Leloir pathway, depicted in figure 1, are conserved between *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *Saccharomyces cerevisiae* usually finds galactose in the form of the monosaccharide or the disaccharide melibiose, which is cleaved extracellularly by the secreted α-galactosidase Mel1. Galactose enters the cell via the high affinity galactose permease Gal2. In its natural habitat *Kluyveromyces lactis* finds galactose predominantly in the form of lactose, which enters the cell through the Lac12 permease. Lactose is cleaved by the intracellular β-galactosidase Lac4. In both yeasts, intracellular galactose is phosphorylated by the galactokinase Gal1. The galactose-1-phosphate uridyltransferase Gal7 thereupon converts the resulting galactose-1-phosphate and UDP-glucose into glucose-1-phosphate and UDP-galactose. The UDP-galactose 4-epimerase Gal10 regenerates UDP-glucose from UDP-galactose through stereochemical modification at the C4 atom. In the final step glucose-1-phosphate is isomerized to glucose-6-phosphate by the phosphoglucomutase Gal5.

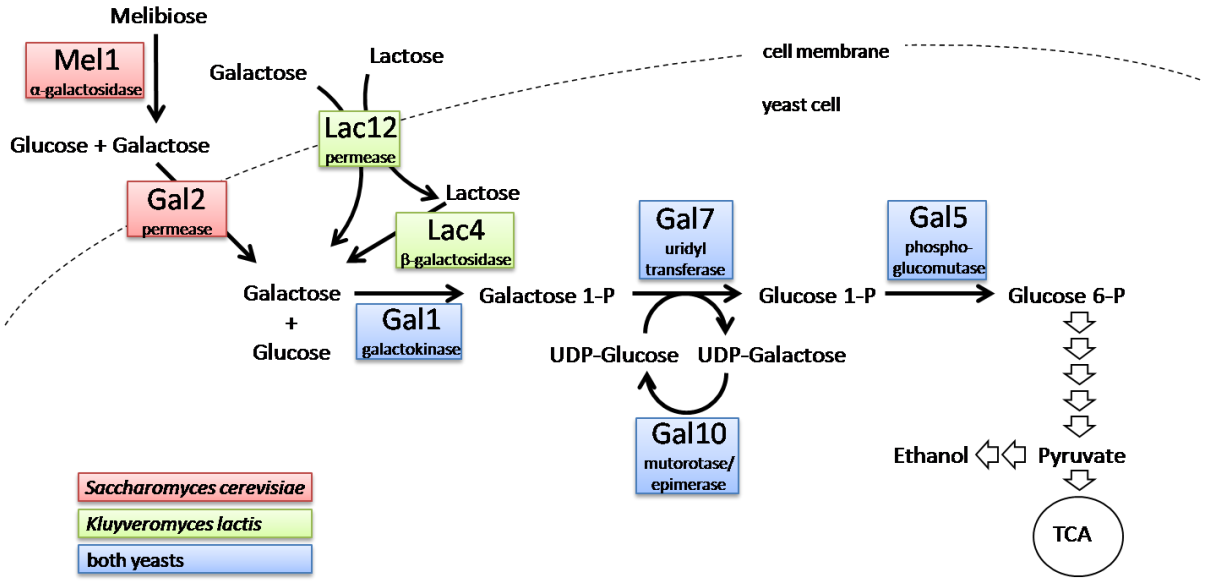

Figure 1: Galactose metabolism in yeast. Schematic view. The enzymes of the Leloir pathway are conserved in both yeasts and are depicted in blue.

1.1.2 Control of gene expression in the Leloir pathway

The expression of the galactose utilizing enzymes is conducted by the activity of three regulatory proteins, which are also conserved between the two yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. The transcriptional activator Gal4 binds to 17 basepair sequences (UAS_{GAL}, CGG-N_{11}–CCG) in the promoters of galactose regulated genes (Giniger et al. 1985; Halvorsen et al. 1990; Bram et al. 1986; Vashee et al. 1993). In the absence of galactose, the activity of Gal4 is repressed by binding of the repressor protein Gal80 to the transcriptional activation domain (AD) of Gal4 (Ma & Ptashne 1987b). Upon galactose induction, the galactose sensor protein Gal3 in *S. cerevisiae*/*Kl*Gal1 in *K. lactis* binds to one molecule of galactose and ATP and adopts a conformation with higher affinity to Gal80 (Sellick et al. 2009; Lavy et al. 2012). The competition between Gal3 and Gal4 for the binding to Gal80 relieves the inhibition of the AD by Gal80 so that general transcription and chromatin remodeling factors can be recruited to the promoter (Keegan et al. 1986; Johnston 1987; Ma & Ptashne 1987). There are three states of induction described for the *GAL* regulatory system: the glucose repressed state, the noninduced state and the induced state. These states are summarized in the following.

Since glucose is preferred to galactose utilization and the galactose switch is associated with the synthesis of the Leloir enzymes, this pathway is repressed in the presence of glucose by the Cys_2His_2-zinc finger protein Mig1, which binds to promoters upstream of glucose repressed genes (Nehlin & Ronne 1990). The precise mechanism of glucose sensing is not fully understood. Under low glucose conditions Mig1 is phosphorylated by the AMP-activated kinase Snf1 (Treitel et al. 1998). This causes the dissociation from the corepressor Ssn6-Tup1 and cytoplasmic localization of the protein. When glucose concentration in the cell is high, Mig1 is dephosphorylated and relocates into the nucleus where it binds to Ssn6-Tup1 again (De Vit et al. 1997). The general repressors Ssn6 and Tup1 recruit several histone deactylases that maintain the chromatin in the condensed, transcritionally inactive form (Treitel & Carlson 1995; Dent & Malave 2006). Although there are some protein homologues involved in glucose repression in *Kluyveromyces lactis*, the catabolite repression is more pronounced in *Saccharomyces cerevisiae*. Since *ScGAL4* expression is directly Mig1-repressed in the presence of glucose, there is a only a low Gal4 protein level in the nucleus (Nehlin et al. 1991; Griggs & Johnston 1991; Lamphier & Ptashne 1992). A study by Ghaemmaghami et al. (2003) determined 166 molecules of transcriptional activator per cell under these conditions. Furthermore, a lower expression of Gal2 permease in glucose grown cells and the elimination of preexisting Gal2 proteins by proteolytic degradation leads to a low concentration of the inducer molecule galactose in the cell (Horak & Wolf 1997). Glucose repression in *Kluyveromyces lactis* is variable between different strains. Some strains exhibit almost no repression, some show a weak repression (about twofold upon glucose addition) whereas others display a strong glucose repression (50-100fold). Two basepair changes in the promoter of the *KlGAL4* gene are responsible for this observation. The non-repressing variant *KlGAL4-1* carries a tyrosin and a cytosine nucleobase 192 bp and 178 bp upstream from the transcription start, whereas the repressed variant *KlGAL4-2* carries a cytosine and a guanine nucleobase in these positions (Kuzhandaivelu et al. 1992; Zachariae et al. 1993). This altered promoter sequence could be the binding site for a repressor protein or lead to a weaker *Kl*Gal4-binding to the promoter. It was furthermore shown, that the repression-type of a *K. lactis* strain depends on the types of expressed hexose transporters. Low glucose response strains carry the *RAG1* gene whereas glucose repressible strains rather exhibit *KHT1* and *KHT2* gene expression (Weirich et al. 1997).

On respiratory carbon sources like glycerol or raffinose, but in the absence of galactose/lactose, there is only a basal *GAL/LAC* gene expression. In this non-induced state the activity of Gal4 is only regulated by the binding of Gal80 to the AD. In *K. lactis* basal gene expression is higher than in *S. cerevisiae*. In contrast to *ScGAL4*, the *KlGAL4* gene is autoregulated and contains a *Kl*Gal4 binding site in its own promoter (Zachariae & Breunig 1993). The concentration of *Kl*Gal4 is therefore tightly regulated by the carbon source. Whereas glucose in the medium holds the *Kl*Gal4 concentration below a certain threshold, the absence of this sugar suffices to elevate the number of activator molecules and thereby basal gene expression.

Under inducing conditions, that means when galactose/lactose is present in the medium, the Gal4 concentration is increased about twofold in *K. lactis*. It was shown that introduction of a second *KlGAL4* gene copy into *K. lactis* results in about four- to fivefold increase in *Kl*Gal4 protein level, but only a twofold increase in β-galactosidase activity and no growth advantage on galactose (Kuger et al. 1990). Even more, a higher copy number of the *KlGAL4* genes is not tolerated by *K. lactis* and an excess of activator can be toxic for the cells (Breunig 1989). To keep the *Kl*Gal4 concentration in a narrow range, *KlGAL80* has two KlGal4 binding sites in its promoter and in the induced state the repressor concentration is increased about 50 fold, resulting in an efficient negative feedback loop (Zachariae & Breunig 1993). Galactose induction in *K. lactis* as well as in *S. cerevisiae* leads to the dissociation of the repressor Gal80 from the Gal4-AD through direct interaction with *Sc*Gal3 or *Kl*Gal1 (Suzuki-Fujimoto et al. 1996; Zenke et al. 1996). Although data with high spatial and temporal resolution are accumulating, the detailed molecular mechanism of induction is still not fully understood. In the past, several experimental observations lead to the introduction of an “allosteric activation model”. According to this model the binding of the galactose- and ATP-charged Gal3/1 to Gal80 leads to a conformational change in the Gal4-Gal80 complex that would release the AD from repression and make it accessible for interaction with the transcriptional machinery without complete dissociation of the repressor (Leuther & Johnston 1992; Platt & Reece 1998; Bhaumik et al. 2004). Evidence that argued against such a ternary complex formation and which lead to the establishment of the “dissociation model” mainly came from the observation that Gal3 is located exclusively in the cytoplasm, and tethering the protein outside the nucleus does not impair induction of the *GAL* genes (Peng & Hopper 2000; Peng & Hopper 2002). The finding that Gal80-dimerization and binding of a Gal80-monomer to Gal3 utilize the same interaction

surfaces, but binding of Gal80 to the Gal4-AD uses features that are unique to the Gal80-dimer form, seemed to prove this model (Pilauri et al. 2005). However, most recent publications have shown that Gal3 is located throughout the cell and that there is no fast redistribution, neither of Gal3 nor Gal80, between nucleus and cytoplasm upon induction or repression of *GAL* gene expression (Wightman et al. 2008; Jiang et al. 2009; Egriboz et al. 2011). But since ternary complex formation could be neither confirmed nor rejected, this issue remains subject of debate.

1.2 The proteins constituting the galactose genetic switch

1.2.1 The repressor protein Gal80

The structures of the repressor and inducer proteins of the galactose transcriptional switch have recently been solved for both yeasts, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. The *Kl*Gal80 crystal structure was solved by Thoden et al. (2007) and is accessible in the protein database (pdb entry: 2NVW). Additionally, they obtained a structure of the *Kl*Gal80 protein in complex with the 22 amino acid C-terminal domain of *Kl*Gal4 (Thoden et al. 2008, pdb entry: 3E1K). The crystal structure of *Sc*Gal80 in complex with a 21mer peptide of the *Sc*Gal4 activation domain comprising amino acids 854-874 was solved in the same year by another group (Kumar et al. 2008, pdb entry: 3BTS). The repressor molecules of *S. cerevisiae* and *K. lactis* have an amino acid sequence identity of 58 % and similarity of 85 % over the whole length of the protein and have also a similar three dimensional structure. They belong to the family of glucose-fructose oxidoreductases and have a two-domain structure with the N-terminus consisting of a Rossmann fold, a protein structural motif in proteins that bind nucleotides, especially the cofactor NAD (Rao & Rossmann 1973). Indeed, Kumar et al. (2008) could detect the NAD-bound form of *Sc*Gal80 in their crystal structure analysis and referring to further binding studies in their own group they speculated that NAD slightly stabilizes the interaction between Gal4 and Gal80, whereas NADP destabilizes the interaction. No such observations could be made for the *K. lactis* homolog. In her PhD thesis, Doreen Schmidt was able to determine dinucleotide-binding constants for both Gal80 homologs (Doreen Schmidt 2010). She showed that both proteins bind NADP with higher affinity than NAD, and that they prefer the oxidized over the reduced form. She furthermore measured a higher affinity to

dinucleotides for *Sc*Gal80. Whereas the K_D-values of *Sc*Gal80 were found to be in the micromolar range, the binding constants of *Kl*Gal80 are in the millimolar range.

1.2.2 The inducer of the galactose genetic switch – *Sc*Gal3/*Kl*Gal1

*Sc*Gal3/1 and *Kl*Gal1 belong to the GHMP-kinase (galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase) family of enzymes (Bork et al. 1993), and many of them are already structurally described (Zhou et al. 2000; Yang et al. 2002; Badger et al. 2005). The structure of *Sc*Gal3 in the apo form as well as the galactose/AMPPNM bound form in complex with Gal80 was solved very recently (Lavy et al. 2012, pdb entry: 3V5R and 3V2U). This group showed that the structure of *Sc*Gal3 is very similar to the that of the galactokinase *Sc*Gal1 (Thoden et al. 2005), with which it shares about 70 % amino acid sequence identity and more than 90 % similarity. From the comparison between the apo and complexed form of the protein they concluded, that Gal80 interacts with the more "closed" conformation of Gal3, assuming that upon galactose and ATP binding the N- and C-terminal domain of *Sc*Gal3/*Kl*Gal1 move towards each other like closing lips (Menezes et al. 2003). They could also show that Gal3 and Gal80 interact via a complex network of hydrogen bonds in which water molecules are involved and that the lip regions are the main interacting regions. The C-terminal lip thereby shows a stronger interaction and remains associated with Gal80 even at low ligand concentrations while the N-terminal lip dissociates, implying that the two proteins can also weakly associate at high protein concentrations in the absence of galactose and ATP. Modeling the Gal4-AD binding surface detected by Kumar et al. (2008) into their Gal3-complexed Gal80 structure, they concluded that the binding surface of Gal3 on Gal80 is different from that of the Gal4-AD.

In the case of the *K. lactis* homolog, Menezes and his colleagues suggested a conservation of the overall topology with other members of the GHMP-kinase family using a 3D modeling approach (Menezes et al. 2003), but the structure to this day is not solved and there is no structure available in the protein database.

Functionally *Kl*Gal1 is able to complement Gal1 as well as Gal3 deletion mutants in *Saccharomyces cerevisiae* (Meyer et al. 1991). But neither *Sc*Gal1 nor *Sc*Gal3 are able to substitute for Gal1 in *Kluyveromyces lactis* (Zenke et al. 1996).

1.2.3 The transcriptional activator Gal4 – a member of the Zn(II)2Cys6-cluster protein family

The transcriptional activator Gal4 is a protein of approx. 100 kD and consists of 881 amino acids in *S. cerevisiae* (Laughon et al. 1984) and 865 amino acids in *K. lactis* (Wray et al. 1987). The two proteins are conserved (about 24 % sequence identity and 54 % sequence similarity, see also figure 3) and can functionally substitute each other (Riley et al. 1987; Salmeron & Johnston 1986). The activator of the *GAL*/*LAC*-regulon belongs to the family of Zn(II)2Cys6-cluster proteins. In contrast to the Cys_2His_2-protein family, that includes hundreds of zinc-finger proteins in all eukaryotes, the zinc-cluster proteins are exclusively fungal. They constitute one of the largest families of transcription factors in yeast and are involved in the control of various cellular processes like sugar metabolism, gluconeogenesis and respiration, amino acid metabolism, chromatin remodeling, nitrogen utilization, stress response and many more.

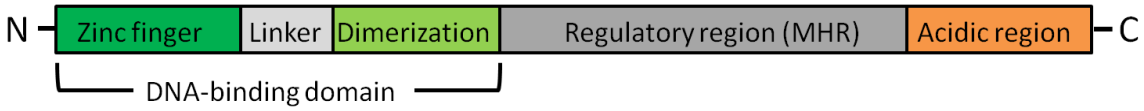

Figure 2: Domain structure of Zn(II)2Cys6-cluster proteins. The N-terminal DNA-binding domain consists of the Zn(II)2Cys6- cluster, a linker and the dimerization domain. The middle homology region (MHR) is thought to be involved in the regulation of protein activity. The Acidic region at the C-terminus constitutes the so called activation domain (AD) and is the binding site for general transcription factors.

As depicted in figure 2, these proteins consist of distinct structural and functional domains. The zinc-finger, which directly contacts the DNA, is further devided into two substructures that are formed by three cysteine residues surrounded by basic amino acids on both sides. The two substructures are separated by a loop of variable length ($CX_2CX_6CX_{5-12}CX_2CX_{6-8}C$) (Schjerling & Holmberg 1996). Together these six cysteine residues coordinate two Zn^{2+} ions. Solution structures of the *Sc*Gal4 DNA-binding element comprising the residues 1-65 were solved 20 years ago in NMR experiments (Baleja et al. 1992; Kraulis et al. 1992). The authors showed that the residues 9-40, that include the six cysteine residues, form a well defined, compact globular cluster.

The linker region has no conserved structure. In the NMR-structure of the *Sc*Gal4-DBD the residues 41-66 show considerable conformational mobility in the absence of DNA, whereas in complex with DNA the residues 50-63 form a coiled-coil dimerization element (Marmorstein et

al. 1992). The linker region of zinc-cluster proteins may therefore contribute to sequence specific DNA-binding of a distinct zinc-cluster protein. It might also be responsible whether a dimer binds to direct, inverted or everted DNA repeat sequences (Gal4 binds to inverted CGG triplets spaced by 11 basepairs (CGG-N_{11} -CCG) (Vashee et al. 1993)). It was shown that substitution of Zn-clusters between different proteins does not affect DNA targeting while linker exchange does (Mamane et al. 1998; Reece & Ptashne 1993).

The dimerization domain contains short heptad repeats similar to those found in leucine zipper proteins and folds into a coiled-coil structure which is highly conserved between Zn-cluster proteins (Schjerling & Holmberg 1996). DNA-independent dimerization of *Sc*Gal4 is possible when the residues 50-106 (corresponding to res. 134-181 in *Kl*Gal4) are included in the structure (Hidalgo et al. 2001). The solution structure of the DNA-binding domain from *Kl*Gal4 is very similar to that of *Sc*Gal4 and was solved by Gardner et al. (1995). A structure of the complete *Sc*Gal4 DNA-binding and dimerization domain (residues 1-100) in complex with DNA was solved only a few years ago (Hong et al. 2008).

Many, but not all, Zn-cluster proteins contain a so called “middle homology region (MHR)” in their regulatory domain. This region is less conserved and up to now no specific function could be addressed to the MHRs of *Sc*Gal4 (residues 326-402) and *Kl*Gal4 (residues 471-544) (see figure 3). In extensive deletion experiments Ma and Ptashne found that about 80 % of the *Sc*Gal4 protein between DNA-binding and activation domain can be deleted without drastic loss of transcriptional activity (Ma & Ptashne 1987). Stone and Sadowski proposed that the internal part of the Gal4 protein might be involved in glucose repression (Stone & Sadowski 1993). Another group reasoned that this region serves as a spacer between DNA-binding and activation domain that enhances the transcriptional activity of the protein (Ding & Johnston 1997). And indeed all available data suggest that the MHRs of zinc-cluster proteins might play a role in the regulation of the transcriptional activity. So does the deletion of large parts of the internal region, including the MHR, of Leu3p (*S. cerevisiae*) and qa1F*Ncr* (*Neurospora crassa*) convert the proteins into constitutive activators (Friden et al. 1989; Zhou et al. 1990; Giles et al. 1991). Deletion of the MHR in Hap1, a heme and oxygen responsive transcription factor, also leads to constitutive activity of the protein (Pfeifer et al. 1989). Schjerling and Holmberg furthermore suggested that the MHR assists in the *in vivo* recognition of the DNA binding site by reducing the affinity of the protein to similar but “wrong” binding sites (Schjerling &

Holmberg 1996). But besides the MHR, there is still a large internal region in Gal4 and other zinc-cluster proteins whose function is up to now unassigned. Unpublished data from K. Melcher show that a particular miniScGal4 variant in which the activation domain and specific residues from the internal region (aa 680-725, see also figure 5) are deleted is unable to interact with the repressor, while a similar variant exhibiting the residues 680 to 725 retains Gal80 interaction.

The C-terminal activation domains of zinc-cluster proteins are generally not well defined. As typical for transcription factor activation domains they are unstructured and flexible in the absence of an interaction partner and it is supposed that a structure is induced merely upon interaction with a specific target protein. The human transcriptional activator c-Myc for example, which is involved in cell growth and differentiation, has an unstructured N-terminal activation domain that becomes α-helical upon interaction with the TATA binding protein (TBP) (McEwan et al. 1996). Another example is the cAMP-regulated basic leucine Zipper (bZIP) transcriptional activator CREB (CRE binding protein). It has a bipartide activation domain consisting of a constitutive domain (Q2) and a kinase inducible part (KID domain), whereof the latter undergoes a coil to helix transformation upon binding to the co-activator protein CBP (Radhakrishnan et al. 1997; Parker et al. 2004). In the case of *Kl*Gal4 it was observed that from nine residues of the activation domain (Asp 854 to Ile 860, corresponding to residues 862-870 in *Sc*Gal4), which display a common sequence motif in many transcriptional activators, seven residues seem to be helical in the co-crystal with *Kl*Gal80 (Thoden et al. 2008).

While the target proteins of the activation domains are often highly conserved, there is no apparent sequence conservation between different ADs. What they have in common are stretches of bulky hydrophobic amino acids interspersed by acidic residues that force the hydrophobic parts into an aequous environment. These parts are able to contact other exposed hydrophobic surfaces while the charges of the hydrophilic residues further stabilize the interaction and confer binding specificity (Melcher 2000).

To prevent the activation domains from being constitutively active, they are regulated by masking. As already mentioned the Gal4-AD is suppressed by the binding of Gal80 in the absence of galactose. *Sc*Gal4 variants lacking the 28 C-terminal amino acids are insensitive to repression by Gal80, showing that these residues are involved in the interaction to the repressor (Johnston et al. 1987; Ma & Ptashne 1987b). Masking by repressor proteins is also

found in the case of the transcriptional activator p53, a mammalian tumorsupressor protein that coordinates induction of cell cycle arrest and apoptosis upon DNA damage. The N-terminal activation domain of this protein is bound by the repressor protein MDM2, whose protein levels are often increased in tumor cells (Kussie et al. 1996). Another example is E2F, a transcription factor in mammalian and plant cells that regulates the transcription of genes involved in DNA synthesis and cell cycle progression. Several types of cancer are associated with overexpression of this protein. The activity of the protein is regulated by the binding to the Retinoblastoma protein which masks the activation domain and inhibits transcription of the target genes (Lee et al. 2002; Hiebert et al. 1992). Transcriptional activation domains can also be masked by intramolecular interactions as in the case of the yeast transcription factor Leu3. Leu3 is involved in branched chain amino acid biosynthesis and ammonia assimilation, and under low α-IPM (alpha-isopropylmalate) concentrations there is an interaction between the internal region of the protein and the AD that prevents *LEU* gene expression. During leucine starvation, α-IPM, which is an intermediate in leucine biosynthesis, accumulates and prevents interaction between the internal region and the AD (Wang et al. 1999; Wang et al. 1997). Intramolecular interactions between the middle region and the AD are also responsible for suppression of Cha4, a transcription factor in yeast that activates the genes for serine and threonine utilization (Wang et al. 1999).

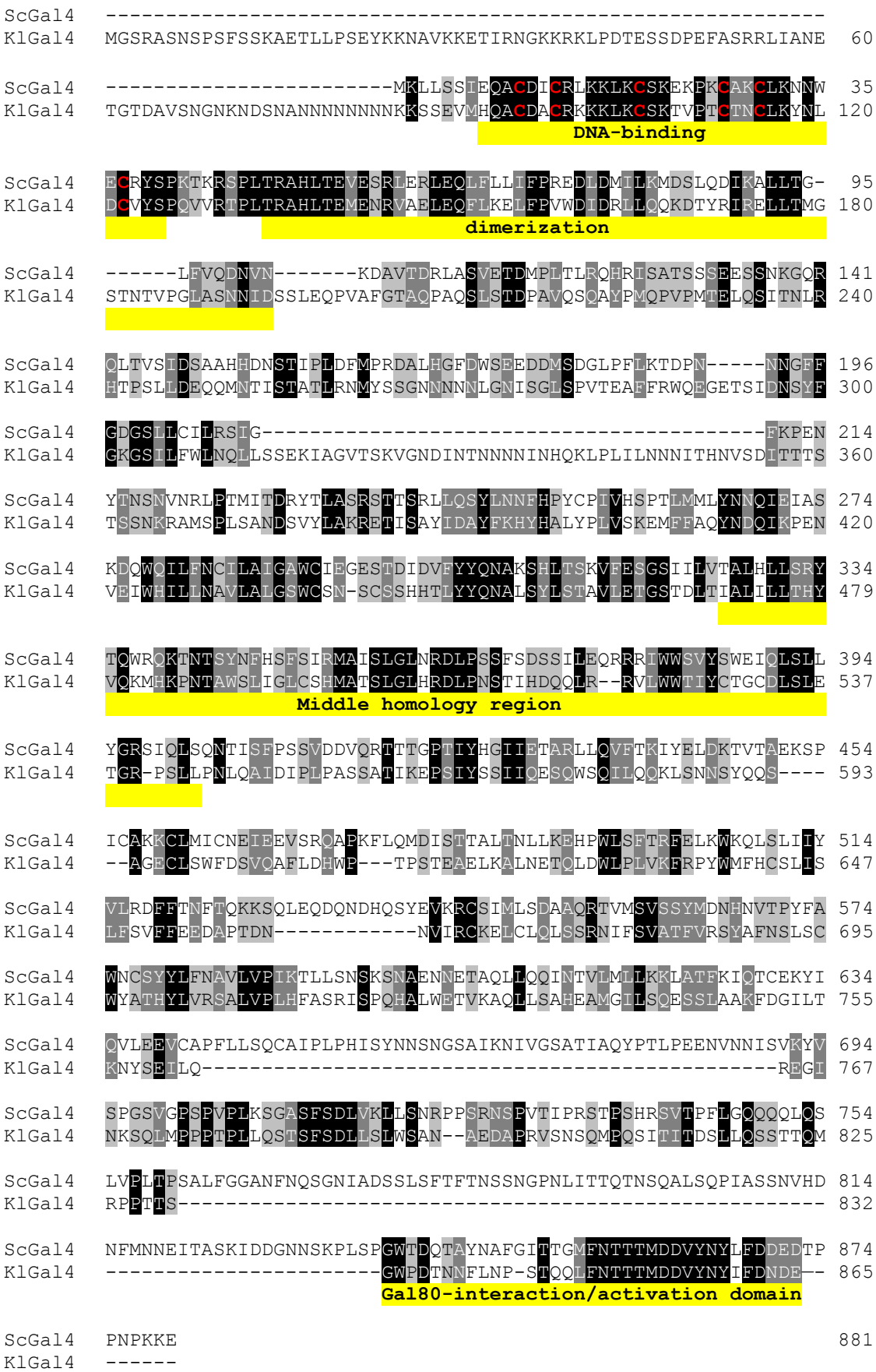
ScGal4 ---------- 0
KlGal4 MGSRASNSPSFSSKAETLLPSEYKKNAVKKETIRNGKKRKLPDTESSDPEFASRRLIANE 60
ScGal4 ----------MKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNW 35
KlGal4 TGTDAVSNGNKNDSNANNNNNNNNKKSSEVMHQACDACRKKKLKCSKTVPTCTNCLKYNL 120
DNA-binding
ScGal4 FCRYSPKTKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTG- 95
KlGal4 DCVYSPQVVRTPLTRAHLTEMENRVAELEQFLKELFPVWDIDRLLQQKDTYRIRELLTMG 180
dimerization
ScGal4 ------LFVQDNVN-------KDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQR 141
KlGal4 STNTVPGLASNNIDSSLEQPVAFGTAQPAQSLSTDPAVQSQAYPMQPVPMTELQSITNLR 240
ScGal4 QLTVSIDSAAHHDNSTIPLDFMPRDALHGFDWSEEDDMSDGLPFLKTDPN-----NNGFF 196
KlGal4 HTPSLLDEQQMNTISTATLRNMYSSGNNNNNLGNISGLSPVTEAFFRWQEGETSIDNSYF 300
ScGal4 GDGSLLCILRSIG----------FKPEN 214
KlGal4 GKGSILFWLNQLLSSEKIAGVTSKVGNDINTNNNNINHQKLPLILNNNITHNVSDITTTS 360
ScGal4 YTNSNVNRLPTMITDRYTLASRSTTSRLLQSYLNNFHPYCPIVHSPTLMMLYNNQIEIAS 274
KlGal4 TSSNKRAMSPLSANDSVYLAKRETISAYIDAYFKHYHALYPLVSKEMFFAQYNDQIKPEN 420
ScGal4 KDQWQILFNCILAIGAWCIEGESTDIDVFYYQNAKSHLISKVFESGSIILVTALHLLSRY 334
KlGal4 VEIWHILLNAVLALGSWCSN-SCSSHHTLYYQNALSYLSTAVLETGSTDLTIALILLTHY 479
ScGal4 TQWRQKTNTSYNFHSFSIRMAISLGLNRDLPSSFSDSSILEQRRRIWWSVYSWEIQLSLL 394
KlGal4 VQKMHKPNTAWSLIGLCSHMATSLGLHRDLPNSTIHDQQLR--RVLWWTIYCTGCDLSLE 537
Middle homology region
ScGal4 YGRSIQLSQNTISFPSSVDDVQRTTTGPTIYHGIIETARLLQVFTKIYELDKTVTAEKSP 454
KlGal4 TGR-PSLLPNLQAIDIPLPASSATIKEPSIYSSIIQESQWSQILQQKLSNNSYQQS---- 593
ScGal4 ICAKKCLMICNEIEEVSRQAPKFLQMDISTTALTNLLKEHPWLSFTRFELKWKQLSLIIY 514
KlGal4 --AGECLSWFDSVQAFLDHWP---TPSTEAELKALNETQLDWLPLVKFRPYWMFHCSLIS 647
ScGal4 VLRDFFTNFTQKKSQLEQDQNDHQSYEVKRCSIMLSDAAQRTVMSVSSYMDNHNVTPYFA 574
KlGal4 LFSVFFEEDAPTDN------------NVIRCKELCLQLSSRNIFSVATFVRSYAFNSLSC 695
ScGal4 WNCSYYLFNAVLVPIKTLLSNSKSNAENNETAQLLQQINTVLMLLKKLATFKIQTCEKYI 634
KlGal4 WYATHYLVRSALVPLHFASRISPQHALWETVKAQLLSAHEAMGILSQESSLAAKFDGILT 755
ScGal4 QVLEEVCAPFLLSQCAIPLPHISYNNSNGSAIKNIVGSATIAQYPTLPEENVNNISVKYV 694
KlGal4 KNYSEILQ----------REGI 767
ScGal4 SPGSVGPSPVPLKSGASFSDLVKLLSNRPPSRNSPVTIPRSTPSHRSVTPFLGQQQQLQS 754
KlGal4 NKSQLMPPPTPLLQSTSFSDLLSLWSAN--AEDAPRVSNSQMPQSITITDSLLQSSTTQM 825
ScGal4 LVPLTPSALFGGANFNQSGNIADSSLSFTFTNSSNGPNLITTQTNSQALSQPIASSNVHD 814
KlGal4 RPPTTS---------- 832
ScGal4 NFMNNEITASKIDDGNNSKPLSPGWTDQTAYNAFGITTGMFNTTTMDDVYNYLFDDEDTP 874
KlGal4 ----------GWPDTNNFLNP-STQQLFNTTTMDDVYNYIFDNDE-- 865
Gal80-interaction/activation domain
ScGal4 PNPKKE 881
KlGal4 ------

Figure 3: Sequence alignment of *Sc*Gal4 and *Kl*Gal4. The alignment was done with the ClustalW2 program, available online at http://www.ebi.ac.uk/Tools/services/web_clustalw2/toolform.ebi. 'black' indicates positions which have a single, fully conserved residue; 'dark grey' indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW; 'light grey' indicates that one of the following 'weaker' groups is fully conserved: ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY; domains were marked basing on the following publications: DNA-Binding domain, (Gardner et al. 1995); dimerization domain, (Hidalgo et al., 2001); Middle homology region, (Schjerling & Holmberg, 1996); Gal80-interaction and activation domain, (Leuther et al. 1993). The Zn^{2+}-coordinating cysteine residues are marked red.

1.3 Posttranslational modifications of Gal4 and Gal80 influence the transcriptional activation

Not only inter- and intramolecular interactions, but also posttranslational modifications play an important role in the regulation of transcription factors. There is a broad spectrum of posttranslational modifications (PTMs) known which impact transcription factor regulation like acetylation, cleavage of inhibitory domains, disulfide bridge formation and methylation, to name just a few. The most important modification is the phosphorylation/dephosphorylation of serine, threonine or tyrosine residues, and the mechanisms by which this modification regulates transcription factor function can be (i) the control of the length of time that transcription factors spend in the nucleus, (ii) the targeting of transcription factors or their coregulators for proteolytic degradation, (iii) the modulation of protein-protein interactions between transcription factors, coregulators and the factors of the basal transcriptional machinery, (iv) the regulation of transcription factor DNA binding and (v) the modification of the chromatin structure (reviewed by Whitmarsh & Davis, 2000). Another modification which is important in gene regulation is the ubiquitination of lysine residues. Besides modulation of the stability of a transcription factor by labeling it for proteasomal degradation other modes of operation have been discovered. These include the activation of transcription factors by labeling precursor proteins for proteasomal processing, recruitment of proteasomal subunits to the chromatin or proteasome independent processes like the initiation of endocytosis (reviewed by Conaway et al. 2002).

1.3.1 Phosphorylation and monoubiquitination are required for full *Sc*Gal4 activity

Although no PTMs of the *K. lactis* activator are known, several phosphorylation sites and one site of ubiquitination where identified for the *Sc*Gal4 protein. It was shown that Gal4 isolated

from *Saccharomyces cerevisiae* cells under non-inducing conditions is unphosphorylated, whereas phosphorylation could be detected in cells grown in medium containing galactose (Parthun & Jaehning 1992). Sites of phosphorylation where identified as Ser691, Ser696, Ser699 and Ser837, and two other not further characterized sites were predicted to be at the N-terminus (aa 1-238) and C-terminus (701-768) (Sadowski et al. 1996; Sadowski et al. 1991). The same group also showed that only phosphorylation at serine 699 is required for full transcriptional activation of the activator in *GAL80*$^{+}$ cells, but not in cells lacking *gal80*. The phosphorylation was furthermore shown to be an event downstream of transcriptional induction and the mutation of Ser699 to alanine does not completely abolish induction, but makes the process slower. Hirst et al. identified the two RNAPII-holoenzyme associated CDKs Srb10 and Kin28/TFIIH to be responsible for the phosphorylation of the residues Ser699 and Ser837 (Hirst et al. 1999). Srb10 is thereby recruited to the activator by direct interaction with the Gal4-AD (Ansari et al. 2002). All these findings were interpreted that way that Ser699-phosphorylation influences the interaction between the repressor and activator. After Gal3 binding to Gal80 has activated transcription to a basal level, Gal4 is phosphorylated at Ser699 by Srb10, which might prevent Gal80 from a fast reassociation with Gal4 (Leverentz & Reece 2006).

In 2008 the role of monoubiquitination at the N-terminus of the *Sc*Gal4 protein was uncovered. For efficient transcriptional activation, activators have to recruit ATPases to the chromatin. The Gal4-AD can extract the proteasomal ATPases from the 26 S proteasome by contacts with the ATPases Rpt4/Rpt6. This would usually destabilize the Gal4-DNA-complex, because the ATPases would treat Gal4 as substrate for unfolding. But when the DNA-binding domain is monoubiqitylated, the monoubiquitin-residues contact the ATPases Rpt1/Rpn1 and this contact destabilizes the Gal4-AD-Rpt4/Rpt6 contacts (Archer, Burdine, et al. 2008). Monoubiquitylation at the DBD is therefore necessary for efficient promoter binding under inducing conditions and prevents stripping of the activator from the DNA by ATPases. The ATPase-complex can probably subsequent to the contact with Gal4 fulfill its function in transcriptional activation. Gal4 lacking the residues 842 to 853 of the activation domain is sensitive to stripping by ATPases. Such a Gal4-variant was shown to be not ubiquitylated and these residues therefore maybe a recognition side for the E3-ligase (Archer, Delahodde, et al. 2008). The site of ubiquitylation was

identified as the lysine at position 23, and ubiquitylation of this residue was shown to be dependent on the phosphorylation of the adjacent residue serine 22 (Ferdous et al. 2008).

1.3.2 *Kl*Gal80 is a target of phosphorylation

Contrary to the posttranslational modifications of the Gal4 protein in *S. cerevisiae*, the *Kluyveromyces lactis* Gal80 but not the *Saccharomyces cerevisiae* Gal80 protein is subject to phosphorylation/dephosphosphorylation. Zenke et al. (1999) have shown that the phosphorylation status of the *K. lactis* repressor is dependent on the carbon source. Whereas *Kl*Gal80 is (hyper)phosphorylated in non-inducing medium, it is non- or underphosphorylated in inducing medium. The inhibition of phosphorylation or dephosphorylation of *Kl*Gal80 in galactose grown cells is dependent on *Kl*Gal1. The galactokinase negatively influences the level of phosphorylated protein maybe by an influence of galactose metabolism on the activity of the *Kl*Gal80-kinase/-phosphatase (Zenke et al. 1999). An impact of this phosphorylation on *Kl*Ga4-*Kl*Gal80 interaction is discussed.

1.4 Aims of the work

There are two models illustrating the so called “galactose switch”, which describes the release of Gal4-repression by Gal80. According to the “dissociation model” the interaction of Gal3/1 with Gal80 upon galactose induction leads to the dissociation of Gal4 and Gal80, setting the activation domain (AD) of Gal4 free for interaction with general transcription factors. The “allosteric model” supposes the formation of a (eventually only temporary) ternary complex consisting of Gal4, Gal80 and Gal3/1 upon galactose induction. This interaction would lead to a conformational change within the Gal4 protein that also sets the Gal4-AD free for interaction with the transcriptional machinery. The aim of this PhD thesis was to analyze the fate of Gal80 upon Gal4 activation with regard to the different activation models and conformational transitions that may occur at the Gal4-Gal80 interface at the molecular level. Since questions regarding „Conformational transitions in macromolecular interactions“ are addressed in the Graduiertenkolleg 1026, my project was embedded in this Research Training Group.

The allosteric model is supported by several observations in *Saccharomyces cerevisiae*. Parthun & Jaehning (1992) for example found that *Sc*Gal4 purified from galactose induced yeast cells was associated with *Sc*Gal80. Platt & Reece (1998) could observe a tripartite *Sc*Gal4-*Sc*Gal80-

*Sc*Gal3 complex *in vitro*. The formation of a ternary complex also postulates an alternative binding site for Gal80 on the Gal4 protein beyond the established binding site at the C-terminus. To identify such a potentially secondary binding site in this work, a β-galactosidase filter assay and Chromatin immunoprecipitation (ChIP) analyses should be performed with *Sc*Gal4 and *Sc*Gal4-deletion variants. The results obtained in these experiments rather approved the dissociation model. The dissociation model was previously supported by the observation, that *Sc*Gal3 and *Sc*Gal80 can shuttle between the nucleus and cytoplasm (Peng & Hopper 2000; Wightman et al. 2008), and that tethering *Sc*Gal3 outside of the nucleus does not impair *GAL*-gene induction (Peng & Hopper 2002). Analyses of the *GAL*-switch in *K. lactis* also favored the dissociation model (Anders et al. 2006). But since it was shown that *Kl*Gal80 is exclusively nuclear and there is no shuttling upon galactose induction (Anders et al. 2006), the formation of a ternary complex cannot be excluded. To find new evidence for one or the other activation model, the transcriptional switch should be reconstituted *in vitro*. Therefore the *K. lactis* proteins *Kl*Gal4, *Kl*Gal80 and *Kl*Gal1 should be recombinantly expressed and purified in *E. coli*. It was aimed to develop a suitable protocol for *Kl*Gal4 expression in this work. The development of a protocol for Gal4-purification is also an important step for further investigations of the Gal4 structure and the Gal4-Gal80 interaction.

2 Materials and Methods

2.1 Materials

The table below enlists materials and chemicals and their suppliers as they are not further mentioned in this section.

Chemical	Supplier
30 % Acrylamide-Bisacrylamide solution (37,5:1)	Roth
Adenosine 5'-diphosphate sodium salt (ADP)	Sigma-Aldrich
Agar-Agar, Kobe I	Roth
Agarose	Serva
L-amino acids	Roth
Ammonium actetate (CH_3COONH_4)	Roth
Ammonium-peroxo-*di*-sulfate (APS)	Roth
Ampicillin	AppliChem
Calcium chloride ($CaCl_2$)	Riedel-de Haën
Chloramphenicol	Roth
Coomassie Brilliant Blue G250	Serva
Deoxycholic acid (sodium salt)	Sigma-Aldrich
Ethidium bromid	Roth
Ethylendiaminetetraacetic acid (EDTA)	Roth
5-Fluoro Orotic Acid (5-FOA)	ForMedium, UK
D(+)-galactose	ForMedium, UK
GeneRuler^{TM} DNA Ladder Mix (# SM 0333)	Fermentas, Thermo Scientific
D(+)-glugose	Roth
Glycerol	Serva
Glycin	AppliChem
Hydrochloric acid (HCl)	Roth
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Roth
Lactose	Roth
Lithium chloride (LiCl)	Merck
6x loading buffer for DNA	Fermentas, Thermo Scientific

Magnesium chloride ($MgCl_2$)	Roth
Manganese(II) chloride ($MnCl_2 \cdot 4H_2O$)	AppliChem
2-Mercaptoethanol	Roth
Milk powder	Roth
3-(N-morpholino)propanesulfonic acid (MOPS)	Roth
Natriumhydroxid (NaOH)	Roth
Nicotinamide adenine dinucleotide (NADP)	AppliChem
2-Nitrophenyl-ß-D-galactopyranosid (ONPG)	AppliChem
Nonidet P-40	AppliChem
Phenol-chloroform-Isoamylalcohol (PCI) (25:24:1)	Roth
Potassium acetate (CH_3CO_2K)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogenphosphate (KH_2PO_4)	Roth
Potassium hydroxide (KOH)	Roth
Prestained Protein Ladder (#SM 0672)	Fermentas, Thermo Scientific
Sodiumacetate	Roth
tri- sodium citrate-dihydrate	Roth
Soiumchloride (NaCl)	Roth
Sodiumdodecylsulfate (SDS)	Roth
Tryptone	Serva
Tween 20	Roth
5-Bromo-4-chloro-3-indolyl-D-galactopyranosid (X-Gal)	ForMedium, UK
Yeast extract	Serva
Yeast nitrogen base (YNB)	Difco, USA

2.2 Yeast strains and growth media

The *S. cerevisiae* strain MaV103 (*MATα, leu2-3,112, trp1-901, his3Δ200, ura3-52, ade2-101, gal*4Δ, *gal*80Δ, *cyh2*^{R}, *can*1^{R}, *GAL1::HIS3@LYS2, GAL1::lacZ, SPAL10::URA3@ura3)* was used for yeast-two-hybrid (Y2H) analysis. This *gal4Δ gal80Δ* strain has an integrated LACZ-reporter gene under the control of the GAL1-promoter (Vidal et al. 1996).

The I4G80Myc strains (*S. cerevisiae*) used and constructed in this work for chromatin immune precipitation experiments were all isogenic to the FI4G10 strain. This strain is derived from FI4 (Schöninger 2000) and has the non-functional *sin4-10* allel (*MATα, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, sin4-10, GAL2, KlLAC4*$^{+}$*LAC12*$^{+}$*TRP*$^{+}$). The parental strain of FI4 was FY1679-06C (*MATα, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, GAL2*) from EUROSCARF. Gene replacements in *S. cerevisiae* were made with *URA3* from *K. lactis*, which was amplified with specific knock out primers from the plasmid YDpKlURA3.

I4G80Myc strain	**FI4 deviating genotype**	**origin**
I4G80Myc	GAL80-3Myc HIS3^{+}	lab Langhammer
I4G80Myc g4Δ	GAL80-3Myc HIS3^{+} *gal4Δ::URA3*	this work
I4G80Myc mG4 (miniGal4 (Wu et al. 1996), aa 1-100, 840-881)	GAL80-3Myc HIS3^{+} *gal4Δ::ura3Δ::miniGAL4*	this work
I4G80Myc mG4#7 (miniGal4#7, aa 1-100, 680-881)	GAL80-3Myc HIS3^{+} *gal4Δ::ura3Δ::miniGAL4#7*	this work
I4G80Myc mG4#9 (miniGal4#9, aa 1-100, 725-881)	GAL80-3Myc HIS3^{+} *gal4Δ::ura3Δ::miniGAL4#9*	this work

The *Kluyvermyces lactis* strains used and constructed in this work were all isogenic to the strain JA6 strain, which was obtained by crossing the wildtype strain CBS2360 and the strain SD11 (Breunig & Kuger 1987). *LAC9* is synonymous with *KlGAL4*.

strain	**(JA6 deviating) genotype**	**origin**
JA6	*α ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2*	(Breunig & Kuger 1987)
DL9	*lac9Δ::ScURA3*	(Kuger et al. 1990)
JA6/2-2	*LAC9-2 LAC9-2-ScURA3*	(Kuger et al. 1990)
YCZ_KlGal4 wt	*lac9Δ::Scura3Δ::LAC9-2*	this work
YCZ_KlGal4-77	*lac9Δ::Scura3Δ::LAC9-2-77*	this work
YCZ_KlGal4-67	*lac9Δ::Scura3Δ::LAC9-2-67*	this work
YCZ_KlGal4-57	*lac9Δ::Scura3Δ::LAC9-2-57*	this work

Strains were grown in rich medium (YEP: 1 % (w/v) yeast extract, 2 % (w/v) bacto-peptone) or synthetic complete medium (SC: 0.67 % (w/v) yeast nitrogen base, supplemented with 11.2 mg/l Ade, 38.4 mg/l Ura, 38.4 mg/l His, Trp, Arg and Met, 14.4 mg/l Tyr, 57.6 mg/l Leu, Ile, Val, and Thr, 48 mg/l Phe, and 28.8 mg/l Lys from a 20x stock solution) at 30°C. For selection of plasmid-containing cells the transformants were grown in synthetic complete medium lacking distinct nucleobases/amino acids. Carbon sources were added in the following concentrations: glucose 2 % (w/v), galactose 2 % (w/v), glycerol 3 % (w/v), ethanol 2 % (v/v) and sodium acetate 2 % (w/v). For solid media the above liquid media were supplemented with 2 % (w/v) agar. All media were autoclaved at 121 °C for 20 min. before use.

Selection for reversion from uracil prototrophy to auxotrophy was performed on SC plates containing uracil, 2 % glucose and 0.06 - 0.1 % (w/v) 5-fluoroortic acid (FOA). For monitoring of β-galactosidase activity, plates were supplemented with 40 µg/ml X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) from a 20 mg/ml stock solution in DMF.

2.3 *Escherichia coli* strains and growth media

***E.coli* strain**	**Genotype**	**origin**
DH5αF'	F'(Φ80d*lacZ*ΔM15), Δ(*lacZYA-argF'*)U169, *recA1*, *endA1*, *hsdR17 (*r_k^- m_k+), *supE44*, *thi-1*, *gyrA*, *relA1*	Invitrogen
Rosetta(DE3)- pLysS	F- ompT hsdSB(r_B^- m_B^-) gal dcm (DE3) pLysSRARE (CamR)	Novagen (Merck)

Strains were usually grown in LB (lysogeny broth)-medium (0.5 % yeast extract, 1 % tryptone, 0.5 % sodium chloride) at 37°C. For plasmide selection the medium was supplemented with antibiotics (50 or 100 mg/l ampicillin, 35 mg/l chloramphenicol or 10 mg/ml kanamycin). Solid LB-medium was supplemented with 2 % (w/v) agar.

For protein production freshly transformed Rosetta cells were grown in LB-medium supplemendted with Cam (35 µg/ml), Amp (100 µg/ml), 200 mM M KH_2PO_4 and 1.5 % lactose at 30°C or TB (terrific broth)-medium at (1.2 % tryptone, 2.4 % yeast extract, 0.4 % (v/v) glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) with 100 mg/l ampicillin and 35 mg/l chloramphenicol at 20°C.

2.4 Plasmids

yeast-plasmid	**description**	**origin**
YDpKlURA3	yeast disruption plasmid for the amplification of the auxotrophic markergene *URA3*, contains *bla* gene for propagation in *E. coli*	Jablonowski et al. 2001
pGBD-C3-Gal4 (1-841)	2µ-plasmid, *GAL4stop841* gene (aa 1-841) under control of the *ADH1* promoter, *TRP1* marker	K. Melcher
pGBD-C3-miniGal4-7-stop841	2µ-plasmid, mini*GAL4-7stop841* gene (aa 1-237, 412-422, 680-841) under control of the *ADH1* promoter, *TRP1* marker	K. Melcher
pGBD-C3-miniGal4-9-stop841	2µ-plasmid, mini*GAL4-9stop841* gene (aa 1-168, 727-841) under control of the *ADH1* promoter, *TRP1* marker	K. Melcher

pVP16-80	2µ-plasmid, *GAL80* fused to *VP16* activation domain under control of the *ADH1* promoter, *LEU2* marker	K. Melcher
YCp50ScGal4	gene bank plasmid containing *ScGAL4, URA3, bla* marker	lab Breunig
pLAC9-2	pBR322 derivative containing the *LAC9-2* gene	Kuger et al. 1990
pCL9	CEN-plasmid for the expression of KlGal4 in *K.lactis*	W. Zachariae
***E.coli*-plasmid**		
pJET 1.2	cloning vector for blunt end ligation of PCR products, *bla* marker gene for Ampicillin resistance	Fermentas, Thermo Scientific
pET-15b	expression vector for N-terminal His_6-tagged protein expression, *bla* marker	Novagen (Merck)
pETNHG80	plasmid for the expression of N-terminal His_6-tagged *Kl*Gal80 protein, *bla* marker	L. Kapp
pETNHG1	plasmid for the expression of N-terminal His_6-tagged *Kl*Gal1 protein	Anders 2006
pGEX-6-P1	Expression vector for N-terminal GST-tagged protein expression, *bla* marker	GE Healthcare
pRJR229	Expression plasmid for miniGal4 (aa 1-100, 840-881), *bla* marker	Wu et al. 1996

The following table summarizes plasmids that were constructed in this work. The correct insertion of a fragment into a vector was proven by test-digestions with suitable restriction enzymes. The sequences of the particular primers are listed in section 2.4.

plasmid	**description**
pETNHG4	Vector for expression of N-terminal His_6-tagged *Kl*Gal4 in *E. coli*. The *KlGAL4* gene was cloned as BamHI-HindIII fragment into pET-15b after amplification with the primer pair *BamHILAC9-2*/*HindIIILAC9-2rv* from pLAC9-2 (Kuger et al. 1990).
pETNHG4-NHG80	Vector for co-expression of N-terminal His_6-tagged *Kl*Gal4 and N-terminal His_6-tagged *Kl*Gal80 in *E. coli.* KlGAL80 was amplified with the primer pair *HindIIIKlGAL80neu*/*EcoRIKlGAL80rv* and subcloned into pJET 1.2. The HindIII-EcoRI fragment was cloned into pETNHG4.

pGSTG4	Vector for expression of N-terminal GST-tagged *Kl*Gal4 in *E. coli*. The *Kl*GAL4 gene was cloned as BamHI-EcoRI fragment into pGEX-6-P1 after amplification with the primer pair *pGex KlGal4 fw/pGex KlGal4 rv* from pLAC9-2 (Kuger et al. 1990)
pGSTG4-NHG80	Vector for co-expression of N-terminal GST-tagged *Kl*Gal4 and N-terminal His_6-tagged *Kl*Gal80 in *E. coli*. *Kl*GAL80 was amplified with the primer pair *KlGal80XmaI fw*/*KlGal80NotI rv* and subcloned into pJET 1.2. The XmaI-NotI fragment was cloned into pGSTG4
pCL9-77	CEN-plasmid for the expression of KlGal4-77 in *K. lactis*. *KlGAL4-77* was amplified with the strategy described in section 2.5. The *LAC9*-gene of pCL9 was replaced by *KlGAL4-77* as Eco91I/BspTI- fragment.
pCL9-67	CEN-plasmid for the expression of KlGal4-67 in *K. lactis*. *KlGAL4-67* was amplified with the strategy described in section 2.5. The *LAC9*-gene of pCL9 was replaced by *KlGAL4-67* as Eco91I/BspTI- fragment.
pCL9-57	CEN-plasmid for the expression of KlGal4-57 in *K. lactis*. *KlGAL4-57* was amplified with the strategy described in section 2.5. The *LAC9*-gene of pCL9 was replaced by *KlGAL4-57* as Eco91I/BspTI- fragment.

2.5 Oligonucleotides and PCR strategies

Oligonucleotides were purchased from Eurofins MWG (Ebersberg) and solved in distilled water to a concentration of 100 pmol/µl. For PCR-reactions dilutions with a concentration of 2 pmol/µl were made.

Knock out primers were used to amplify the URA3 gene from YDpKlURA3. The nucleotides complementary to URA3 are underlined, the long overhangs are complementary to bases in the target gene KlGAL4. The PCR product was used to knock out GAL4 in the yeast strain I4G80Myc, the resulting strain was termed I4G80Myc g4Δ.

knock out primer	**Sequence (5'→3' direction)**
GAL4KO 2 left	AAAGCTCAAGTGCTCCAAAGAAAAACCGAAGTGCGCCAAGTGTCTG AAGACGGCCAGTGAATTCCCGG
GAL4KO 2 right	CACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCAT TTTACTGCAGGTCGACGGATCC

The following primers were used to amplify the mini*GAL4* gene (miniGal4 aa 1-100, 840-881) from the plasmid pRJR229. To create a PCR product with suitable 5' and 3' ends for homologous recombination at the *gal4Δ::URA3* locus in I4G80Myc g4Δ, three subsequent PCR reactions were performed.

PCR reaction	**#1**	**#2**	**#3**
forward primer	miniGAL4 L1	miniGAL4 L2	miniGAL4 L3
sequence (5'→3')	CCATCATTTTAAGAGAGGACAGAGAAGCAAGCCTCCTGAAAGATGAAGCTACTGTCTTCTATC	CCCCAGATTTTCAGCTTCATCTCCAGATTGTGTCTACGTAATGCACGCCATCATTTTAAGAGAGG	CTTCGGGCCTTTTTCTGTTTTATGAGCTATTTTTTCCGTCATCCTTCCCCAGATTTTCAGCTTC
reverse primer	miniGAL4 R	miniGAL4 R2	
sequence (5'→3')	TTTACTCTTTTTTTGGGTTTGGTGGGGTATCTTCATCATCGAATAGATAGTTATATACATCATCCATTGT	TGCACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTTTACTCTTTTTTTGGGT	
template	pRJR229	PCR product #1	PCR product #2

The primers in the next table were used to create a GAL4ΔAD (aa 1-841) PCR fragment suitable for homologous recombination at the *gal4Δ::URA3* locus in I4G80Myc g4Δ. Two subsequent PCR reactions were necessary.

PCR reaction	**#1**	**#2**
forward primer	miniGAL4 L3	
sequence (5'→3')	See previous table	
reverse primer	Gal4dAD R1	Gal4dAD R2
sequence (5'→3')	ACCAGGTGACAGTGGTTTTGAATTATTACCATCATCAATTTTACTAGCCGTGATTTCATTATTCATGAAG	CACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTTTAACCAGGTGACAGTGGTTTTG
template	YCp50ScGAL4	PCR product #1

Mini*GAL*4#7- (1-100, 680-881) and #9- (1-100, 725-881) variants for homologous recombination at the *gal4Δ::URA3* locus in I4G80Myc g4Δ were amplified with the following fusion-PCR strategy. Template of the first PCR reaction was YCp50ScGAL4. 10 -20 ng of the PCR products from PCR#1 were used for the second reaction.

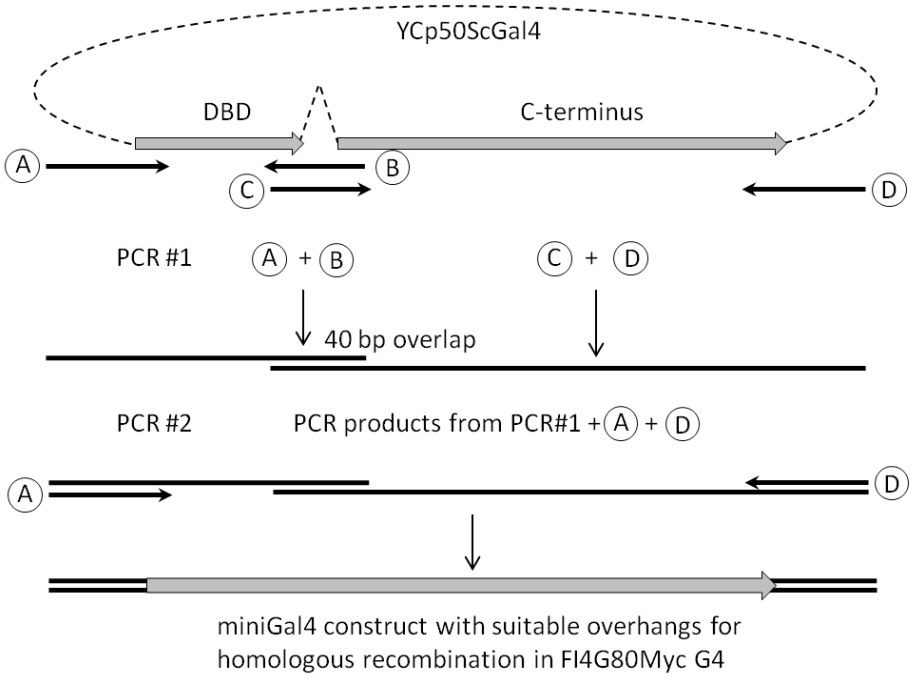

Figure 4: Fusion PCR strategy. The figure shows the order of subsequent PCR reactions and the template(s) and primers used to generate *miniGAL4*-variants.

primer	***miniGAL4#7***	***miniGAL4#9***
A sequence (5'→3')	miniGAL4 L3 see previous table	
B sequence (5'→3')	miniGal4#7 DBD rv TCCGGAAGAGTAGGGTATTGATTCA CATTATCTTGTACAA	miniGal4#9 DBD rv GAGTTACGAGAGGGTGGACGATTCACA TTATCTTGTACAA
C sequence (5'→3')	miniGal4#7 AD fw TCCGGAAGAGTAGGGTATTGATTCA CATTATCTTGTACAA	miniGal4#9 AD fw GAGTTACGAGAGGGTGGACGATTCACA TTATCTTGTACAA
D sequence (5'→3')	miniGAL4 R2 See previous table	

KlGAL4 deletion variants for integration into the *K. lactis* strain DL9 and for cloning into the pCL9-vector were amplified with the fusion PCR strategy illustrated above (figure 4). The plasmid pCL9 served as template. The following primers were used.

primer	***KlGAL4-77***	***KlGAL4-67***	***KlGAL4-57***
A sequence (5'→3')	pCL9 BspTI CCTTAAGTGTAATGTATGGGTGTGATCTCTGTCCTCCGCC		
B sequence (5'→3')	KlGal4 381 up CTTTTAGCGAGATATAC AGAGTCTGAATCGATAT TATTCG	KlGal4 472 up GTGCTATGGTTAAATCT GTGGATGAATCGATATT ATTCGA	KlGal4 557 up TAGTGGCAGATGAAGCT GGTAATGAATCGATATT ATTCGA
C sequence (5'→3')	KlGal4 381 down CGAATAATATCGATTCA GACTCTGTATATCTCGC TAAAAG	KlGal4 472 down TCGAATAATATCGATTC ATCCACAGATTTAACCA TAGCAC	KlGal4 557 down TCGAATAATATCGATTC ATTACCAGCTTCATCTG CCACTA
D sequence (5'→3')	pCL9 Eco91I TGGTTACCTACAATGTGCATGATGGACACTGTTCGTACCA		

The PCR protocol for the fusion PCR is illustrated in the following table. Since Pfu-polymerase was used, the amplification time X was 2 min/kb.

temperature	**time**	
94 °C	5'	
94 °C 60 °C 72 °C	1' 3' X + 30''	4x
94 °C 65 °C 72 °C	1' 1' X	25x
72 °C	5'	

The next table summarizes primers used for control PCR and sequencing reactions regarding FI4G80Myc-derivate and *Kl*Gal4-variant construction.

primer	**sequence (5'→3' direction)**
GAL4 FW	ACTTCGGGCCTTTTTCTGTT
GAL4 RV	TCCCTGTAGTGATTCCAAACG
GAL4L1	CAGGGATGCTCTTCATGGAT
GAL4L2	GGTCTTCGAGTCAGGTTCCA
GAL4L3	GGAACACCCTTGGCTATCCT
GAL4L4	TCACAGTGTGCAATCCCATT
GAL4L5	CAGTCACGCCTTTTCTAGGG
GAL4L6	AGGGCAGTAGGGGTGAAAAT
GAL4L7	GGTCCCTATTCAAGCCCAAT
GAL4R1	TTTGTGCTGCATCGCTTAAC
GAL4R2	ACGTTCGATCCAAACCCTTT
GAL4R3	CCGCGTCCTTTGAGACAG
GAL4R4	TTGTACAAATAATCCTGTTAACAATGC
GAL4R5	TCTGACAGAAGTGGAATCAAGG
GAL4R6	CACTGATATTGTTGACATTTTCCTC
ScURA1	TCTGTGCTCCTTCCTTCGTT
ScURA2	GATGACAAGGGAGACGCATT
LAC9-2 L1	TTGTTGTTGTTGGCATTGCT
LAC9-2 R1	GCGGATAACAATTCCCCTCT
LAC9-2 L2	TCCCAAACTGGGAAAAGTTC
LAC9-2 R2	AATGAAACTGGCACTGATGC
LAC9-2 L8	TGCCACCTGACGTCTAAGAA
LAC9-2 R8	AGGCATCAACAAAAGCCAAC

The following primers were used in Chromatin immunoprecipitation (ChIP) experiments.

Primer	**sequence (5'→3' direction)**
Gal1 up	CCTTCTCTTTGGAACTTTCAG
Gal1 down	GGGCCAGGTTACTGCCAATT
Real time PCR primer	
RT-GAL1UAS1 left	CGTTCCTGAAACGCAGATGT
GAL1UAS1_4_R	GCCAGGTTACTGCCAATTTT

The primers in the next table were used to construct the plasmids for recombinant protein expression (see 2.3).

primer	**sequence (5'→3' direction)**	**template-/target -/product vector**
BamHILAC9-2neu	TCGGGATCCACTATACGAAATGGGTAG	pLAC9-2/pET-15b/pETNHG4
HindIIILAC9-2rv	GGGAAGCTTATTGACTACGGAAAAGAG	pLAC9-2/pET-15b/pETNHG4
HindIIIKlGAL80neu	GATAAGCTTGGAGGATCGAGATCTCGA	pETG80BH/pETNHG4/ pETNHG4-NHG80
EcoRIKlGAL80rv	AAGGAATTCGCGGGATATCCGGATATA	pETG80BH/pETNHG4/ pETNHG4-NHG80
pGex KlGal4 fw	AGGATCCATGGGTAGTAGGGCCTCCAA	pLAC9-2/pGEX-6-P1/pGSTG4
pGex KlGal4 rv	GCGGAATTCTTACTCGTCGTTATCAAAT	pLAC9-2/pGEX-6-P1/pGSTG4
KlGal80XmaI fw	TCCCCGGGGAAATAATTTTGTTTAAC	pETG80BH/pGSTG4/ pGSTG4-NHG80
KlGal80NotI rv	ATGCGGCCGCTTATATCATTATTTTC	pETG80BH/pGSTG4/ pGSTG4-NHG80

For Electrophoretic Mobility Shift Assays (EMSA) 5'-biotinylated oligonucleotides were dissolved in bidest water to a concentration of 100 pmol/µl. For annealing a dilution containing 1 pmol/µl of each of the complementary oligonucleotides was heated up to 95°C for 5 min. and slowly cooled down in the switched off thermoblock.

Oligonucleotide	sequence (5'→3' direction)
EMSA Gal4 CZ-1	BIO-AGCTTCCCGGGAAGCGCTTCCCGGG
EMSA Gal4 CZ-2	BIO-GATCCCCGGGAAGCGCTTCCCGGGA

2.6 Synthetic peptide (AD-22)

A peptide consisting of the 22 C-terminal amino acids of KlGal4 (amino acid sequence: TQQLFNTTTMDDVYNYIFDNDE) was provided by Dr. F. Bordusa (Max Planck Research Unit for Enzymology of Protein Folding, Halle (Saale)). It was produced by solid phase synthesis using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) strategy.

2.7 Phenotypical characterization of yeast

A single yeast colony was resuspended in sterile water and diluted to an optical density (A_{600}) of 0.4 and serial dilutions with the factor 10^{-1} were made. 5 µl of the yeast cell suspension were dropped on selective (X-gal-) plates with the appropriate carbon source and amino acid/nucleobase mix and incubated at 30°C for 2-3 days.

2.8 β-galactosidase filter assay

The galactose analogon X-gal is an organic compound which consists of galactose linked to a substituted indole molecule and yealds insoluble blue compounds when hydrolyzed by β-galactosidase. This substrate is often used to visualize β-galactosidase activity in yeast cells, but it can usually not enter the cell membrane of *S. cerevisiae*. Therefore *S. cerevisiae* cells from selective plates were replicated on a sterile nitrocellulose filter. The filter was subsequently placed on selective medium and incubated at 30°C o/n. The filter replicate was then dunked in liquid nitrogen for 30 sec. and thawed at room temperature to make the cells permeable. Then it was placed with the yeast cells upside on a sterile sheet of Whatman paper soaked in X-gal reaction solution (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0, 0.27 % (v/v) β-mercaptoethanol, 490 µM X-gal (from X-gal stem solution in N,N-DMF)) and incubated in a closed petri dish at 30°C up to 24 h.

2.9 Transformation procedures

2.9.1 Preparation and transformation of chemocompetent *E.coli* cells

200 ml LB medium supplied with 20 mM $MgSO_4$ in a 500 ml shaking flask was inoculated with 100 µl of an *E. coli* (DH5α or Rosetta (DE3)pLysS) preparatory culture and shaked at 37°C and 160 rpm to a densitity A_{600} of about 0.3. The culture was devided into 4 x 50 ml and pelleted for 10 min. at 4000 rpm and 4°C. The cell pellets were resuspended carefully in 20 ml ice cold TfbI buffer and incubated on ice for 30 min. The cells were pelleted again united in 4 ml ice cold TfbII. 100 µl aliquots of the competent E.coli cell suspension were stored at -70°C.

TfbI 30 mM Potassiumacetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2 \cdot 4\ H_2O$, 15 % Glycerol, pH 5.8 (dilute acetic acid)

TfbII 10 mM MOPS, 75 mM CaCl2, 10 mM RbCl, 15 % Glycerol, pH 6.5 (KOH)

100 µl of the chemocompetent *E.coli* cells were thawed and incubated with 0.1-0.5 µg plasmide-DNA or 20 µl of a ligation mixture on ice for 20-30 min. The cells were exposed to a 42°C heat shock for 90 seconds and subsequently cooled on ice for 5 min. Cells transformed with a ligation mixture were incubated with LB medium on a shaker at 37°C prior to plating on LB agar plates containing antibiotics. Transformed *E.coli* cells were pelleted for 3 min. at 4000 rpm, resuspended in 100 µl LB medium and variable volumes were plated.

2.9.2 Preparation and transformation of chemocompetent yeast cells

50 ml YEPD main culture was inoculated with an 3 ml o/n culture and grown to an optical density A_{600} of 0.5 for *K.lactis* or 0.8 for *S.cerevisiae* at 30°C in a shaking flask. Cells were pelleted and resuspended in 2 ml PLAG solution. After addition of 250 µl of 10 mg/ml RNA prepared from *E.coli* 200 µl aliquots were prepared and frozen at -70°C.

PLAG 40 % PEG400, 0.1 M LiAc, 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 15 % glycerol (v/v)

~ 0.5 µg plasmid-DNA or 1-5 µg linear DNA was added to the frozen cells and incubated at 37°C and 500 rpm for 30 min. on a thermomixer, followed by 1 h incubation at 42°C. The whole cell suspension was plated on selective medium and incubated at 30°C.

2.10 Preparation and manipulation of DNA

2.10.1 Plasmid isolation from *E.coli*

Plasmids were prepared in a small scale with the GeneJET™ Plasmid Miniprep Kit (Fermentas, Thermo Scientific) according to the manufacturer's instructions.

2.10.2 Isolation of chromosomal DNA from yeast

Cells from 2 ml YEPD over night culture were collected and washed with 500 µl H_2O. The cells were lysed by vortexing (3 min.) with 200µl Breaking buffer, 200 µl glass beads and 200 µl Phenol/Chloroform/Isoamylalcohol. After addition of 400 µl TE buffer the suspension was centrifuged at 14000 rpm for 5 min. The aequeous phase was applied to Ethanol precipitation and nucleic acids were pelleted at 14000 rpm for 5 min. The pellet was carefully resuspended in 200 µl H_2O and incubated with 7µl of an 1mg/ml RNAse A solution at 37°C for 15 min. A second ethanol precipitation took place by adding 500 µl Ethanol and Ammoniumacetate to a final concentration of 6 mM and centrifugation at 14000 rpm for 5 min. The dry DNA pellet was resuspended in a variable volume H_2O.

Breaking buffer 2% TritonX 100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0

2.10.3 General enzymatic manipulation of DNA

General recombinant DNA techniques were performed according to the protocols of the suppliers of enzymes and reagents. Restriction enzymes and most other DNA modifying enzymes were purchased from Fermentas (Fermentas, Thermo Scientific). Control PCR reactions were performed with the Roche PCR Master Mix (Roche).

2.10.4 DNA sequencing

Cycle sequencing reactions were performed on high purity plasmid DNA using the ABI dRhodamine Termination Cycle Sequencing kit according to the manufacturer's protocol. Products were analysed on an ABI Abiprism 377 DNA sequencer.

2.11 Chromatin Immunoprecipitation

2.11.1 Crosslinking of proteins and cell harvest

I4G80Myc derived yeast cells were grown in 50 ml sc-medium containing 2 % raffinose to an OD_{600} between 0.8-1.0, then galactose was added to a final concentration of 0.5 % as indicated. For preparation of DNA for qRT-PCR cells were pregrown in 2 % raffinose containing sc-medium to an OD_{600} of about 0.5. Then cultures were shifted to sc-medium with 2 % raffinose and 0.5 % galactose and harvestet at different time points. Proteins and DNA were crosslinked by addition of formaldehyde to a final concentration of 1.4 %. After 15 min. glycin was added to a final concentration of 125 mM and incubated for 5 min. The cells were harvested by centrifugation (5', 4000 rpm, 4 °C) and washed twice with 20 ml ice cold TBS buffer (150 mM NaCl, 20 mM Tris/HCl, pH 7.6). The washed cells were frozen at -70 °C.

2.11.2 Cell lysis and chromatin shearing

Cells were resuspended in 400 µl ice cold Lysis buffer with protease-inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche) and vortexed at 4°C (6x 3min.) with 500 µl glass beads. After addition of further 100 µl Lysis buffer the cell suspension was separated from the glass beads and the chromosomal DNA was sheared into 250-500 bp fragments by sonication (Diagenode bioruptor, power setting H, interval 30''/30'', 10 min.). The suspension was cleared by centrifugation at 14000 rpm for 5 min. (2x). The clear lysate could be stored at -70°C.

2.11.3 Chromatin precipitation

To prevent high backround in the precipitation step, the cell lysate was incubated with a 50 % Protein A-sepharose (Protein A sepharose-4B, Invitrogen) suspension for 1 hour. 1 mg of whole cell protein was filled up to a final volume of 200 µl with ice cold lysis buffer and incubated with 0.5 µg antibody (c-Myc (A-14) or Gal4 (DBD) X; Santa Cruz Biotechnology) over night. Antibody bound protein-DNA-complexes were pricipated by addition of 60 µl 50 % Protein A-sepharose suspension for 1 h. 50 µl of the supernatant was added to 200 µl TE/ 1 % SDS as "total"-DNA control. The sepharose beads were washed successively with Lysis buffer (2x), Lysis buffer 500, LiCl-detergence buffer and TE buffer for 5 min. at room temperature. The sepharose beads were incubated with elution buffer at 65°C and 950 rpm for 10 min. followed by 150 µl TE/ 0.67 % SDS shortly and the "precipitate" as well as the "total" were incubated at 65°C over night to

reverse the crosslink. Protein in the sample was digested by addition of 250 µl Proteinase K solution at 37°C (precipitate 30 min., total 2 h). The chromosomal DNA fragments were extracted twice with 55 µl 4M LiCl and 500 µl Phenol/Chloroform/Isoamylalcolhol (25:24:1, Roth) and precipitated with 1 ml 96 % Ethanol (15', 14000 rpm). The pellets were washed with 750 µl 75 % Ethanol. The precipitates were resuspended in 100 µl TE. The totals were resuspended in 50 µl TE, incubated with RNase A at 37 °C for 30 min. and filled up to a final volume of 1 ml. Totals and precipitates could be stored at -20 °C.

Lysis buffer	0.1 % (w/v) deoxycholic acid (sodium-salt), 1 mM EDTA, 50 mM HEPES/KOH, 140 mM NaCl, 1 % TritonX-100, pH 7.5
Lysis buffer 500	0.1 % (w/v) deoxycholic acid (sodium-salt), 1 mM EDTA, 50 mM HEPES/KOH, 500 mM NaCl, 1 % TritonX-100, pH 7.5
LiCl-detergence buffer	0.5 % (w/v) deoxycholic acid (sodium-salt), 1 mM EDTA, 250 mM LiCl , 10 mM Tris/HCl, 0.5 % NP-40, pH 8.0
Elution Buffer	10 mM EDTA, 1 % (w/v) SDS, 50 mM Tris/HCl, pH 8.0
Proteinase K solution	0.5 µl 20 mg/ml glycogen, 5 µl Proteinase K stem solution, 244.5 µl TE (pH 7.6)
Proteinase K stem solution	20 mg/ml Proteinase K in 50 mM Tris/HCl, 1mM $CaCl_2$, pH 8.0
TE buffer	10 mM Tris/HCl, 1 mM EDTA, pH 7.6

2.12 Analysis of ChIP samples with quantitative Real Time PCR

Quantitative Real Time PCR analysis was performed with the Bio-Rad MyiQ™2 Real-Time PCR System and the iQ™5 software version 2.1 using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas/Thermo Scientific) in 96-well PCR-plates (Eppendorf; sealing sheets, Thermo Scientific). The reaction volume was 15 µl per well.

Component	Concentration of stem solution	Concentration/volume in reaction mixture
SYBR Green Master Mix	2x	1x
forward primer	2 µM	0.3 µM
reverse primer	2 µM	0.3 µM
fluorescein	1 µM	10 nM
template	-	4 µl
nuclease free water	-	1.1 µl

PCR-protocol:

temperature	time	
95 °C	10’	
95 °C 55 °C 72 °C	10’’ 15’’ 30’’	40x

A melting curve was recorded following the PCR protocol to control the quality of the PCR products and exclude the occurrence of smaller fragments. Recording of the melting curve started at 50 °C, the temperature was elevated in 0.5 °C steps every 10 seconds for a number of 101 cycles.

The DNA levels were quantified according to the standard curve method. Therefore a serial dilution of a certain “total”-sample covering the area of concentrations of the ChIP samples was prepared for every run. The qRT-PCR program then allows an automatic calculation of the starting quantities (SQ) in the samples by comparing the Ct values of the samples and standards. The relative amounts of precipitated DNA could therefore be calcultated as the ratio of SQ (precipitate)/SQ(total).

2.13 Determination of galactose concentration in yeast culture supernatants

The galactose concentrations of yeast culture supernatants were determined with the ENZYTEC™ Lactose/D-Galactose kit according to the supplier's instructions. The assay volumes were reduced to 825 µl.

2.14 Preparation and manipulation of proteins

2.14.1 Extraction of proteins from yeast

Whole cell extracts were prepared by glass-bead disruption. Typically the cell pellet from a 50 ml culture grown to mid- to late-logarithmic phase was washed and resuspended in 400 µl of icecold B60-buffer. 300 µl of 0.4 mm glass beads were added and cells were disrupted (3 x 4 min., 4°C) in a Braun homogenizer at maximum speed. Debris was removed from the extracts by 5 min. centrifugation (20.000 x g, 4°C) in an Eppendorf cooled tabletop centrifuge, supernatant was transferred in a new tube and centrifuged for further 20 min. The supernatants were kept on ice until further processing.

B60 buffer	50 mM HEPES/KOH pH 7.3, 60 mM Potassiumacetate, 5 mM Magnesiumacetate, 0.1 % Triton X-100, 10 % glycerol (v/v), 1 mM NaF, 20 mM glycerolphosphate, 1 mM DTT

2.13.2 Determination of protein concentration

Concentrations of soluble protein in extracts were determined by the method of Bradford (1976) in 1:5 diluted Bio-Rad Protein Assay dye reagent concentrate (Bio-Rad). Bovine serum albumin (BSA) was used as standard in concentrations from 0.625 to 20 µg/ml.

2.14.3 SDS-Polyacrylamid Gel Electrophoresis (SDS-PAGE) and Western Blot analysis

SDS-PAGE was performed according to the standard method of Lämmli (1970) with the Bio-Rad Mini-PROTEAN equipment. Usually 10 % acrylamide gels were cast and run in Lämmli buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) at 160 V. Samples were mixed with 5x SDS-sample buffer

(0.5 M Tris/HCl pH 6.8, 50 % glycerol, 20 % SDS, 25 % β-mercaptoethanol, 0.02 % bromophenol blue) prior to application to the gel.

Component	**Resolving gel**	**Stacking gel**
Water	4.8 ml	3.1 ml
resolving gel buffer (2 M Tris pH 8.8)	1.9 ml	
stacking gel buffer (0.5 M Tris pH 6.8)		1.25 ml
acrylamide (30 %)	3.33 ml	0.66 ml
SDS (20 %)	50 µl	25 µl
APS (20 %)	50 µl	30 µl
TEMED	5 µl	3 µl

After electrophoretic separation the proteins on the gel were stained with Colloidal Coomassie G250 solution (20 % ethanol, 2 % phosphoric acid, 10 % ammonium sulphate, 0.01 % G250) or transferred to a nitrocellulose membrane (Amersham Hybond-ECL, GE) with the “Bio-Rad Mini Trans-Blot Cell” in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 1 h at 100 V. After blotting the membrane was blocked in TBS-T/ 5 % milkpowder (TBS-TM) and incubated sequentially with primary and secondary antibody in the appropriate dilution (in TBS-TM). The membrane was washed between each step with TBS-T buffer. Proteins were detected with the ECL Plus Western Detection System (GE Healthcare) on an X-ray film (CEA). Kodak developer and fixer were purchased from Sigma-Aldrich.

Antibodies

Primary antibody	**Secondary antibody**
GST (B-14) (Santa Cruz Biotechnology)	anti-mouse IgG (Santa Cruz Biotechnology)
His-probe (H3) (Santa Cruz Biotechnology)	anti-mouse IgG (Santa Cruz Biotechnology)
GST-LAC9HX (Zachariae et al. 1993)	anti-rabbit IgG (Santa Cruz Biotechnology)

2.14.4 Purification of the *Kl*Gal4-*Kl*Gal80 complex

The frozen cells were resuspended in ice-cold buffer A supplemented with 1 mM PMSF and broken by sonication (Branson Sonifier S-250A, USA). After centrifugation (10000 rpm, 20 min) the supernatant was incubated for about 1 h with the double stranded DNA-oligo UASg3-4 (annealing of UASg3 5'-CTAGGAGCGGGTGACAGCCCTCCGAAT and UASg4 5'-CTAGATTCGGAGGGCTGTCACCCGCTC (Salmeron et al. 1989)) , which contains a high affinity binding site for *Kl*Gal4. After a second centrifugation step (25000 rpm, 1 h) the clear supernatant was applied to a HisPrepFF 16/10 (GE Helthcare) column at a flow rate of 2 ml/min. The column was washed with 20 % buffer B, which equates approximately 100 mM imidazole. The protein-complex was eluted with 40 % buffer B (about 300 mM imidazole).
The highest peak fractions (UV 280 nm) were applied to a 5 ml GSTrap FF column (GE Helthcare) (0.3 ml/min.), the column was washed with 3 CV buffer C and the protein eluted with buffer C including 10 mM reduced glutathione (2 ml/min).

Buffer A	20 mM HEPES, 0.5 M NaCl, 20 mM imidazole, 0.1 M $MgCl_2$, 1 mM EGTA, 0.1 % (v/v) Tween20, pH 7.2
Buffer B	20 mM HEPES, 0.5 M NaCl, 0.5 M imidazole, 0.1 M $MgCl_2$, 1 mM EGTA, 0.1 % (v/v) Tween20, pH 7.2
Buffer C	20 mM HEPES, 0.1 M NaCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 µM $ZnCl_2$, pH 8.6

2.14.5 Purification of *Kl*Gal1

N-terminal His_6-tagged *Kl*Gal1 was purified using a HisPrepFF 16/10 column according to the conditions of the *Kl*Gal4-*Kl*Gal80 purification. Instead of incubation with the oligo UASg3-4 the supernatant was incubated with 0.3 % streptomycin sulfate for nucleic acid precipitation. Following the affinity purification the protein was applied to gelfiltration (HiLoad 16/600 Superdex 200 pg, GE Helthcare) using buffer D. The purified protein was dialyzed against buffer D including 10 % glycerol and frozen in liquid nitrogen.

Buffer D	20 mM HEPES, 0.1 M NaCl, pH 7.0

2.14.6 Colorless Native PAGE

Native 180 x 190 x 1 mm gradient gels were composed as follows:

Component	footgel	3% res. gel	12% res. gel	stacking gel
bidest H_2O	0.138 ml	7.27 ml	1.97 ml	5.0 ml
0.5 M Bistris pH 7.0	0.1 ml	1.5 ml	1.5 ml	1.0 ml
30 % acrylamide solution	0.5 ml	1.55 ml	6.1 ml	1.3 ml
2 M ε-aminocaproic acid	0.25 ml	3.75 ml	3.75 ml	2.5 ml
87 % (v/v) glycerol	-	0.89 ml	1.6 ml	-
10 % (w/v) APS	10 µl	70 µl	70 µl	90 µl
TEMED	2 µl	7 µl	7 µl	9 µl

The gradient gel was cast with the help of a gradient mixer (Hoefer SG50).
Samples were mixed with 4x CN-sample buffer (60 % glycerol, 200 mM Bistris pH 7.0, 0.1 % Ponceau S) and centrifuged at 13000 rpm for 5 min. at 4°C prior to application on the gel. Electrophoresis was done at 100 V in a cold room.

Cathode buffer: 50 mM Tricine, 15 mM Bistris
Anode buffer: 50 mM Bistris pH 7.0
Marker proteins: Thyroglobulin (669 kD, 7.6 mg/ml), Ferritin (440 kD, 5 mg/ml), Aldolase (158 kD, 5.2 mg/ml), Albumin (66 kD, 6.5 mg/ml)

Gels were either stained with Colloidal Coomassie G250 solution or single stripes were cut out and transferred to a denaturing gel for 2D-electrophoresis.

2.14.7 2D-Gel electrophoresis

180 x 190 x 1.5 mm SDS- gels were composed as follows:

Component	10 % resolving gel	5 % stacking gel
Bidest H_2O	22.5 ml	8.7 ml
30 % acrylamide	16.0 ml	2.1 ml
2 M Tris pH 8.8	9.0 ml	-
1 M Tris pH 6.8	-	1.58 ml
10 % SDS	480 µl	125 µl
10 % APS	200 µl	100 µl
TEMED	20 µl	10 µl

Stripes of the CN-gel were denatured in 100 ml denaturing buffer (90 ml Lämmli buffer, 10 ml 10 % SDS, 1 ml β-mercaptoethanol) for 5 min. at 60°C in a water bath prior to application to the SDS-gel. 10 µl protein marker (PageRuler™ Prestained Protein Ladder, Fermentas) was applied to the gel on a small slice of Whatman paper, shoved into the stacking gel. The CN-gel stripe was fixed with 1 % Agarose (Tris pH 6.8) containing bromophenol blue. Electrophoresis was performed at 60 V in Lämmli buffer.

2.15 Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed with the help of the LightShift® Chemoluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's instructions. A test-reaction usually consisted of the following components:

Component	volume	concentration in the reaction
H_2O	ad 20 µl	
10x BP	2 µl	1x
$MgCl_2$ (100mM)	2 µl	10 mM
Poly dIdC (200 ng/µl)	5 µl	50 ng/µl
EDTA (200mM)	0,5	5 mM
unlabeled DNA (2 pmol/µl)	2 µl	200 fmol/µl
Protein (6 µg)	x	300 ng/µl
Biotin-labeled DNA (10 fmol/µl)	2 µl	1 fmol/µl

3 Results

3.1 Functional determination of mini*Sc*Gal4-variants

Analysis of the *Sc*Gal4-protein have shown that about 80 % of the internal region of the protein can be deleted without complete loss of transcriptional activity (Ma & Ptashne 1987a). Ding and Johnston created so called miniGal4 variants consisting of variable N- and C-terminal parts of the protein and tested their ability to activate β-galactosidase reporter gene expression in yeast (Ding & Johnston 1997). The variant miniGal4-7, comprising the residues 1-237, 412-424 and 680-881, exhibited about 66 % of the wild-type protein activity. The variant miniGal4-9, consisting of residues 1-168 and 727-881 had a lower activity of about 47 % of that of the wild-type protein. Both variants were expressed to nearly wild-type protein levels from a single copy vector and behaved similar to wtGal4 regarding glucose- and Gal80-repression, DNA-binding and interaction with other proteins of the transcriptional machinery. Interestingly in a GST-pulldown assay, K. Melcher found that Gal4 variants lacking the well characterized Gal80 binding site overlapping the activation domain (Gal4stop841) were still able to interact with the inhibitor protein Gal80. Specifically the miniGal4-7 variant lacking the activation domain (miniGal4-7stop841, see figure 5) like full length Gal4stop841 but not miniGal4-9stop841 (miniGal4-9 lacking the activation domain) could pull down Gal80 in that assay (unpublished data). He confirmed this finding in a yeast-two-hybrid (Y2H) assay with β-galactosidase as the reporter gene in cells expressing the truncated miniGal4stop841 variants and Gal80 fused to the VP-16 activation domain. Unfortunately in his assay the miniGal4-7stop841 variant showed a very high reporter gene activity even in the absence of Gal80, but this activity increased significantly upon VP16-Gal80 expression. Because the miniGal4-7stop841 variant possesses residues, which are missing in the miniGal4-9stop841 variant, it might comprise for a second Gal80 binding site on the Gal4 protein that could play a role in the activation process.

3.1.1 All AD-truncated (mini)Gal4 variants induce transcriptional activation in a yeast-two-hybrid assay

We entered into a cooperation with K. Melcher who provided the plasmid constructs and first tried to verify the Y2H assay results. The respective AD-truncated (mini)Gal4 variants were tested for Gal80 interaction in a yeast-two-hybrid coupled β-galactosidase filter assay.

Therefore the VP16-Gal80 coding plasmid pVP16-80 and a (mini)Gal4stop841-coding plasmid (see in the materials and methods section) were co-transformed into the Y2H-strain MaV103 and transformants were grown on selective medium. A dilution series of the yeast suspension was spotted on selective medium and replicated on a nitrocellulose filter. β-galactosidase activity could be detected on the filter after addition of X-gal containing solution.

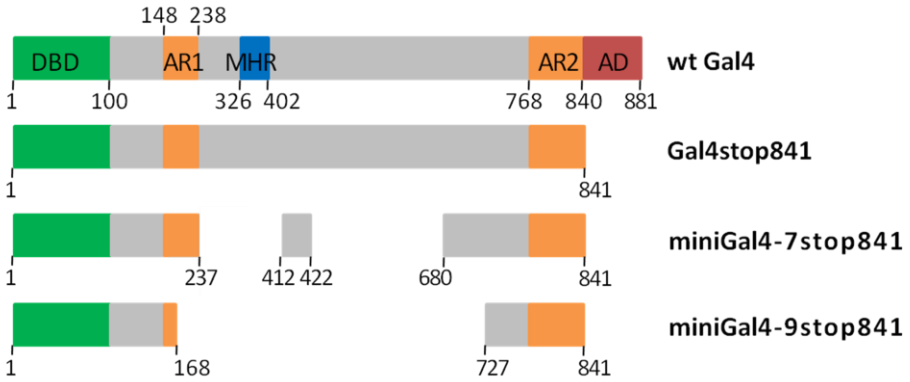

Figure 5: Schematic view of (mini)Gal4stop841 variants. The numbers indicate the positions of amino acid residues. DBD, DNA binding domain; AR1/2; activating region 1/2; MHR, middle homology region; AD, activation domain.

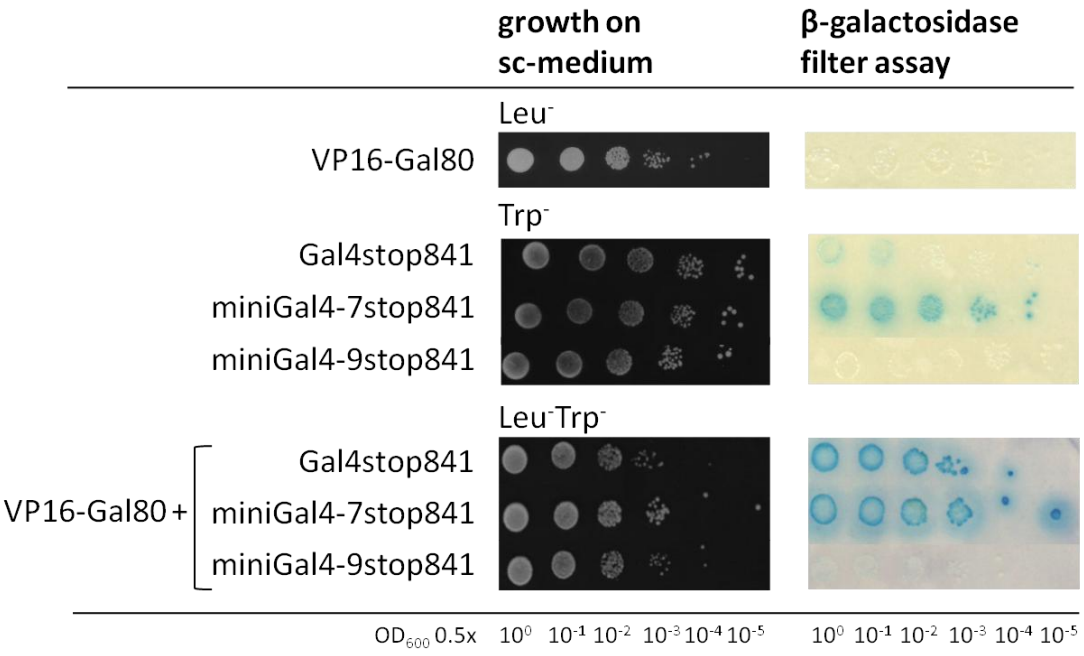

Figure 6: β-galactosidase filter assay. MaV103 cells were transfected with the indicated plasmids were grown on sc-medium lacking the amino acids leucine, tryptophane or both for 3 days (left hand side). Replicates of the cells were made on a sterile nitrocellusose filter and incubated at 30°C o/n (right hand side). The β-galactosidase activity (blue precipitate of colour) was detected 24 h after cell permeabilization and X-Gal addition.

As can be seen in figure 6, miniGal4-7stop841 and to a low extend Gal4stop841 are able to activate β-galactosidase expression even in the absence of VP16-Gal80, an observation that is consistent with the Y2H assay made by K. Melcher. There is no β-galactosidase activity in the cells expressing the miniGal4-9stop841 variant in the absence of VP16-Gal80. Co-expression of VP16-Gal80 and Gal4stop841 or miniGal4-7stop841 leads to clearly enhanced β-galactosidase expression. In the cells co-expressing VP16-Gal80 and the miniGal4-9stop841 variant a very weak blue signal could be detected after 24 hours. A weak interaction of miniGal4-9stop841 with VP16-Gal80 can therefore be assumed.

Because of the apparent activity of the miniGal4-7stop841 and Gal4stop841 constructs in the absence of Gal80, this assay seems to be inappropriate to monitor the presence of a hypothetical secondary binding site for Gal80 on the Gal4 protein.

3.1.2 Reconstruction of miniGAL4#7/miniGAL4#9 and transformation into the *S. cerevisiae* strain I4G80Myc

For further studies *S. cerevisiae* strains expressing different Gal4-deletion variants from their natural locus should be constructed. Gene replacements were made at the *gal4Δ::URA3* locus in the strain I4G80Myc g4Δ which comprises a *MYC*-tagged *GAL80* gene. In addition, this strain carries the *LAC4* and *LAC12* genes of *Kluyveromyces lactis*, which are also regulated by Gal4 and serve as Gal4 activity reporters in a plate assay. The expression of the Lac12 permease allows for transport of the lactose-analogon X-gal into the cells without permeabilization and *LAC4* encodes a β-galactosidase.

A strain expressing an AD-truncated but otherwise full length Gal4 protein (Gal4ΔAD) was constructed first. The correct place of insertion was confirmed by PCR-reactions using chromosomal DNA. Unfortunately sequencing showed that a missing guanosine nucleotide at position 2513 leads to a slightly altered C-terminus. Instead of '…G_{839}- W_{840}- T_{841}' the protein ending is '…$V_{839}K_{840}M_{841}N_{842}R_{843}R_{844}Y_{845}$'. Nonetheless the strain carrying this truncated Gal4ΔAD variant (I4G80Myc G4ΔAD) was used for further analysis, because the established Gal80-binding site is either way deleted in this protein. The growth of I4G80Myc, I4G80Myc g4Δ and I4G80Myc G4ΔAD was then tested on X-gal plates containing either glucose or galactose as carbon source (see figure 7). The strains grow well on glucose plates. On galactose plates Gal80 dissociates from the AD of wtGal4 and *LAC*-gene expression becomes visible. The control strain harbouring the *gal4*-deletion does not grow on galactose plates because of the absence of *GAL*-

gene expression. The Gal4ΔAD expressing strain does also not grow on galactose plates. Contrary to the observation made in the β-galactosidase filter assay there is no activation function of Gal4ΔAD visible in a strain expressing this protein from its chromosomal locus. It is expected that miniGal4-proteins lacking the activation domain would neither be able to grow on galactose plates. Therefore miniGal4-variants were constructed which are not truncated for the activation domain. Since the miniGal4-7stop841 and miniGal4-9stop841 variants used above differ markedly in their central region, it is difficult to say which of them are responsible for their different ability to interact with Gal80 or their ability to activate transcription. The most obvious candidate residues are the 47 amino acids 680 to 725, which include the phosphorylation site Ser699. These residues are present in miniGal4-7stop841, but not in the miniGal4-9stop841 protein. Therefore the new miniGal4#7 and miniGal4#9 variants, which were constructed with the help of a fusion PCR strategy (see materials and methods, figure 4) differ only in the length of their C-terminus. The new proteins consist of the N-terminal 100 residues spanning DNA-binding and dimerization domain and residues 680-881 (miniGal4#7) or 725-881 (miniGal4#9), respectively (see figure 7). The resulting strains were termed I4G80Myc mG4#7 and I4G80Myc mG4#9. For control experiments I4G80Myc g4Δ was also transformed with the smallest known active and regulatable miniGal4 variant consisting of the residues 1-100 + 840-881, leading to the strain I4G80Myc mG4. This miniGal4 variant was described by Wu et al. (1996) and has an activity of about 30-40 % of that of the full length Gal4 protein when expressed from a low copy number plasmid in yeast.

The correct sequence and place of insertion was tested and could be confirmed for all miniGal4-constructs.

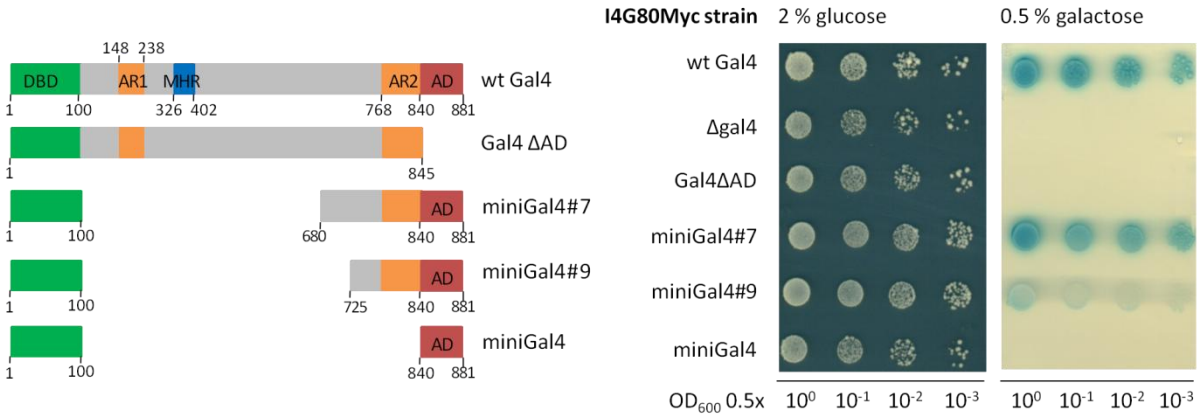

Figure 7: Reconstructed (mini)Gal4-variants. The left hand side of the figure shows the (mini)Gal4-variants which are expressed in the I4G80Myc strains. On the right, the growth of the different strains on sc-medium containing 2% glucose/X-gal or 0.5 % galactose/X-gal after 3 days at 30°C is shown. The blue precipitate in the galactose grown cells indicates *GAL*-gene expression.

The strains expressing miniGal4#7 or miniGal4#9 do both grow on glucose. They also grow on galactose containing plates. Therefore both variants are able to activate *GAL*-gene expression and expression of the integrated *LAC*-genes, visible by the blue precipitate in the cells. However the miniGal4#9 expressing strain seems to grow worse than the miniGal4#7 strain on galactose and the blue signal is weak. Unfortunately there was no growth detectable of the cells expressing miniGal4 on galactose plates, either because of the relatively short time of incubation (3 d) or because this variant in poorly expressed or unstable in the yeast cells.

3.1.3 Qualitative analysis of (mini)Gal4-DNA-binding and (mini)Gal4-Gal80- binding

Since none of the Gal4 variants were detectable in a Western Blot, probably due to the low protein concentration, the expression of the proteins was demonstrated in a qualitative Chromatin immunoprecipitation (ChIP) experiment. In preparation of the experiment the yeast strains were grown in 2% raffinose containing medium to an OD_{600} between 0.8-1.0, then galactose was added to a final concentration of 0.5 % and cells were grown for additional two hours before crosslinking. Chromatin immunoprecipitation was performed with either an antibody against the DNA binding domain of Gal4 or anti-myc antibody to see whether the different Gal4 variants interact with Gal80 (control = no antibody). For PCR reactions primers were used that bind within the *GAL1* promoter and amplify a product of about 240 basepairs. If a Gal4 variant is expressed in the cells and binds to the *GAL1* promoter, then this region should be enriched in the ChIP and a PCR product should be detected. This was the case for all Gal4 variants under inducing as well as non-inducing conditions. The control with the *gal4*-deleted

strain shows that the signal depends on the presence of Gal4 and neither the Gal4-DBD-antibody nor the Myc-antibody gave high background.

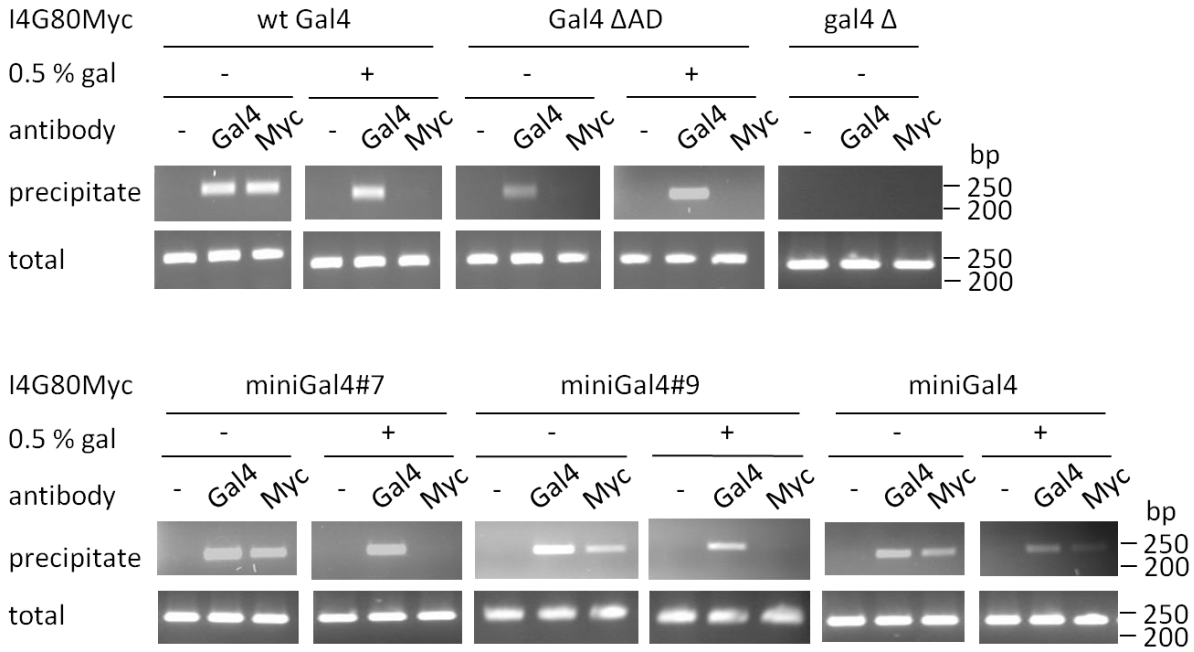

Figure 8: Qualitative ChIP analysis. The ethidium bromide stained agarose gels show the PCR-products from initial ChIP-analysis. For antibody-precipitation 1 mg of total protein (5 µg/µl) and 0.5 µg antibody (c-Myc (A-14) or Gal4 (DBD) X; Santa Cruz Biotechnology) was used. The PCR-reactions were performed with 4 µl of the precipitate respectively 1 µl total DNA from the sample before precipitation. The primers selected for the PCR reactions bind within the *GAL1* promoter.

The ChIP with the antibody against Myc-tagged Gal80 should show whether Gal80 binds to a specific Gal4-variant under inducing and non-inducing conditions. If Gal80 binds to Gal4, then it should be crosslinked with the activator at the *GAL1* promoter, leading to an enrichment of the region in the ChIP assay. It can be seen in figure 8, that Gal80 binds to wtGal4 as well as all miniGal4-variants under non-inducing conditions, but not to Gal4ΔAD. Contrary to the β-galactosidase filter assay, there is no Gal4-Gal80 interaction detectable when the activation domain of Gal4 is deleted. This finding argues against an interaction of Gal80 with different residues besides the established Gal80 binding site within the 40 C-terminal amino acids of Gal4.

Under inducing conditions Gal80 cannot be found at the *GAL1* promoter in all I4G80Myc-strains. It seems unlikely that Gal4, Gal80 and Gal3 form a stable tripartite complex while Gal4 is in the active state. This result therefore supports the dissociation model rather than the allosteric activation model.

3.1.4 Analysis of the Gal80-binding activity of the (mini)Gal4-variants at several time points after induction

To determine the dissociation respectively re-association of the different Gal4 variants with Gal80 after galactose induction, ChIP analyses at different time points after galactose addition in combination with quantitative realtime PCR were performed. For this purpose the different I4G80Myc strains were grown in 2 % raffinose containing sc-medium to OD_{600} 0.5. Then cultures were shifted to sc-medium with 2 % raffinose and 0.5 % galactose and harvestet after 1, 6, 18 and 24 hours. All experiments were run in triplicate and the binding of myc-tagged Gal80 to the *GAL1* promoter in the different I4G80Myc strains was determined relative to the value measured in the wildtype Gal4-strain under non-inducing conditions (timepoint = 0 h). Additionally the galactose consumption was determined by an enzymatic assay which is based on the conversion of D-galactose and NAD^+ to D-galactonic acid and NADH + H^+ by the enzyme β-galactose dehydrogenase. The production of NADH + H^+ leads to an increase of the extinction at 340 nm. The galactose concentration is therefore proportional to the NADH + H^+ production and could be determined in the supernatants of all yeast cultures. The concentration at 0h was supposed to be 0.5 %.

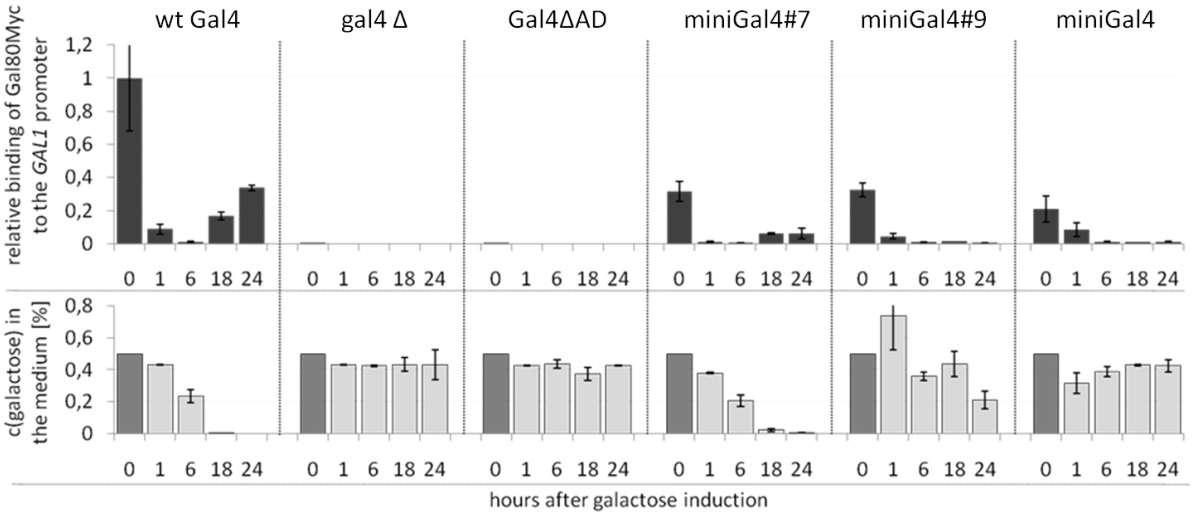

Figure 9: Quantitative ChIP analysis and determination of galactose consumption. Upper panels: relative binding of Gal80 to the *GAL1* promoter in different I4G80Myc derivate strains at different time points after galactose addition. Cells were pregrown in sc-medium containing 2 % raffinose and shifted to medium containg 2 % raffinose and 0.5 % galactose. The binding of Gal80 to the promoter is indicated relative to the value measured in the wt Gal4 strain at 0h which was set 1.0; lower panels: galactose concentration in the media of the I4G80Myc derivate strains at different time points after galactose induction.

Figure 9 shows that in the strain expressing wt Gal4 there is almost no Gal80 detectable at the *GAL1*-promoter one hour after galactose induction. The Gal80 signal reappears 18 hours after induction and increases further within the next 6 hours. It is obvious that galactose is entirely depleted at the time of recurrence of the Gal80 signal. In the Gal4ΔAD-strain there is no Gal80 signal detectable at the promoter at all, just as in the *gal4*-deleted strain. There is also no galactose consumption in these cells. Although the overall Gal80 signal in the miniGal4#7 strain is weaker than in the wildtype strain, the pattern of the Gal80 signal as well as the galactose consumption over the time is similar in both strains. This is not the case in the miniGal4#9- and the miniGal4-strain. In both strains there is no Gal80 observable at the promoter after 18 or 24 hours, and there is only a small or no decrease, respectively, in galactose concentration in the medium over time. A reason for this observation might be that these miniGal4-variants have such a low activity that complete galactose turnover would take much longer than 24 hours.
The results from this experiment show that binding of Gal80 to the Gal4 protein is strictly dependent on the presence of the activation domain. There is also no hint that Gal80 binds to an alternative site upon galactose addition. If ternary complex formation occurs, it can be only temporary and cannot be detected in the ChIP-analysis. Nevertheless the region between the residues 680 and 840 seems to be important for proper transcriptional activation.

3.2 Purification of the *Kl*Gal4-*Kl*Gal80 complex

More and more structural details of two of the key regulatorary proteins of the *GAL*-switch, the repressor Gal80 and the galactose sensor Gal3/1, and their interactions are accumulating (Kumar et al. 2008; Thoden et al. 2008; Lavy et al. 2012). In contrast to that, knowledge about the Gal4 protein and interactions between the activator and Gal80 is still fragmentary. One reason for this poor data record is that structural information of *Sc*- as well as *Kl*Gal4 is limited to the DNA binding- and activation domain. Recombinant expression and purification of the full length activator from *E. coli* has not been reported thus far. There are only some hints in the literature that the protein tends to aggregate upon expression in bacteria (Chasman & Kornberg 1990). Several attempts have been made in the past to purify the Gal4 protein from yeast. Using a quite complex and extensive protocol Chasman and Kornberg (1990) purified small amounts of *Sc*Gal4 (10 µg from a 300 ml culture) from an overproducing yeast strain transformed with a low copy number plasmid (Chasman & Kornberg 1990). Expression from a

high copy number plasmid yielded about 5-10 fold less protein, indicating that overexpression of the protein above a certain level is disadvantageous for the cells. Interestingly they co-purified the activator bound to the repressor Gal80, showing that the two proteins constitute a stable complex *in vivo*. The same observation was made by Parthun and Jaening when they purified also very small amounts of *Sc*Gal4 protein from yeast (Parthun & Jaehning 1990). The amounts obtained from expression in the natural host are too low for structural studies, and purification of high protein amounts is very elaborate and time-consuming. It is known that the amount of Gal4 protein in *K. lactis* is higher than in *S. cerevisiae*, but it is still not sufficient for purification. Overexpression of *Kl*Gal4 in the milkyeast is also disadvantageous for cell viability (Breunig 1989). Thus, to further address the question how the activator and the repressor Gal80 interact and how this interaction is relieved I have tried to express *Kl*Gal4 recominantly in *E. coli*. Recombinant expression and purification of the *K. lactis* repressor *Kl*Gal80 in *E. coli* was shown to be more effective than the *S. cerevisiae* homolog and a protocol for purification of *Kl*Gal80 is already established in our group (Anders et al. 2006).

3.2.1 Expression and purification of *Kl*Gal4 as N-terminal His_6-tagged protein (NH*Kl*Gal4)

The His_6-(Hexahistidine)-tag is the most commonly used affinity tag for protein purification. The tag is small, has a low metabolic burden and purification of His_6-tagged proteins by immobilized metal affinity chromatography (IMAC) is quite inexpensive. Therefore in a first attempt to purify the *Kl*Gal4 protein, the *KlGAL4*-gene was cloned into the pET15b-vector for expression with an N-terminal His_6-tag (*NHKlGAL4*) and transformed into the expression strain Rosetta(DE3)-pLysS. 50 ml LB-starter cultures were transferred into 500 ml induction medium (LB + Cam (35 µg/ml) + Amp (100 µg/ml) + 200 mM KH_2PO_4 + 1.5 % lactose) and incubated overnight at 30 °C and 140 rpm. The cells were harvested by centrifugation for 20 min. at 4000 rpm (Beckman Avanti J25, rotor JA10) and frozen at -70 °C. For this initial experiment buffers and purification conditions were adopted from the protocol for *Kl*Gal80 purification described by A. Anders (Anders et al. 2006; Anders 2006). Figure 10 shows the analysis by Coomassie staining and Western Blot after affinity chromatography on a Ni-NTA (Ni(II)-nitrilo-triacetic acid)- matrix. After Coomassie staining, there was only a weak band of NH*Kl*Gal4 protein visible at the estimated molecular weight of about 100 kD in the whole cell lysate. The Western Blot with an antibody against the His_6-tag revealed a signal at the expected size of 100 kD and additional bands probably

representing degradation products. Some NH*Kl*Gal4 was also detected in the insoluble fraction (not shown). Figure 10 shows, that only poor amounts of full length NH*Kl*Gal4 can be enriched via IMAC. The protein recovery was very poor and the elution fraction presumably includes degradation products.

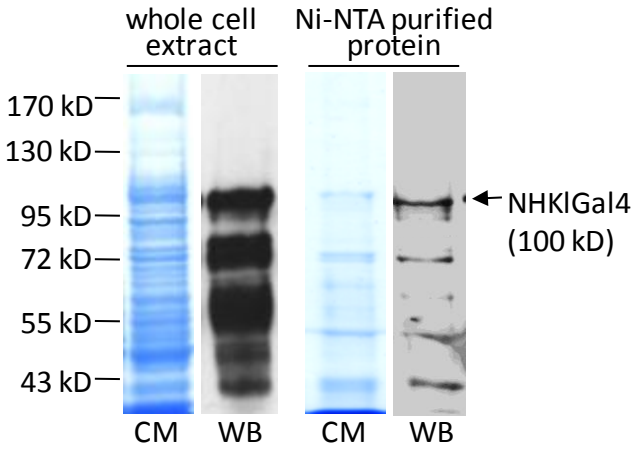

Figure 10: Coomassie stain (CM) and Western Blot (WB) analysis of NHKlGal4-purification steps. 50 µg protein from the whole cell extract and 20 µl of the elution fraction from Ni-NTA affinity chromatography were applied to a 7.5 % SDS-polyacrylamide gel for Coomassie staining. For Western Blot analysis 12.5 µg protein from the whole cell extract and 5 µl of the elution fraction from Ni-NTA purification were applied to the gel. The Western Blot membranes were incubated with an antibody against the His_6-tag.

NH*Kl*Gal4 seems to be unstable. One reason for this instability might be the very flexible C-terminal domain which is probably unfolded in the absence of an interaction partner. Therefore I decided to co-express and purify NH*Kl*Gal4 with the repressor protein *Kl*Gal80.

3.2.2 Co-expression and purification of NH*Kl*Gal4 and NH*Kl*Gal80

For co-expression in *E. coli NHKlGAL80* was amplified from pETNHG80 and cloned downstream of the *NHKlGAL4*-gene in pETNHG4 in order to construct the bicistronic expression vector pETNHG4-NHG80 (see Materials and Methods section 2.4). The vector should allow the simultaneous expression of both proteins from the same plasmid. Cell culture and purification conditions were identical to the previous experiment. The Coomassie stained SDS-polyacrylamide gel in figure 11 shows that there is a band at 100 kD, which is the expected size of the NH*Kl*Gal4 protein and also a band at 54 kD, which is the size of the NH*Kl*Gal80 protein. The Western Blot membrane which was incubated with anti-His_6-antibody also exhibits bands at 100 and 54 kD. In contrast to the solely expressed NH*Kl*Gal4 protein, there are two distinct His_6-signals. Degradation products are hardly visible. Therefore, I concluded that the co-expression of the transcriptional activator with the repressor *Kl*Gal80 indeed led to a stabilization of the protein. Unfortunately IMAC purification primarily lead to an excessive

enrichment of NH*Kl*Gal80, indicating that the purification procedure only partially enriched the stochiometric *Kl*Gal4-*Kl*Gal80 complex.

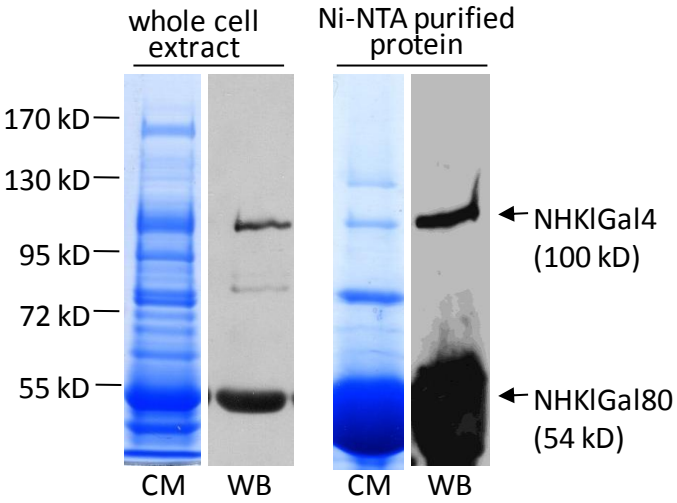

Figure 11: Coomassie stain (CM) and Western Blot (WB) of NH*Kl*Gal4-NH*Kl*Gal80 purification steps. 50 µg protein from the whole cell extract and 20 µl of the elution fraction from Ni-NTA affinity chromatography were applied to a 7.5 % SDS-polyacrylamide gel for Coomassie staining. For Western Blot analysis 12.5 µg protein from the whole cell extract and 5 µl of the elution fraction from Ni-NTA purification were applied to the gel. The Western Blot membranes were incubated with an antibody against the His_6-tag.

To get rid of excessive Gal80 and other impurities that are visible on the Coomassie stained gel, size exclusion chromatography (SEC) was performed subsequent to the IMAC purification. The Superdex 200 10/300 GL column (GE Healthcare) which was used for gel filtration was calibrated with proteins from the Gel Filtration HMW Calibration Kit (GE Healthcare) according to the manufacturer's instructions. A complex of a *Kl*Gal4-dimer bound to two molecules of *Kl*Gal80 (*Kl*$Gal4_2$-*Kl*$Gal80_2$) has an expected size of roughly 300 kD and should run between 10.8-11.2 ml using the Superdex 200 10/300 GL column. As shown in figure 12, most of the protein eluted in one peak at about 8.4 ml, which is the determined void volume of the column.

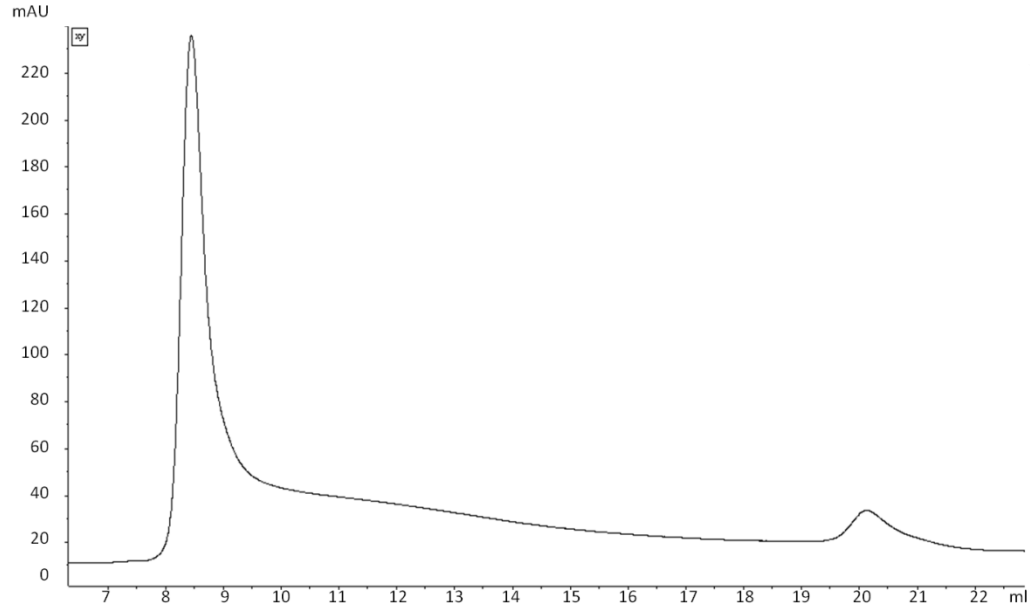

Figure 12: Size exclusion chromatography of the NHKlGal4-NHKlGal80 complex. Elution profile (λ = 280 nm) of the Ni-NTA purified NH*Kl*Gal4-NH*Kl*Gal80 complex using SEC (column: Superdex 200 10/300 GL, GE Healthcare).

The elution profile of SEC analysis indicates that there is no Gal4-Gal80 complex of the expected size of a heterotetramer. The finding that there is only one peak at the void volume rather indicates that most of the protein is aggregated or bound in complexes larger than 600 kD, which is the upper exclusion limit of the Superdex column.

3.2.3 Expression and purification of N-terminal GST-tagged *Kl*Gal4 (NG*Kl*Gal4)

One possibility to improve recombinant protein expression in *E. coli* and to enhance the solubility and stability of recombinant proteins is the fusion to the 26 kD glutathione S-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum* (Nygren et al. 1994; Smith & Johnson 1988) to the N-terminus of the protein. Since in previous studies the activation domain of Gal4 and other *Sc*Gal4-fragments (Gal4 (1-147), Gal4 (1-147)-VP16) have successfully been fused to GST (Leuther & Johnston 1992; Sil et al. 1999; Archer et al. 2008), the *KlGAL4*-gene was cloned into the pGEX-6-P1 vector for N-terminal GST-tagged protein expression (*NGKlGAL4*). As one can see in figure 13, the GST-tag led to an increased protein amount in the whole cell extract compared to His_6-tagged *Kl*Gal4. Unfortunately the binding to the glutathione-sepharose column was very inefficient, and only low amounts of NG*Kl*Gal4 protein could be recovered from the cell extract.

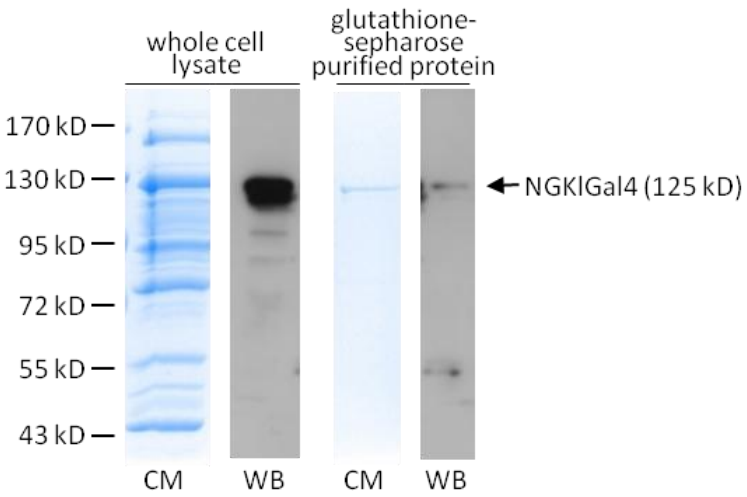

Figure 13: Coomassie stain (CM) and Western Blot (WB) of NH*Kl*Gal4 purification steps. 50 µg protein from the whole cell extract and 20 µl of the elution fraction from glutathione-sepharose affinity chromatography were applied to a 7.5 % SDS-polyacrylamide gel for Coomassie staining. For Western Blot analysis 12.5 µg protein from the whole cell extract and 5 µl of the elution fraction from the purification were applied to the gel. The Western Blot membranes were incubated with an antibody against the GST-tag.

3.2.4 Co-expression and purification of N-terminal GST-tagged *Kl*Gal4 (NG*Kl*Gal4) with NH*Kl*Gal80

Since co-expression of NH*Kl*Gal4 with NH*Kl*Gal80 worked quite well, this strategy was also used to purify GST-tagged *Kl*Gal4 with NH*Kl*Gal80. Therefore the *NHKlGAL80* gene was cloned downstream of *NGKlGAL4* into the pGSTG4 vector to yield the bicistronic expression vector pGSTG4-NHG80. Both proteins could be detected on the Coomassie stained gel and Western Blot but as for NG*Kl*Gal4 alone, recovery of the co-expressed proteins by glutathione-sepharose purification was very poor (see figure 14). Therefore purification was performed in two steps (figure 15). Buffers for purification were modified as described in materials and methods (2.13.4). Firstly, the whole cell lysate was applied to a Ni-NTA matrix and the bound complex was eluted with buffer containing 300 mM imidazole. The eluate was subsequently applied to a glutathione-sepharose column. As shown in figure 15, the binding of the NG*Kl*Gal4-NH*Kl*Gal80-complex to the glutathione-sepharose column is clearly improved after removal of the bulk of *E.coli*-proteins from the cell lysate. Further improvements could be achieved by changing the pH value of the buffer for IMAC purification from 8.0 to 7.2 because NH*Kl*Gal80 is predicted to have a pI of 8.0 and NG*Kl*Gal4 a pI of 6.1. Furthermore the buffer conditions were changed as indicated in the material and methods section. The new HEPES based buffer did not negatively influence protein stability and would be suitable for future crosslinking studies with aminoreactive crosslinkers. The protocol was then upscaled and on average 2.2 mg of protein-complex could be purified from 0.5 l main culture. The main impurities that are visible on the

Coomassie stained gel after both purification steps were analysed by mass spectrometry (S. Schaks, AG Sinz) and identified as the *E. coli* chaperon DnaK which has a molecular weight of 69 kD and the 60 kD *E. coli* chaperonine Ch60. As visible in figure 15, there is always a weaker, slightly faster migrating band below the 125 kD NG*Kl*Gal4-band which might be a degradation product of the recombinant activator, since it can also be detected on the GST-antibody probed Western Blot membrane.

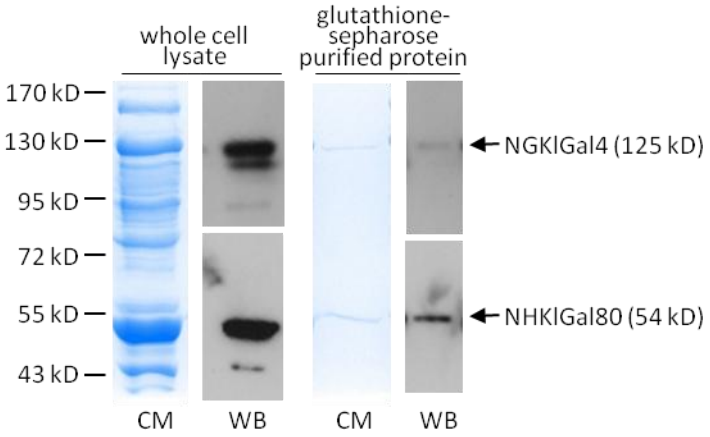

Figure 14: Coomassie stain (CM) and Western Blot (WB) of NG*Kl*Gal4-NH*Kl*Gal80 purification with glutathione-sepharose chromatography. 50 µg protein from the whole cell extract and 20 µl of the elution fraction from glutathione-sepharose affinity chromatography were applied to a 7.5 % SDS-polyacrylamide gel for Coomassie staining. For Western Blot analysis 12.5 µg protein from the whole cell extract and 5 µl of the elution fraction from the purification were applied to the gel. The Western Blot membranes were incubated with an antibody against the GST-tag (upper Western Blot membranes) or His6-tag (lower Western Blot membranes).

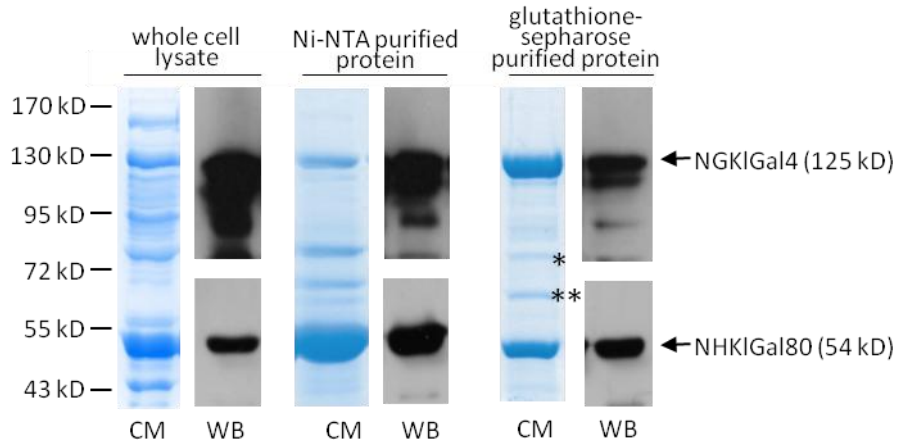

Figure 15: Coomassie stain (CM) and Western Blot (WB) of NG*Kl*Gal4-NH*Kl*Gal80 purification with Ni-NTA chromatography followed by glutathione-sepharose chromatography. 50 µg protein from the whole cell extract and 20 µl of the elution fraction from the chromatography steps were applied to a 7.5 % SDS-polyacrylamide gel for Coomassie staining. For Western Blot analysis 12.5 µg protein from the whole cell extract and 5 µl of the elution fraction from the purification steps were applied to the gel. The Western Blot membranes were incubated with an antibody against the GST-tag (upper Western Blot membranes) or His_6-tag (lower Western Blot membranes). *DnaK; **Ch60

To remove residual impurities, a final SEC step was performed using the Superdex 200 10/300 GL column. A complex consisting of a NG*Kl*Gal4-dimer and two NH*Kl*Gal80 proteins has an estimated molecular weight of 360 kD and should elute at 10.3-10.5 ml. But no sharp peak could be observed in the elution profile at this volume (figure 16). A broad peak around the void volume at 8.4 ml indicates that most of the protein is either aggregated or bound in complexes larger than 600 kD. There are also several smaller proteins visible in the elution profile, but they are diffusely distributed over the whole range of separation.

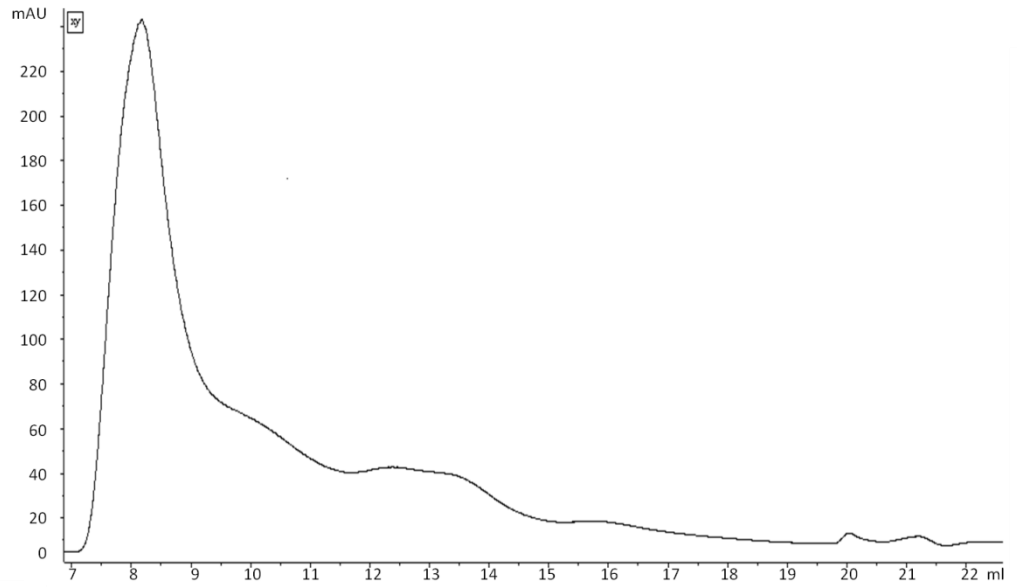

Figure 16: Size exclusion chromatography of the NGKlGal4-NHKlGal80 complex. Elution profile (λ = 280 nm) of the Ni-NTA purified NG*Kl*Gal4-NH*Kl*Gal80 complex using SEC (column: Superdex 200 10/300 GL, GE Healthcare).

3.3 Biochemical characterization of the purified NG*Kl*Gal4-NH*Kl*Gal80 complex

3.3.1 Molecular weight determination of the purified *Kl*Gal4-*Kl*Gal80 complex

Figure 16 shows that it is not possible to determine the size of the purified *Kl*Gal4-*Kl*Gal80 complex by size exclusion chromatography.

Another method for molecular weight determination is analytical ultracentrifugation (AUC). AUC was performed by H. Lilie with a Beckman Optima XL-A (Beckmann Coulter, USA) centrifuge using the An50Ti rotor. The analysis showed that the protein is not aggregated. However weight determination was not possible. The sedimentation velocity could not be determined because the complex was too big and heterogeneous.

To distinguish between complexes of different size in the heterogeneous protein mixture, it was applied to Colorless Native Polyacrylamide Gel Electrophoresis (CN-PAGE). Therefore 30 µg of the glutathione sepharose purified protein were loaded onto a native gel with an acrylamide gradient of 3-12 %. The proteins were separated in a cold room at 100 V for 48 h. The long electrophoresis time ensures that all proteins migrate into the gel up to the concentration were the polyacrylamide network becomes too close meshed and the proteins cannot migrate further. The migration distance of a protein or protein complex is therefore mainly a function of the molecule size and shape and not the net charge. The molecular weight can be approximated from a calibration curve calculated from marker proteins loaded onto the same gel.

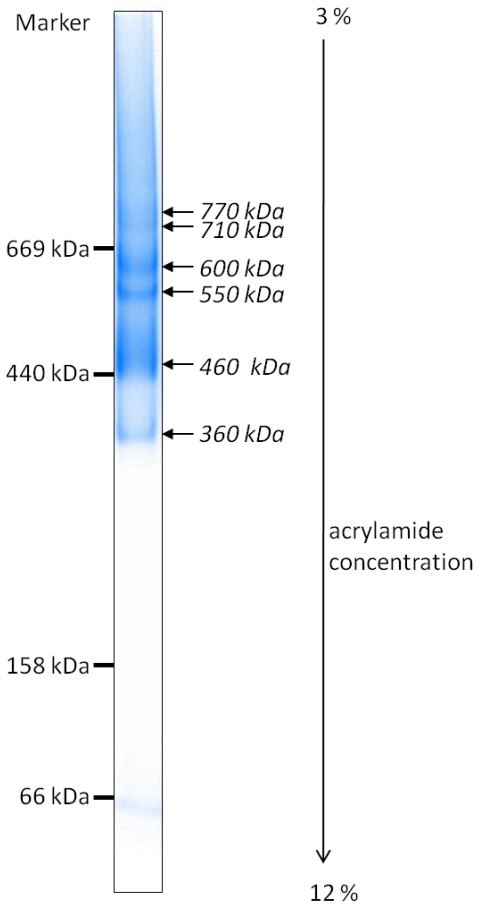

Figure 17: CN-PAGE of the purified NG*Kl*Gal4-NH*Kl*Gal80 complex. 30 µg of the gluthathione purified NG*Kl*Gal4-NH*Kl*Gal80 protein complex were applied to a native gradient polyacrylamide gel (3-12%). Distinct bands were visualized by Coomassie staining after 48 h electrophoresis time.

Marker proteins: Thyroglobulin, 669 kD, 7.6 mg/ml; Ferritin, 440 kD, 5 mg/ml; Aldolase, 158 kD, 5.2 mg/ml; Albumin , 66 kD, 6.5 mg/ml.

The CN-PAGE was reproduced several times. Six distinct bands could be separated and the approximate molecular weights were determined (see figure 17). It is very likely that higher molecular weight complexes present in the protein solution, which did not migrate into the gel, remained undetected. To see whether all of the complexes separated in the CN-gel consist of

NG*Kl*Gal4 and NH*Kl*Gal80, a stripe of the CN-gel was excised and applied to SDS-PAGE. After electrophoresis the proteins in the denaturing SDS-gel were blotted on a nitrocellulose membrane.

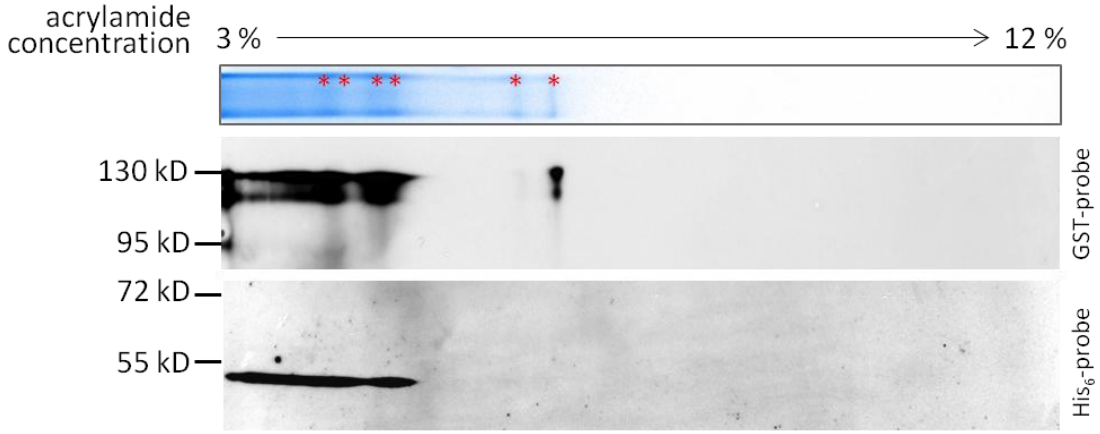

Figure 18: Western Blot analysis of the CN-PAGE separated protein complexes. A stripe of the CN-gel (top) was denatured and applied to a 10 % SDS-polyacrylamide gel which was blotted onto a nitrocellulose membrane. The membrane was probed with antibodies against the GST-tag (upper membrane) and the His_6-tag (lower membrane).

The GST-antibody probed Western Blot shows that all complexes comprise of GST-tagged *Kl*Gal4, but the 460 kD complex only to a small amount. This complex furthermore includes no NH*Kl*Gal80, so it can be assumed that it primarily consists of protein impurities. The smallest (360 kD-) complex does also not contain any NH*Kl*Gal80, so it is either purely composed of NG*Kl*Gal4 or the activator protein and other unknown components. As in the Western Blots from the purification steps (figure 14 & 15) there are always two NG*Kl*Gal4-signals, one at 125 kD which is consistent with the calculated molecular weight of full-length NG*Kl*Gal4, and a lower band migrating slightly faster. This lower molecular weight NG*Kl*Gal4 protein might be a degradation product and is additionally engaged in complex formation.

It can furthermore not be excluded that other protein impurities from the purified *Kl*Ga4-*Kl*Gal80 fractions are involved in complex formation of the bigger complexes and influence their migration behavior.

3.3.2 DNA binding behavior of the NG*Kl*Gal4-NH*Kl*Gal80 complex

To test whether the purified protein complex is able to bind to the Gal4-specific binding site, an electrophoretic mobility shift assay in mini-gel format was performed. 6 µg total protein (corresponding to appx. 20 pmol of hypothetically homogenous NG*Kl*$Gal4_2$-NH*Kl*$Gal80_2$

complex) from the affinity chromatography purification were applied to one binding reaction, containing 20 fmol of biotinylated DNA. After electrophoretic separation the molecules were blotted onto a nitrocellulose membrane. Free and protein bound DNA could be detected by addition of horseradish-peroxidase coupled streptavidin.

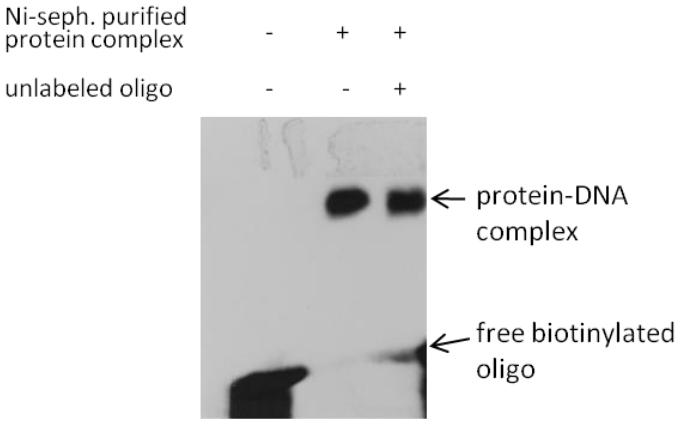

Figure 19: EMSA with purified NG*Kl*Gal4-NH*Kl*Gal80 protein. 6 µg of total protein (corresponding to 20 pmol of homogenous NG*Kl*Gal4$_2$-NH*Kl*Gal80$_2$) were added to 20 fmol biotinylated oligo harbouring the specific binding site for the transcriptional activator. The unlabeled oligonucleotide had the same sequence as the biotinylated oligonucleotide and was added to the reaction to a tenfold excess over the labeled oligo (200 fmol).

It could be clearly distinguished between a free and protein bound oligonucleotide-form (figure 19), and a low quantity of biotinylated oligo could be displaced by addition of the non-biotinylated DNA to the binding reaction. Outcompetition is incomplete because of the great excess of protein over the unlabeled DNA (appx. 100fold). Anyway the DNA-binding activity of the protein complex demonstrates that the DNA-binding domain of NG*Kl*Gal4 can be assumed to be folded correctly.

3.3.3 Removal of the GST-tag

Although large affinity-tags are often advantageous for enhanced protein expression and solubility, they are sometimes troublesome for further downstream applications. As shown in the Colorless Native PAGE, there is conformational heterogeneity of the NG*Kl*Gal4-NH*Kl*Gal80 complex, and this heterogeneity might be caused by the GST-tag. Glutathione-S-transferase itself is a protein that dimerizes in solution. Fusion of a GST-tag to a protein that also forms dimers can lead to the formation of large oligomers. Hoping to reduce the number of complexes for further studies I tried to remove the GST-tag from the *Kl*Gal4-protein. PreScission Protease (GE Healthcare) was added to the recombinant protein complex according to the manufacturer's protocol. Unfortunately this step led to an immediate precipitation of the

protein (within a few minutes). Therefore the GST-tag cannot be removed from the *Kl*Gal4 protein. The protein complex in this form is therefore not suitable for crystal structure determination. Other approaches for analysis of the *Kl*Gal4-*Kl*Gal80 interaction surface like crosslinking studies have to be tested. Independent from that the functional assays which were aimed in this work could be performed.

3.4 *In vitro* reconstitution of the transcriptional switch

3.4.1 A peptide consisting of the 22 C-terminal amino acid residues of *Kl*Gal4 is able to displace the repressor from the activator

As previously shown for *S. cerevisiae* the C-terminus of Gal4 is the interaction site for factors of the transcriptional machinery as well as Gal80 (Ding & Johnston 1997, Johnston 1987; Ma & Ptashne 1987b). *Sc*- as well as *Kl*Gal80 were co-crystallized with a 21- or 22-mer peptide, respectively, consisting of the C-terminal *Sc*-/*Kl*Gal4 amino acids and it could be shown that at least some of these residues make contacts with the repressor (Thoden et al. 2008; Kumar et al. 2008). In her PhD thesis D. Schmidt could show that a peptide consisting of the 22 C-terminal residues (AD-22) of the *Kl*Gal4-AD has an impact on the binding of Gal80 to dinucleotides, showing that AD-22 interacts with the repressor (Doreen Schmidt 2010). Anders et al. (2006) showed that this peptide also has a negative impact on the interaction between *Kl*Gal80 and *Kl*Gal1 (Anders et al. 2006). In this work it should be tested if AD-22 is able to displace the recombinant NH*Kl*Gal80 protein from the recombinant NG*Kl*Gal4 protein in the purified complex. If AD-22 is able to outcompete the repressor from the NG*Kl*Gal4-NH*Kl*Gal80 complex, then the 22 C-terminal residues of the *Kl*Gal4 protein are sufficient to bind *Kl*Gal80. To test this, I established a competition experiment. For this experiment 1 mg of NG*Kl*Gal4-NH*Kl*Gal80 complex eluted from the Ni-sepharose column was coupled to a gluthatione-sepharose spin column (GST Spin Trap™, GE Healthcare). The AD-22 peptide dissolved in binding buffer (buffer A, see 2.12.4) was used to for a first elution (2x150 µl). In the final elution step 10 mM reduced glutathione (300 µl) was used to recover the glutathione-sepharose coupled NG*Kl*Gal4, eventually associated with NH*Kl*Gal80 protein.

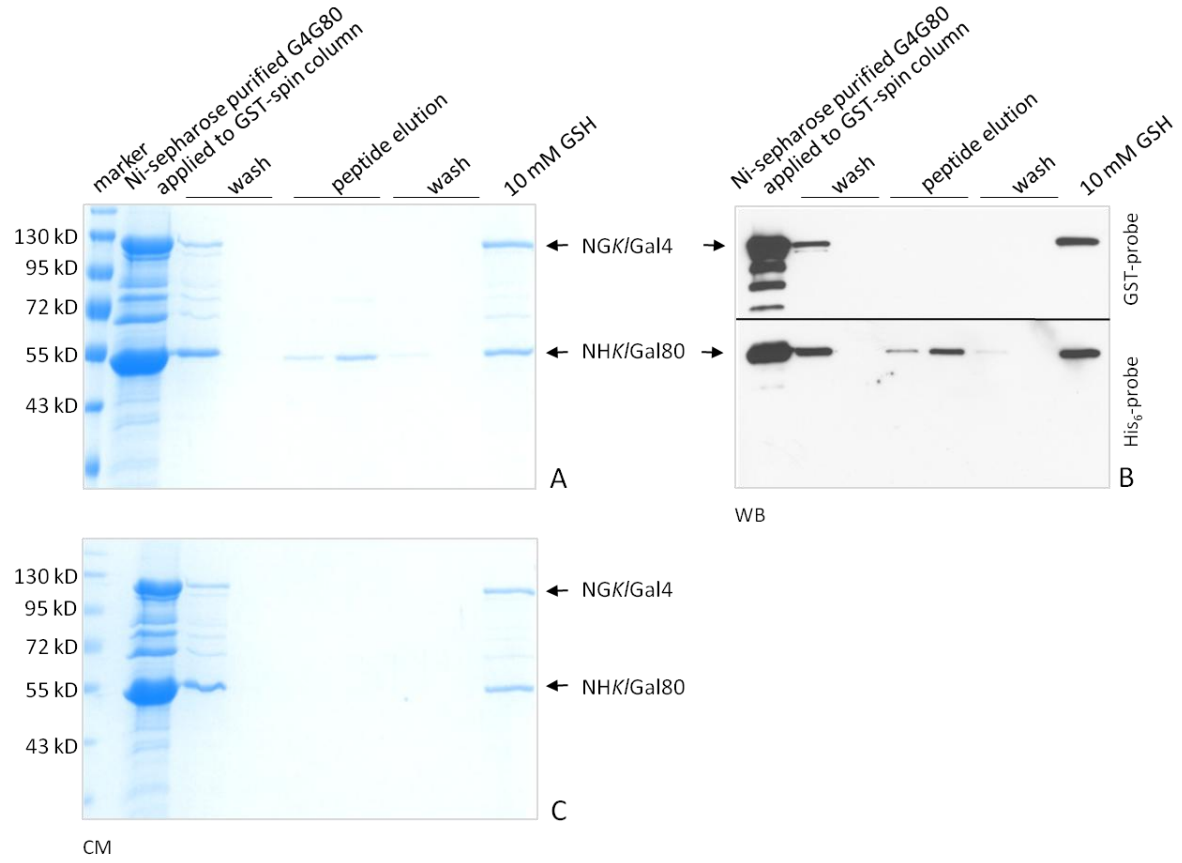

Figure 20: Displacement of NH*Kl*Gal80 from the recombinant protein complex by the AD-22 peptide. 1 mg of protein complex from Ni-sepharose chromatography was coupled to a GT-Spin Trap column. Elution steps were performed with AD-22 peptide dissolved in wash buffer (2 mg/ml ≙ 744 µM) and reduced glutathione dissolved in wash buffer (10 mM). 15 µl of each elution fraction was applied to a 10 % SDS-polyacrylamide gel for Coomassie staining (A+C) and 5 µl for Western Blot analysis (B); A: competition experiment with AD-22 peptide; C: control, elution without AD-22 peptide; B: Western Blot of the AD-22 competition experiment, the upper membrane was probed with anti-GST antibody to detect NG*Kl*Gal4, the lower membrane was probed with anti-His_6 antibody to detect NH*Kl*Gal80.

As shown in figure 20, NH*Kl*Gal80 is detectable in the peptide elution fraction where NG*Kl*Gal4 remained attached to the column. Thus AD-22 in a 744 µM peptide solution is able to displace the repressor from *Kl*Gal4. However, the displacement was incomplete since a large fraction of NH*Kl*Gal80 remained in the complex and could only be co-eluted with NG*Kl*Gal4. This result is not unexpected since Gal4 and Gal80 interact as dimers and the affinity of *Kl*Gal80 to full length *Kl*Gal4 is much higher than to the AD-22 peptide, probably due to cooperative interaction (Anders et al. 2006). It is also possible that additional residues of *Kl*Gal4 besides the 22 C-terminal amino acids contribute to the interaction with *Kl*Gal80. The smallest fully active and regulatable miniGal4 variant in *S. cerevisiae* indeed possesses a C-terminal domain consisting of 42 residues (Wu et al. 1996). Since the Colorless Native gel showed that there are different

species of *Kl*Gal4-*Kl*Gal80 complexes in the purified protein solution, these species might have different *Kl*Gal4-*Kl*Gal80-binding affinities due to unknown interactions between the complexes. It might also be that some of the complexes are not dissociable because they are protected by surrounding complexes. Nevertheless the results shown in figure 20 strongly indicate that the 22 C-terminal residues of the activator are sufficient to bind the repressor and constitute the preferential *Kl*Gal80-interaction site.

3.4.2 Recombinant *Kl*Gal1 can dissociate the *Kl*Gal4-*Kl*Gal80 complex

According to the allosteric activation model Gal4 and Gal80 remain associated and form a transcriptionally active tripartite complex with Gal3/1 in the presence of galactose. In electrophoretic mobility shift assays Platt & Reece demonstrated that the constitutive active Gal3-mutant $Gal3^c$ can bind to DNA-bound (mini)Gal4-Gal80 complexes (Platt & Reece 1998). In more recent studies it was observed that Gal3 and Gal80 remain equally distributed between cytoplasm and nucleus upon induction, so it cannot be ruled out that at least some Gal4 and Gal80 remain associated (Wightman et al. 2008; Egriboz et al. 2011). According to the dissociation model of the galactose switch, competition between Gal4 and Gal3/1 for the binding to Gal80 leads to the dissociation of the repressor from the activator. Several *in vivo* analyses have supplied evidence for this model. The ChIP-data generated in this work also strongly indicate that Gal80 dissociates from the promoter upon galactose induction in *S. cerevisiae* (see chapter 3.1). It is not known whether other factors besides Gal3/1 are involved in the dissociation process. To test whether *Kl*Gal1 is sufficient to dissociate *Kl*Gal4 and *Kl*Gal80 I have performed dissociation experiments with the purified NG*Kl*Gal4-NH*Kl*Gal80 complex and recombinantly expressed and purified NH*Kl*Gal1. Therefore 1 mg of total protein (≙ 2.8 nmol of NG*Kl*$Gal4_2$-NH*Kl*$Gal80_2$ complex) from the Ni-sepharose purification was loaded onto a GST SpinTrap™ column (GE Healthcare). Postulating a homogeneous NG*Kl*$Gal4_2$-NH*Kl*$Gal80_2$ complex, this would correspond to approximately 690 µg NG*Kl*$Gal4_2$ and 310 µg of NH*Kl*$Gal80_2$ protein. According to the manufacturer's information up to 500 µg GST-fusion protein can be bound to the column depending on the molecule size. It is therefore expected that maximally 725 µg of the applied protein complex (corresponding to approximately 2.0 nmol of the 360 kDa NG*Kl*$Gal4_2$-NH*Kl*$Gal80_2$ complex) have bound to the column, but because of the large size and heterogeneity of the complex it is assumingly less. The NH*Kl*Gal1 protein was purified as described in the materials and methods section (protocol modified from Anders et al. 2006) and

had a concentration of 475 µg/ml (8.2 nmol/ml; NH*Kl*Gal1 ≈ 58 kDa). To easily detect the eluted NH*Kl*Gal80 on a Coomassie stained SDS-polyacrylamide gel the elution volume should be as small as possible. Therefore elution was performed twice with each 110 µg purified NH*Kl*Gal1 in 300 µl buffer D (supplied with 25 mM $MgCl_2$), corresponding to a concentration of approximately 6.3 nmol/ml. There were no ligands added to the inducer, because it was previously shown that *Kl*Gal1 and *Kl*Gal80 are able to associate upon overexpression of the inducer in the absence of galactose and ATP (Zachariae, 1994). The influence of the ligands on the dissociation should be tested later in an independent dissociation experiment. A final elution step was performed with 300 µl buffer containing 10 mM reduced glutathione to recover NG*Kl*Gal4 with the retained NH*Kl*Gal80 and eventually associated NH*Kl*Gal1.

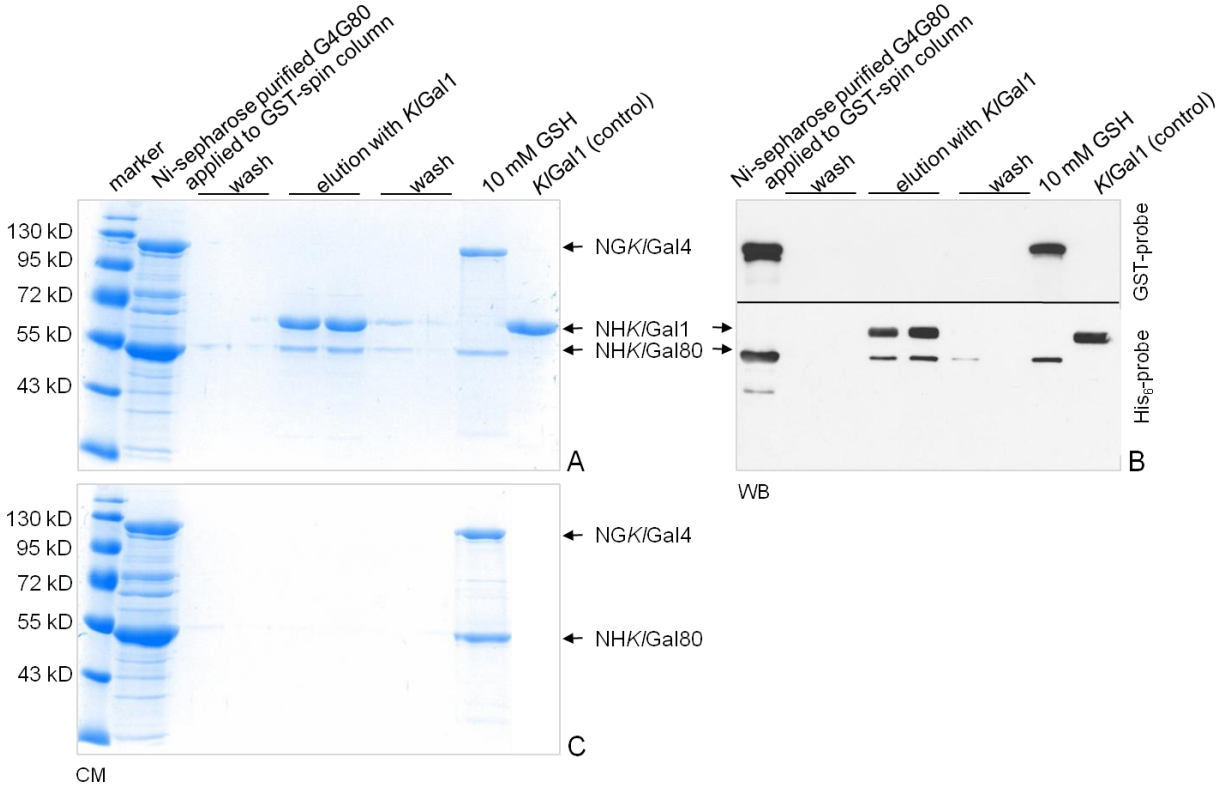

Figure 21: Dissociation of the NG*Kl*Gal4-NH*Kl*Gal80 complex by NH*Kl*Gal1. 1 mg of NG*Kl*Gal4-NH*Kl*Gal80 protein complex from Ni-sepharose chromatography was coupled to a GT-Spin Trap column. Elution steps were performed with NH*Kl*Gal1 protein solution (6.3 nmol/ml) and reduced glutathione dissolved in wash buffer (10 mM). 15 µl of each elution fraction was applied to a 10 % SDS-polyacrylamide gel for Coomassie staining (A+C) and 5 µl for Western Blot analysis (B); A: dissociation experiment with NH*Kl*Gal1; C: control, elution without NH*Kl*Gal1 (buffer D + 25 mM $MgCl_2$ only); B: Western Blot of the NH*Kl*Gal1 dissociation experiment, the upper membrane was probed with anti-GST antibody to detect NG*Kl*Gal4, the lower membrane was probed with anti-His_6 antibody to detect NH*Kl*Gal80 and NH*Kl*Gal1.

The different fractions of the dissociation experiment are depicted in figure 21. Clearly the NH*Kl*Gal1 protein eluted a large fraction of NH*Kl*Gal80 protein from the column. Some NH*Kl*Gal80 protein was retained by NG*Kl*Gal4 (detected in the GSH-elution fraction) and was not stripped by association with NH*Kl*Gal1. Eventually a few more elution steps would have been necessary for complete dissociation. It was shown in *S. cerevisiae* that a 20-30 fold excess of Gal3 over Gal80 is necessary to alleviate repression (Platt & Reece 1998) and in *K. lactis* there is a 3-6 fold excess of Gal1 over Gal80 present in the nucleus upon galactose induction (Anders et al. 2006). In the dissociation experiment there was no, or eventually only a small excess of NH*Kl*Gal1 over NH*Kl*Gal80. Maybe a higher concentrated NH*Kl*Gal1 solution would be sufficient to elute the whole *Kl*Gal80 protein. It is furthermore expected that the addition of ligands increases the eluted NH*Kl*Gal80-fraction.

To estimate the relative amount of NH*Kl*Gal80 eluted by NH*Kl*Gal1 and the amount of NH*Kl*Gal80 retained by NG*Kl*Gal4 (coeluted with NG*Kl*Gal4 in the 10 mM GSH-elution), quantitative Western Blots were made. To furthermore see which influence the addition of ligands to NH*Kl*Gal1 has on the eluted NH*Kl*Gal80 fraction, the experiment was repeated with 25 mM galactose and 1 mM ADP in the NH*Kl*Gal1 containing buffer. ADP was used instead of ATP to avoid an enzymatic turnover of galactose without an impact on the ability of *Kl*Gal1 to interact with *Kl*Gal80 (shown by Zenke et al. 1996). The elution experiment was performed twice and 5 µl of the elution fractions from the NH*Kl*Gal1-elution and the GSH-elution were applied to SDS-PAGE and Western Blot analysis. The chemoluminescence-intensities of the His_6- and GST- signals were recorded and quantified by the AlphaEase® FC Imaging System (Alpha Innotech). The software of the system assigns a value in percentages to every signal on the membrane in such way that all signals together sum up to 100 %. The bars in figure 22 represent the relative His_6-luminescence signals of NH*Kl*Gal80 which were calculated as follows:

$$rel.signal\ intensity\ of\ eluted\ NHKlGal80 = \ signal\ intensity \frac{\%\ NHKlGal80}{\%\ NGKlGal4}$$

The intensities were related to NG*Kl*Gal4, because the Gal4-signal should be the nearly constant in every dissociation experiment. The white bars represent the amount of NH*Kl*Gal80 eluted by NH*Kl*Gal1, the grey bars represent the amount of NH*Kl*Gal80 eluted with 10 mM GSH in the final elution step.

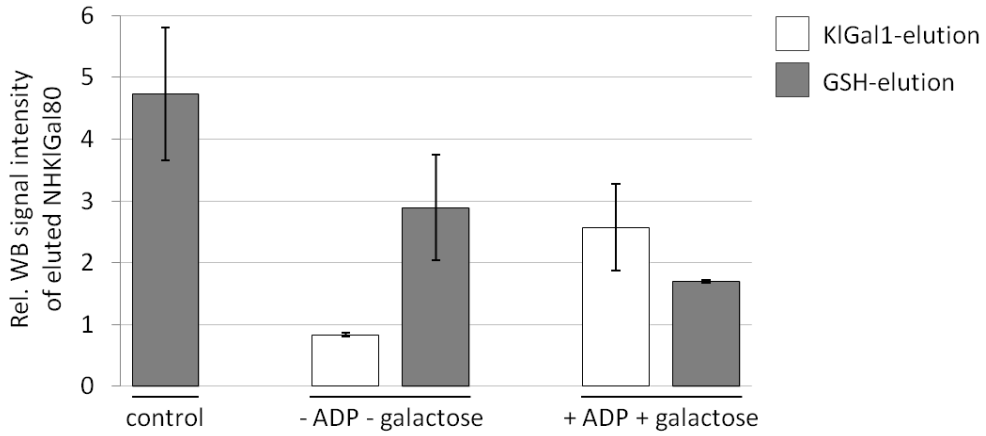

Figure 22: Quantification of NH*Kl*Gal1-eluted NH*Kl*Gal80. 1 mg of Ni-sepharose chromatography purified NG*Kl*Gal4-NH*Kl*Gal80 complex was coupled to a GST SpinTrap™ column and NH*Kl*Gal80 was eluted with NH*Kl*Gal1 protein solution with or without ligands (+/- ADP/galactose). NH*Kl*Gal80 which was retained at the column by NG*Kl*Gal4 (and not eluted by NH*Kl*Gal1) was recovered by elution with 10 mM GSH-solution. Each experiment was performed in duplicate. 5 µl of the NH*Kl*Gal1-elution fractions (white bars) and the GSH-elution (grey bars) were used for Western Blot analysis. The His_6-signal intensities from the dissociation-experiments were quantified by the AlphaEase® FC Imaging System (Alpha Innotech) and normalized to the GST-signal of NG*Kl*Gal4. The control represents the amount of NH*Kl*Gal80 which was co-eluted with NG*Kl*Gal4 by addition of 10 mM GSH without previous NH*Kl*Gal1-elution.

The graphs clearly show that the addition of galactose and ADP to the purified NH*Kl*Gal1 protein increases the ability to dissociate the NH*Kl*Gal80 protein from the recombinant protein complex. The amount of NH*Kl*Gal80 dissociated by NH*Kl*Gal1 in the presence of ligands is increased more than twofold compared to the inducer without ligands while the amount of NH*Kl*Gal80 retained by NG*Kl*Gal4 in the complex decreases. Thus, it can be concluded that *Kl*Gal1 is sufficient to dissociate *Kl*Gal80 and *Kl*Gal4. There are no further factors needed to strip off the repressor from the activator. The observation that *Kl*Gal1 was never retained by the *Kl*Gal4-*Kl*Gal80 complex furthermore precludes the formation of a tripartite complex (at least under the experimental conditions). This strongly supports the dissociation model of the *GAL/LAC* switch.

3.5 Construction and functional analysis of *Kl*Gal4-deletion variants

In the previous chapter it was shown that it is possible to produce recombinant *Kl*Gal4 protein in complex with *Kl*Gal80 in *E. coli* and purify it with affinity chromatography. Unfortunately the GST-tagged protein is not suitable for downstream applications like crystal structure determination, because the NG*Kl*Gal4-NH*Kl*Gal80 complex is too heterogenous.

Different approaches are thinkable to overcome the problem of heterogeneity. The most obvious one is the use of another tag which also enhances the yield, stability and solubility of the recombinant protein, but does not dimerize itself. Alternatively eukaryotic expression systems (f. e. *Pichia pastoris*, insect cells, etc.) could be used. Another approach is the expression of smaller, eventually more stable variants of the protein in combination with a small tag like hexahistidine. Therefore I tried to generate smaller *Kl*Gal4 variants that could act as Gal80-regulated transcription activators *in vivo*.

3.5.1 Web based secondary structure analysis of the *Kl*Gal4 protein

In a first step an internet based secondary structure analysis was performed to characterize distinct structural elements and thereby potential functional domains. Such an analysis can show which regions within a protein sequence are likely to have no secondary structure elements and can potentially be deleted without loss of the three-dimensional structure of the whole protein. Altogether eleven open source programs (listed in table 1) were applied and evaluated according to how often a structure was predicted. The result of the analysis is depicted in figure 23. The color-code defining the frequency of a predicted structure is shown in table 2.

Table 1: Progams used for secondary structure prediction of the *Kl*Gal4 protein.

Program	Website	Literature
GOR IV	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html	(Garnier et al. 1996)
HNN	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html	Guermeur, Y; Combinaison de classifieurs statistiques, Application a la prediction de structure secondaire des proteines. PhD Thesis.

PROF	http://www.aber.ac.uk/~phiwww/prof/	(Ouali & King 2000)
PSIPRED	http://bioinf.cs.ucl.ac.uk/psipred/	Bloomsbury Centre for Bioinformatics
PHYRE	http://www.sbg.bio.ic.ac.uk/phyre/	(Kelley & Sternberg 2009)
SOPMA	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html	(Geourjon & Deléage 1995)
PORTER	http://distill.ucd.ie/porter/	(Pollastri & McLysaght 2005)
APSSP2	http://imtech.res.in/raghava/apssp2/	(Raghava 2002)
NetSurfP	http://www.cbs.dtu.dk/services/NetSurfP/	(Petersen et al. 2009)
Jpred3	http://www.compbio.dundee.ac.uk/www-jpred/	(Cole et al. 2008)
SSpro	http://scratch.proteomics.ics.uci.edu/	(Cheng et al. 2005)

Table 2: Color-code used for the evaluation of the secondary structure prediction programs.

yellow	coiled coil; predicted by 10-11 programmes
light green	α-helical conformation; predicted by 2-3 programmes
green	α-helical conformation; predicted by 4-7 programmes
dark green	α-helical conformation; predicted by 8-11 programmes
orange	β-strand conformation; predicted by 2-3 programmes
red	β-strand conformation; predicted by 4-7 programmes

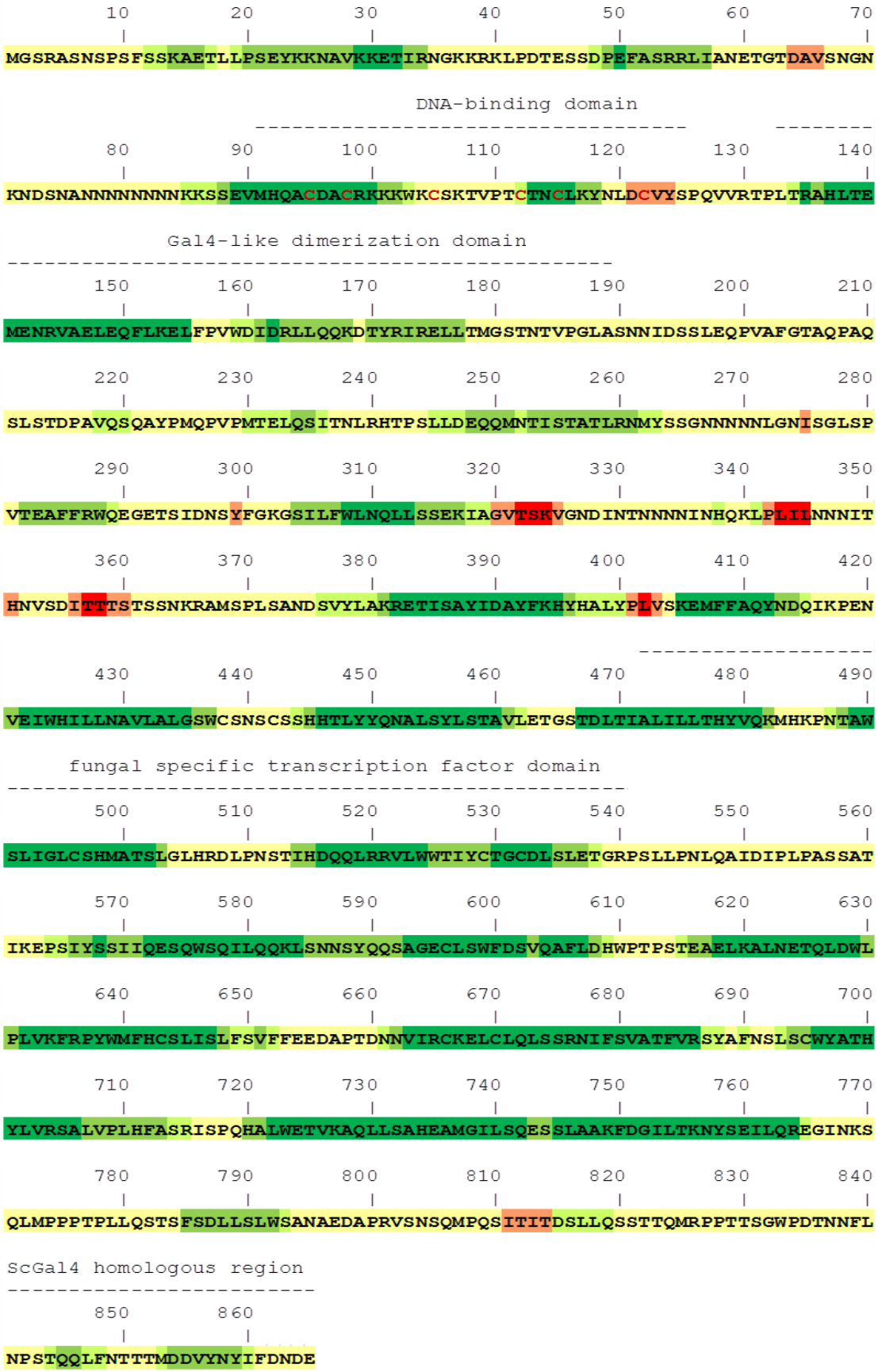

Figure 23: Secondary structure prediction of the *Kl*Gal4 protein. Altogether 11 open source programs were used. The color-code used for evaluation of the prediction results is defined in table 2. The programs used for secondary structure prediction are listed in table 1. The “fungal specific transcription factor domain” correlates with the MHR.

As shown in the prediction results, there are four larger regions where the probability to find a defined secondary structure is rather low. The first of this regions includes the residues 1-90 upstream of the DNA-binding and dimerization domain and there is indeed no function known for this very N-terminal region of the *K. lactis* activator and which is lacking in *Sc*Gal4. The second region encompasses the residues 160-380 and starts within the dimerization domain, which is known to consist of a coiled-coil structure element. The region ends right in front of the moderately conserved middle homology region (MHR), which was predicted to be predominantly α-helical. Following the MHR there is the third region (spanning the residues 540-570) which was predicted to lack a secondary structure. While the region from 570-760 was predicted to be predominantly α-helical, the C-terminus encompassing the residues 765-865, (including the established Gal80-binding and activation domain) appear largely unstructured. This is in good accordance with models of AD properties, which predict that a structure is induced upon interaction with a specific target protein.

3.5.2 Cloning and characterization of *Kl*Gal4-deletion mutants

With the fusion PCR-strategy described in the material and methods section (see figure 4) three *KlGAL4* deletion mutants (figure 24) were created. All variants consist of the complete N-terminus comprising residues 1-190 to ensure proper nuclear localization and DNA binding/dimerization and a portion of the C-terminus of variable length. In the variant with the shortest deletion the potentially unstructured region between the DNA-binding/dimerization domain and residue 380 was deleted. This deletion resulted in a protein variant with an approximate molecular weight of 77 kD (computed with the ExPASy Compute pI/MW tool; http://web.expasy.org/compute_pi/). The second variant was deleted between the residues 190 and 472. In this variant with a predicted molecular weight of about 67 kD the MHR is still intact, but a large region with predominantly α-helical content is eliminated. So far, there is no function known for this region. The variant with the largest deletion has an approximate molecular weight of about 57 kD and does not contain the MHR anymore. This variant consists of the residues 1-190 and 557-865. The *KlGAL4-77, KlGAL4-67* and *KlGAL4-57* genes were integrated at the *Klgal4Δ::Scura3* locus in the *Kl*gal4-deletion strain DL9. As a control the wt *KlGAL4* gene was also used for transformation. The resulting strains were termed YCZ_KlGal4-X. It could be shown in Western Blot analysis that all variants were expressed to same level as the wild type protein in the strain JA6 (see figure 24 B).

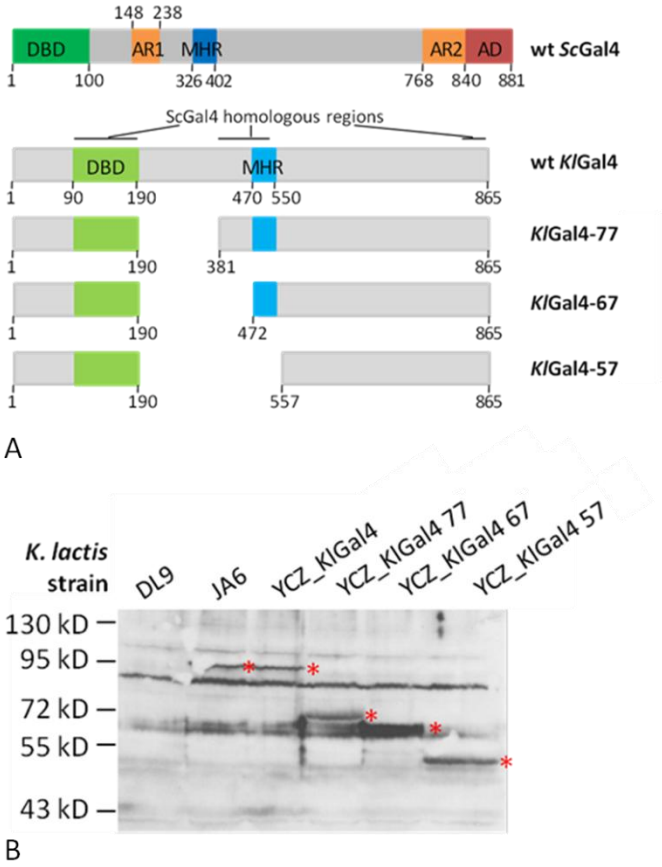

Figure 24: KlGal4-variants.
A: Schematic view of the *Kl*Gal4 domains (compared to *Sc*Gal4) and the deletion variants.
B: Western Blot analysis of yeast strains expressing the different *Kl*Gal4 variants. Crude extracts were prepared from cells grown in YEPD medium to an OD_{600} 0.5 and 30 µg of total protein were applied to a 10 % SDS-polyacrylamide gel. After electrophoresis and blotting the membrane was incubated with the GST-Lac9HX-antiserum. DL9: *Kl*gal4 deletion strain; JA6: wildtype strain and parent strain of DL9; YCZ_KlGal4 X: *Kl*GAL4-derivative strains.

Since all *Kl*Gal4-variants gave similar protein levels *in vivo*, their activity, which was tested by measuring *LAC4* encoded β-galactosidase activity (see figure 25), should reflect their ability to activate transcription. All strains were pregrown in sc-medium containing 2 % glucose to an OD_{600} 0.8 and were then shifted to fresh sc-medium containing either 2 % glucose (repressing medium) or 2 % galactose (inducing medium) and grown for another four hours.

On the left hand side of figure 25 the β-galactosidase activities of the *K. lactis* strains expressing the different chromosomally integrated *Kl*Gal4 variants are shown. Under repressing conditions only background activity (< 150 mU/mg) was detectable in all strains. Under inducing conditions, both the wildtype strain JA6 and the strain YCZ_KlGal4 wt with the integrated wildtype *KlGAL4* gene both showed β-galactosidase activity of approximately 1500 mU/mg, demonstrating that the chromosomal re-integration of the KlGAL4 gene into the DL9 background does not lead to any unexpected side effects. The KlGal4-67 and KlGal4-57 variants were unable to activate *LAC4* gene expression, whereas the *Kl*Gal4-77 variant displays about 60 % of the activity of the wildtype protein. KlGal4-77 and is therefore the first functional mini*Kl*Gal4-protein described so far.

To test whether higher levels of the smaller variants were able to activate transcription a second gene copy of the respective *KlGAL4-X* gene was introduced via a CEN-plasmid. The β-galactosidase activities of the strains with two copies of the *KlGAL4*-variants are displayed on the right hand side of figure 25. Except for the control strain JA6/2-2, which has two chromosomally integrated *KlGAL4* gene copies, there is only background activity in all strains under repressing conditions. The β-galactosidase activity in JA6/2-2 under repressing conditions is about 450 mU/mg, while in the strain YCZ_KlGal4 wt with the second plasmid encoded wildtype gene copy the activity is just 150 mU/mg. The activities after the galactose shift are however similar in both strains (≈ 3000 mU/mg). Interestingly there was β-galactosidase activity detectable in the strain YCZ_*Kl*Gal4-67 with another *KlGAL4-67* gene copy, which was approximately 20 % of the activity measured in the YCZ_KlGal4 wt strain with the plasmid. Obviously *Kl*Gal4-67 is regulated by Gal80-repression under non-inducing conditions and bears some residual activity. This variant therefore represents a second functional mini*Kl*Gal4-variant and might eventually be a good candidate for recombinant expression and purification in order to study *Kl*Gal4-*Kl*Gal80 interaction. However the β-galactosidase activity of the *Kl*Gal4-77 variant could not be enhanced by expression of a second gene copy. The *Kl*Gal4-57 variant shows no significant activity, even in the presence of two gene copies. Perhaps the MHR, which is lacking here, is indispensable for the function of the *Kl*Gal4 protein.

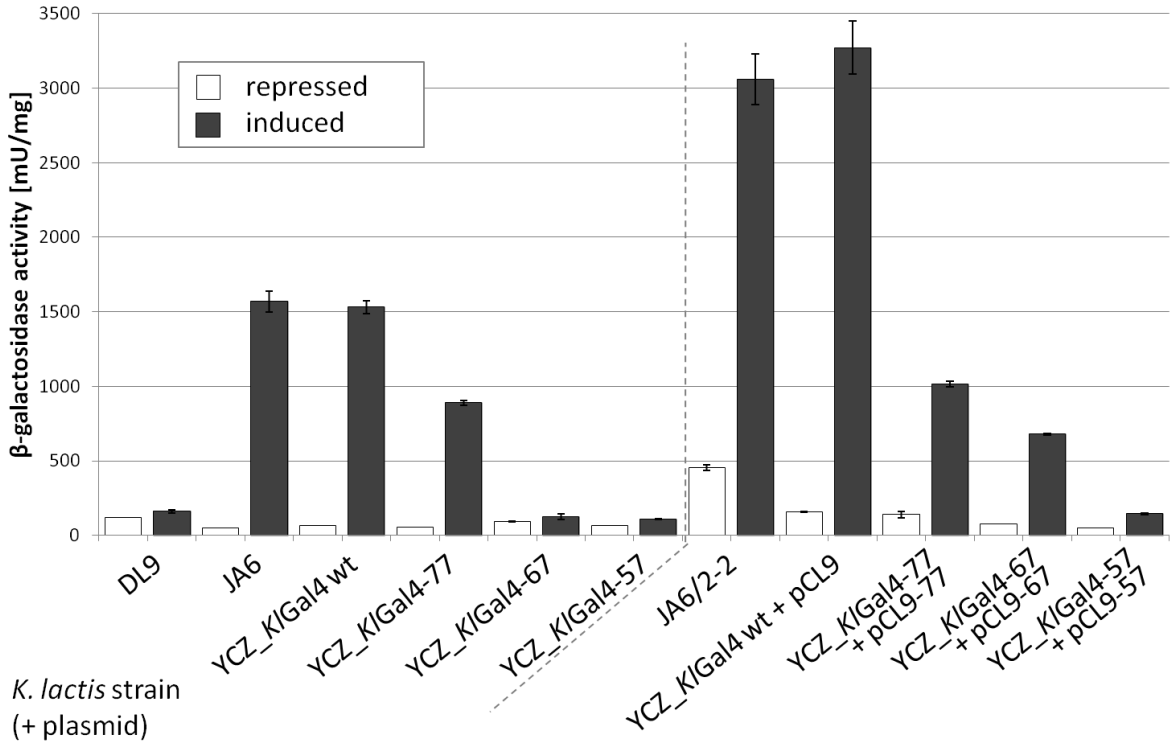

Figure 25: β-galactosidase activities of *K. lactis* cells expressing different *Kl*Gal4 variants. All cells were pregrown in sc-medium with 2 % glucose to an OD_{600} 0.8, shifted to new medium containing either 2 % glucose or 2 % galactose and grown for further 4 h. The CEN-plasmid pCL9 carries the *KlGAL4* gene which was replaced with the particular smaller *KlGAL4-X* gene variants in the pCL9-X derivate plasmids. The experiment was performed in duplicate.

4 Discussion

4.1 Recombinant expression, purification and characterization of the NG*Kl*Gal4-NH*Kl*Gal80 complex

The established Gal80-binding site on the Gal4 protein is located at the C-terminus of the activator and overlaps with the activation domain (Gal4-AD). The existence of protein-protein contacts between Gal4 and Gal80 beyond the established binding site remains up to now only speculative. The hypothesis that additional weaker interactions contribute to the specific interaction between Gal80 and the C-terminus of Gal4 would be quite attractive because it could explain several observations. Sil et al. (1999) found that the fusion protein LexA-Gal4 (aa 225-797), but not LexA-Gal4 (aa 225-534) and LexA-Gal4 (aa 534-797), is able to interact with VP16-Gal80. This could mean that there is an interaction surface formed in the presence of both regions, aa 225-534 and 534-797, which is sufficient to bind Gal80 weakly, while the presence of only one of these regions would be insufficient. It could also explain why in GST-pulldown assays K. Melcher found that the AD-deleted Gal4-variants miniGal4-7stop841 and Gal4stop841, but not miniGal4-9stop841 bound to Gal80. Unlike miniGal4-9stop841 the miniGal4-7stop841 variant contains the residues 168-237, 412-422 and 680-725. Two or more weak interactions between these regions and Gal80 could be sufficient for the Gal80-pulldown. But all the *in vivo* and *in vitro* interaction assays cannot answer the questions regarding additional sites of Gal4-Gal80 interaction in a satisfactory manner.

To find clear evidence for interaction sites between Gal4 and its repressor, structural studies with the full length proteins would be desirable. But all attempts to purify larger amounts of Gal4 proteins have failed so far. Therefore I turned to the *Kluyveromyces lactis* homolog *Kl*Gal4 and its partner *Kl*Gal80. First attempts in this work to purify the *Kl*Gal4 as a His_6-tagged protein were not successful because the yield of soluble protein was very low and the purified protein tended to aggregate very fast. When fused to a SUMO-tag (not shown) only low *Kl*Gal4 expression level was observed. However, when *Kl*Gal4 was fused with GST (NG*Kl*Gal4) and co-expressed with His_6-tagged *Kl*Gal80 (NH*Kl*Gal80) in *E. coli* both proteins were stable and could be purified in a two step affinity chromatography protocol. Cleavage of the GST-tag led to immediate aggregation of the protein, which is why it was left fused to *Kl*Gal4. The protein was able to bind to a Gal4-specific DNA binding site and to *Kl*Gal80, indicating that the protein (or at least the DBD and Gal80-binding site) was correctly folded. The protein yield was indeed

sufficient for further structural studies, but it turned out that the NG*Kl*Gal4-NH*Kl*Gal80 complex was a heterogeneous mixture of a number (six distinct bands could be separated on a native gradient gel) of complexes. The approximately 460 kD complex lacked *Kl*Gal4 as well as *Kl*Gal80. This complex, therefore, must consist of other proteins, probably the chaperons that were co-purified with the NG*Kl*Gal4-NH*Kl*Gal80 complex. It is known for example for DnaK that it can form oligomers in solution (Schönfeld et al. 1995). The complex with the molecular weight of about 360 kD would perfectly fit with a NG*Kl*Gal4$_2$-NH*Kl*Gal80$_2$ tetramer, but since this complex also lacked *Kl*Gal80, *Kl*Gal4 must be in complex with other components. The precise composition of the two smallest complexes, however, should be determined by mass spectrometry. The larger complexes all consist of both proteins, *Kl*Gal4 and *Kl*Gal80. Melcher and Xu (2001) showed that *Sc*Gal80 is able to form tetramers and that Gal80-dimer-dimer interaction on adjacent Gal4-binding sites plays a role in the modulation of Gal80-repression (Melcher & Xu 2001). Oligomerization was also proposed for *Kl*Gal80 by Anders et al. (2006). Interaction of two NG*Kl*Gal4$_2$-NH*Kl*Gal80$_2$-complexes mediated by *Kl*Gal80-tetramerization could therefore lead to one of the larger complexes detected on the native gel.

Another reason for the formation of the large complexes could be the GST-tag, which was used as *Kl*Gal4-fusion partner. The glutathione S-transferase (GST) from *Schistosoma japonicum* is itself a homodimeric protein with about 26 kD per subunit. Under non-reducing conditions it can reversibly form huge aggregates without loss of its catalytical activity (Kaplan et al. 1997). When fused to a protein that also oligomerizes the consequence can be the formation of heterogeneous oligomers. Though GST-fusion was beneficial in enhancing the expression yield and solubility of the protein, it is not very suitable in studies of *Kl*Gal4-*Kl*Gal80 complex formation. There are several other fusion partners, which might be more suitable but have not been tested yet. Good experiences with challenging proteins (i.e. proteins which have proved difficult to express and purify) were for example made with a NusA (N utilization substance A)-tag (Davis et al. 1999). Also the maltose binding protein (MBP) would be worth testing. If none of the tags leads to a satisfying result, maybe eukaryotic hosts like the yeast *Pichia pastoris* or insect cells or cell free expression systems (e.g. wheat germ cell extract) should be tested for expression of the *Kl*Gal4-protein. Eukaryotic expression systems would also have the advantage that they are able to add posttranslational modifications (PTMs), if necessary. Although PTMs have not been identified for the *Kl*Gal4 protein so far, it is very likely that it is a target of modifications as the *Sc*Gal4 protein (see introduction section 1.3.1). PTMs are not only involved

in the regulation of the activity of a protein, they can also have an impact on the stability and conformation of a protein or a certain domain (for a review see Gsponer & Babu 2009). Since only phosphorylation of the serines 22 and 699 in *Sc*Gal4 were shown to have a regulatory role (Leverentz & Reece 2006; Ferdous et al. 2008), the other serine phosphorylations might be relevant for the stability and/or conformation of the protein. An example is the methylation of the β-glycosidase from *Sulfolobus solfataricus*, a PTM that enhances the thermal stability of the protein (Febbraio et al. 2004). The absence of potential PTMs of recombinantly expressed *Kl*Gal4 protein in *E. coli* might therefore have a negative impact on its stability.

Functional analysis of the NG*Kl*Gal4-NH*Kl*Gal80 complex showed that a peptide comprising the 22 residues of the *Kl*Gal4-C-terminus (AD-22) is able to dissociate the NG*Kl*Gal4-NH*Kl*Gal80 complex. The 22 C-terminal residues of *Kl*Gal4 are therefore sufficient to bind the repressor and it can be assumed that this established *Kl*Gal80-binding site is indeed the main *Kl*Gal80-interaction site. The dissociation by AD-22 was, however, not complete. Possible explanations for the incomplete dissociation have already been discussed in the results section (see 3.6.1).
Anders et al. (2006) had determined a dissociation constant of about 1.5 µM for the *Kl*Gal80-AD-22 complex and about 1.0 nM for a *Kl*Gal80-mini*Sc*Gal4 complex. As the reason for this much higher affinity of *Kl*Gal80 to mini*Sc*Gal4 cooperativity was discussed. This cooperativity effect was confirmed when the AD-22 peptide was fused to GST, allowing the protein to form dimers. Since Gal4 and Gal80 interact as dimers, a much higher concentration of the AD-22 peptide in contrast to a protein-dimer is necessary to dissociate a *Kl*Gal4-*Kl*Gal80 complex. With about 744 µM the concentration of AD-22 peptide used in this work was rather high, but if the complex is very stable over time, multiple consecutive peptide elution steps would be necessary to dissociate the NG*Kl*Gal4-NH*Kl*Gal80 complex completely.
Another reason might be that other residues besides the 22 C-terminal amino acids of the *Kl*Gal4-AD might also be involved in complex formation and therefore stabilization. Although up to now there is no evidence that other residues of Gal4 are involved in Gal80-binding, the protein might have an influence on the fold of the activation domain upon Gal80-interaction. It is known that peptides can be structurally ambivalent and can adopt different conformations dependent on their environment (Kuznetsov & Rackovsky 2003). Eventually the AD-22 peptide does not have the proper conformation when separated from its natural protein context, and a

deviation from the fold in the full length protein might be therefore an additional reason for the weak dissociation.

4.2 The *K. lactis* Gal4-Gal80-Gal1 transcriptional switch can be reconstituted *in vitro*

It is shown in section 3.6.2 that the recombinant NG*Kl*Gal4-NH*Kl*Gal80 complex can be dissociated by addition of recombinant *Kl*Gal1 protein. This is the first time that the transcriptional switch was reconstituted *in vitro*. Although the situation *in vivo* might be different, it could be demonstrated here that the dissociation of *Kl*Gal4 and *Kl*Gal80 is mediated only by *Kl*Gal1 and that it does not depend on the presence of other yeast proteins. Furthermore, NH*Kl*Gal1 was never found to be retained by the NG*Kl*Gal4-NH*Kl*Gal80 complex, neither in the absence nor in the presence of the ligands ADP and galactose. This finding argues against the formation of a ternary complex upon induction of transcriptional activation, as proposed for the allosteric activation model. Instead, these results strongly favor the dissociation model of Gal4 activation in *Kluyveromyces lactis*.

In the dissociation experiments it was furthermore shown that the amount of *Kl*Gal80 eluted by *Kl*Gal1 was increased more than twofold in the presence of the ligands ADP and galactose. *In vivo*, the excess of Gal1 over Gal80 in the nucleus was determined to be three- to sixfold upon galactose addition and the K_D-value for *Kl*Gal80-*Kl*Gal1 binding (83 nM) is very high compared to *Kl*Gal4-*Kl*Gal80 binding (1 nM) (Anders et al. 2006). It was therefore expected that a high molar excess of *Kl*Gal1 over *Kl*Gal80 would be necessary to dissociate *Kl*Gal80 from *Kl*Gal4. Unfortunately the exact molar amounts of the *Kl*Gal4 and *Kl*Gal80 proteins could not be determined since the purified complex was not homogeneous, but it was calculated that only a small excess of *Kl*Gal1 over *Kl*Gal80, if any, was present in the experiment (see also figure 21). Despite these circumstances the amount of *Kl*Gal80 eluted by *Kl*Gal1 was really high. The heterogeneity of the NG*Kl*Gal4-NH*Kl*Gal80 complex seems not to interfere with the dissociation by *Kl*Gal1. Therefore, it can be expected that complete dissociation of the recombinant *Kl*Gal4-*Kl*Gal80 complex could be achieved by addition of higher quantities of recombinant *Kl*Gal1 protein in the presence of ADP and galactose.

4.3 *Sc*Gal4 and *Sc*Gal80 dissociate under inducing condition

According to the allosteric model of the yeast galactose genetic switch a conformational change of the Gal4-Gal80 complex upon induction leads to the release of Gal4-repression. During this process Gal3/1 binds to Gal80 and a ternary complex is formed. In this work, the formation of a ternary complex could not be proved for *Kluyveromyces lactis*. But the data that lead to the allosteric model were all based on experiments with the *Saccharomyces cerevisiae* proteins. This were for example electrophoretic mobility shift assays using a Gal3 mutant ($Gal3^{C-322}$) (Platt & Reece 1998) or yeast-two-hybrid experiments using Gal4-variants lacking the classical Gal80 interaction domain (comprising the residues 850-874) (Sil et al. 1999). Immunoprecipitation and yeast-two-hybrid data obtained with Gal4-variants lacking the classical Gal80-interaction site (Gal4-AD) also suggested that Gal80 might bind to an alternative binding site on the Gal4 protein (K. Melcher, unpublished data; see also section 3.1.1). The internal binding site was proposed to be located in the region between the residues 680 and 725.

To investigate the potential Gal80-binding function of the region aa 680-725 ChIP-analyses were performed in this work. Therefore miniGal4#7 and miniGal4#9 variants were constructed based on the miniGal4-variant described by Wu et al. 1996, which consists of the DNA-binding and dimerization domain (DBD, aa 1-100) and the Gal80-binding and activation domain (AD, aa 840-881) (see also 3.1.2). The C-terminus of miniGal4#7 consists of the residues 680-881, the miniGAL4#9 C-terminus of the residues 725-881. The miniGAL4, miniGAL4#9 and miniGAL4#7 gene variants were then used for gene replacement in the *S. cerevisiae* strain I4G80Myc. As a control a strain consisting of a GAL4ΔAD gene was also constructed. None of the Gal4-variants could be detected in a Western Blot, which is not surprising because there are normally only few Gal4-molecules in the *S. cerevisiae* cells (Ghaemmaghami et al. 2003; Ding & Johnston 1997). Nevertheless expression and DNA-binding of all variants could be shown by ChIP with an antibody against the Gal4-DNA binding domain (see figure 8). The binding of Gal80-Myc to the *GAL1*-promoter via the Gal4-protein was analyzed in the ChIP-experiment with the antibody against the Myc-tag. Under non-inducing conditions Gal80 was bound to all Gal4-variants except the Gal4ΔAD variant. Under inducing conditions, Gal80-binding was not detectable, independent from the expressed Gal4-variant (only some background was detected in the miniGal4-strain). Therefore this experiment gave no indication that Gal80 is able to bind to an alternative binding site on the Gal4 protein upon galactose induction, neither in the absence nor in the presence of the AD, at least *in vivo* and when expressed from its natural locus. Of

course it cannot be excluded that alternative binding regions of Gal4 are involved in Gal80 binding under non-inducing conditions. However, such potential interaction regions are apparently not sufficient to bind Gal80 *in vivo* in the absence of the AD.

Surprisingly, the β-galactosidase filter assay with the AD-truncated (mini)Gal4-variants that were performed in this work showed that all of these variants were able to interact with VP16-Gal80 and therefore to induce β-galactosidase reporter gene expression. This is in contrast with the observations from the ChIP-analyses. The variants only differed in the strength of VP16-Gal80 interaction, meaning that the interaction between miniGal4-9stop841 and VP16-Gal80 was weaker than that of Gal4stop841 or miniGal4-7stop841. An alternative explanation for the weak interaction could simply be that miniGal4-9stop841 is expressed to a very low level or that it is unstable in the cells. Unfortunately this could not be shown, because none of the Gal4-variants could be detected in a Western Blot. A conclusion regarding potential alternative Gal80-binding sites was furthermore difficult to make because Gal4stop841 and miniGal4-7stop841 exhibited high background activities even in the absence of VP16-Gal80. This could be due to the activating function of the activating region I (ARI, aa 148-238 (Johnston & Dover 1988; Ma & Ptashne 1987a)), which becomes apparent in the absence of the C-terminal activation domain and which is present in Gal4stop841 and miniGal4-7stop841 but absent in miniGal4-9stop841. But it could also be that due to the AD-truncation other surfaces than the ARI are exposed which are usually buried and that now lead to the recruitment of general transcription factors. Maybe also new VP16-Gal80 interaction sites have been created. It is well known that mutations can alter the interaction between two proteins. So does the single amino acid exchange of Asn342 against any hydrophobic amino acid in Gal11 (a component of the RNAPII-mediator-complex) lead to the Gal11P mutant (P stands for transcription potentiator) which is able to interact with the Gal4-dimerization domain (Gal4-dd) (Hidalgo et al. 2001). Possible conformational changes must be considered when working with (deletion-) mutants, but nevertheless working with such mutants is a common way to elucidate the role of distinct regions or domains of a protein in *in vivo* studies.

In summary, the existence of an alternative biologically relevant Gal80-binding site on Gal4 could not be proved.

4.4 *Sc*Gal80-binding to *Sc*Gal4 correlates with the galactose concentration in the medium

The ChIP-evaluation of Gal80 bound to the promoter at several time points after induction revealed that there is a correlation of Gal80 binding to the promoter and the galactose concentration in the medium. One hour after the galactose shift there is nearly no Gal80 bound to the promoters of the examined cells. In wtGal4 cells the Gal80 protein bound to Gal4 rises again after galactose is completely depleted (visible after 18 hours) and increases further during the time course. This is in good accordance with the observations made by Jiang et al. (2009) in life cell imaging experiments. Thus, galactose concentration in the medium should be thoroughly controlled in Gal4-Gal80 interaction studies to avoid false positive binding results arising from galactose depletion.

However, the quantity of Gal80 bound to Gal4 is not completely restored to the initial level after 24 hours. The same is true for the cells expressing miniGal4#7. One could speculate that this is due to a decreased *Sc*Gal4 protein level. Maybe *Sc*Gal4 is subject to proteolytic degradation upon induction as proposed by Muratani et al. (2005) and it might take a while to fully restore the Gal4-protein level. But it might also be that the protein levels are generally changed due to the higher cell densities after 24 hours.

In the miniGal4#9 expressing cells the galactose concentration drops much slower than in the wtGal4- and miniGal4#7 containing cells. Galactose is not completely depleted during the measured time course and no re-association of Gal80 to Gal4 is observable. In the miniGal4-cells there is not even a decrease in galactose concentration measurable. In contrast to wtGal4 and miniGal4#7, miniGal4#9 and especially miniGal4 are no good transcriptional activators. The efficiency of transcriptional activation seems to decrease linearly with the size of the internal deletion. The region between the residues 680-840 therefore seems to be necessary for efficient transcriptional activation. It cannot be distinguished if specific residues are required or if a spacer with a specific minimal length is needed between DBD and AD.

The ChIP followed by qRT-PCR also revealed a higher quantity of Gal80 in the wtGal4-expressing cells than in the cells expressing miniGal4-variants under non-inducing conditions. A simple explanation would be that the amounts of miniGal4-proteins are less in the respective cells than that of the wildtype protein in the wt-cells. Since Western Blot analysis was unsuccessful, this could be evaluated with qRT-PCR of ChIP-samples immunoprecipitated with a Gal4-antibody. One could also argue that the significantly higher amount of Gal80 bound to wtGal4

might be due to a more efficient binding of Gal80 via one or several additional sites on Gal4 distinct from the established binding domain. A famous example for an effective protein-protein-interaction via multiple low affinity interactions is the binding of Gal11 to Gcn4. The bZIP transcriptional activator Gcn4 (also an acidic activator) has an N-terminal and a central AD that both interact with each of the three Gcn4-activator-binding domains (ABD1-3) of Gal11. The binding can occur in various conformations and orientations and is mediated only via hydrophobic interactions that additively contribute to overall transcriptional activation (Brzovic et al. 2011). This example demonstrates how a gene specific transcriptional activator is able to recruit multiple factors of the transcriptional machinery rather unspecifically. It is therefore possible that weak interactions between Gal4 and its specific binding partner Gal80 add to the strong interaction with the C-terminal domain. However, there is no direct evidence that Gal80 binding to the miniGal4-variants is weaker than binding to the full length Gal4 protein.

4.5 Functional mini*Kl*Gal4 variants can be generated by deletion of specific parts of the internal region

Extensive deletion experiments and functional studies have been performed with the *Sc*Gal4-protein in the past. These studies have shown that large regions of *Sc*Gal4 can be deleted without loss of transcriptional activity. In the miniGal4-variant described by Wu et al. (1996) about 84 % of the of the internal region is deleted. It is remarkable that no such deletion studies have been performed with *Kl*Gal4. It is not known whether the minimal requirements for a functional *Sc*Gal4-variant (DBD + AD) are the same for *Kl*Gal4.

A functional mini*Kl*Gal4-variant might also be interesting for further investigations of the *Kl*Gal4-*Kl*Gal80 interaction. In fact, it is very common to work with separate domains instead of the full length protein in structural analysis. This simplifies the data which is for example generated in crystal structure analysis or crosslinking studies and facilitates the interpretation of the results. As discussed before the deletion of distinct regions can lead to conformational and structural changes in the protein. But especially when the expression and purification of a full length protein is very challenging and when the protein is very unstable, expression of individual domains or deletions within the protein can be helpful. It is usually unstructured regions within a protein that impede recombinant expression, purification and stability of the protein, not to mention crystallization. To find these regions and to decide whether they could be deleted in the *Kl*Gal4-protein, a secondary structure analysis was performed with eleven

independent open source programs. The prediction of secondary structures is of course only based on the primary amino acid sequence of *Kl*Gal4. To predict potentially structured regions (α-helices, β-sheets) and coiled-coil regions the programs use algorithms that integrate many structure respectively disorder parameters like sequence complexity, amino-acid compositional bias, content of bulky hydrophobic amino acids, proportion of particular polar and charged amino acids and so on (Dyson & Wright 2005; see also section 3.5.1 table 1). They cannot substitute for experimental determination of structure elements but they are helpful for a first estimation.

The N-termial region spanning the amino acids 1-90 was predicted to contain if at all only a few short structured residues, but deletion of this region would possibly eliminate the nuclear localization sequence of *Kl*Gal4. I mapped a monopartite NLS to the amino acids 35-45 with the help of the cNLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) with a score of 9 (with scores ranging from 0-10), so that it can be assumed to be there with high probability. Deletion of the 90 N-terminal residues therefore might enhance the stability of recombinantly expressed protein, but makes it unusable for functional studies *in vivo*. The solution structure of the *Kl*Gal4-DBD by Gardner et al. (1995) was indeed solved with a 60 residue peptide consisting of the amino acids 85-144.

Another relatively large potentially unstructured region was predicted in the region between the DNA-binding/dimerization domain and residue 380. This region was deleted in the *Kl*Gal4-77 variant, which could be shown to have about 60 % of the wildtype *Kl*Gal4-activity in a β-galactosidase activity assay. This protein is therefore the first functional mini*Kl*Gal4-variant described so far (although it is still a large protein). The loss of the full activity due to the deletion shows that the region between aa 190 and 381 must have a function, but maybe it is just a linker or spacer function. Unfortunately transformation of a second *KlGAL4-77* gene copy into the strain does not lead to a significant increase in β-galactosidase activity. But expression of the *Kl*Gal4-77 protein in the plasmid transformed strain was not checked by Western Blot. Contrary to that the *Kl*Gal4-67 variant, which did not activate β-galactosidase expression in the YCZ_KlGal4-67 strain, displayed a visibly increased activity in the plasmid transformed strain. In this variant the residues 191 to 471 are deleted. The region from aa 380-470 was predicted to have a large α-helical content and ends right in front of the middle homology region. The measured β-galactosidase activity was very low in contrast to a wildtype *Kl*Gal4-strain transformed with a plasmid encoded second wt*Kl*GAL4-gene copy (only 20 % of the activity

measured in a wt *Kl*Gal4-strain). But nevertheless this variant has some residual activity and is regulatable by galactose induction, indicating that it interacts with *Kl*Gal80 under repressing conditions. *Kl*Gal4-67 is therefore also a functional mini*Kl*Gal4-variant. In *Kl*Gal4-57 the residues 191-556 were deleted, which means that also the MHR is eliminated in this variant. The strain expressing this *Kl*Gal4-variant displayed no β-galactosidase activity, not even when transformed with a second plasmid-encoded gene copy. Therefore the MHR is either essential for the function of the protein or the large internal deletion leads to an improper folding of this variant. It might also be that there is some residual activity that cannot be detected by the β-galactosidase activity assay. Anyway this variant does not represent a (measurably) functional mini*Kl*Gal4 variant, whereas the variants *Kl*Gal4-77 and *Kl*Gal4-67 could be considered for structural studies. Since they are now shown to be functional *in vivo*, further deletion of the unstructured N-terminus could be advantageous.

5 Summary

The galactose genetic switch in *Saccharomyces cerevisiae* and *Kluyveromyces lactis* is conducted by the activity of three proteins, the transcriptional activator Gal4, the repressor Gal80 and the galactose sensing protein Gal3/1. According to the "allosteric model", the formation of a ternary Gal4-Gal80-Gal3/1 complex leads to activation of *GAL/LAC*-gene transcription upon galactose induction. According to the "dissociation model", Gal3/1 and Gal4 compete for the binding to Gal80, leading to dissociation of Gal3/1-bound Gal80 from the activator Gal4.

The results obtained in this work strongly support the dissociation model for both yeasts.

Chromatin immunoprecipitation (ChIP) experiments in *Saccharomyces cerevisiae* revealed that *Sc*Gal80 is bound to *Sc*Gal4 at the *GAL1*-promoter only under non-inducing conditions. It could be shown, that the addition of galactose leads to dissociation of *Sc*Gal80 from the promoter bound *Sc*Gal4. A transfer of the *Sc*Gal80 protein to a different site on *Sc*Gal4 upon transcriptional activation, as it was proposed for the allosteric activation model, could not be observed. The *Sc*Gal80-signal reappeared when galactose in the medium was depleted. A ChIP experiment with a *Sc*Gal4 variant lacking the activation domain showed that Gal80 cannot bind to an alternative binding site on the Gal4 protein.

Competition experiments with a recombinantly expressed and purified *Kl*Gal4-*Kl*Gal80 complex showed, that the complex is dissociated by the addition of recombinant *Kl*Gal1. Ternary complex formation could not be observed. The further addition of the ligands galactose and ADP to *Kl*Gal1 increased the amount of dissociated *Kl*Gal80. The *Kl*Gal4 and *Kl*Gal80 proteins which were used in the competition assays were co-expressed in *E. coli* as GST-tagged and His_6-tagged proteins, respectively, and purified in a two-step affinity chromatography protocol. The co-expression with the repressor increased the stability of the *Kl*Gal4 protein and facilitated its purification. Native gel electrophoresis with the *Kl*Gal4-*Kl*Gal80 complex showed that it forms a number of distinct complexes, probably due to Gal80-tetramerization or to the GST-fusion of *Kl*Gal4. The complex was shown to bind to a specific DNA-binding site in an electrophoretic mobility shift assay (EMSA) and could be dissociated by the addition of a peptide consisting of the 22 C-terminal residues of *Kl*Gal4 (AD-22), confirming that the activation domain and established Gal80-binding site is capable to bind the repressor and represents the main *Kl*Gal80 interaction site on the *Kl*Gal4 protein.

6 Zusammenfassung

Der Galaktose-Switch in *Saccharomyces cerevisiae* und *Kluyveromyces lactis* wird durch die Aktivität dreier Proteine reguliert, den Transkriptionsaktivator Gal4, den Repressor Gal80 und den Galaktosesensor Gal3/1. Es existieren zwei Modellvorstellungen, die diesen Galaktoseschalter beschreiben. Dem „Allosterischen Modell“ zufolge bilden Gal4, Gal80 und Gal3/1 bei Galaktose-Induktion einen trimären Komplex, der zur Aktivierung der *GAL/LAC*-Gene fähig ist. Beim „Dissoziationmodell“ hingegen konkurrieren Gal3/1 und Gal4 um die Bindung an Gal80, was zu einer vollständigen Dissoziation des Gal3/1-Gal80 Komplexes vom Aktivator führt.

Die in der vorliegenden Arbeit erzielten Ergebnisse stützen eindeutig das Dissoziationsmodell, sowohl für *S. cerevisiae* als auch für *K. lactis*.

Chromatin Immunpräzipitations-Experimente (ChIP) in *Saccharomyces cerevisiae* zeigten deutlich, dass *Sc*Gal80 nur unter nicht-induzierenden Bedingungen an *Sc*Gal4 am *GAL1*-Promotor bindet. Unter induzierenden Bedingungen (Galaktose im Medium) konnte kein *Sc*Gal80 am Promotor detektiert werden. Ein Transfer von *Sc*Gal80 zu einer anderen Bindungsstelle an *Sc*Gal4 entsprechend dem allosterischen Modell wurde nicht beobachtet. Eine erneute Bindung von *Sc*Gal80 an *Sc*Gal4 konnte erst wieder nachgewiesen werden, nachdem die Galaktose im Medium aufgebraucht war. Ein ChIP-Versuch mit einer *Sc*Gal4-Variante, deren Aktivierungsdomäne deletiert war, zeigte, dass Gal80 nicht an eine alternative Bindungsstelle am Gal4-Protein binden kann.

In Konkurrenzexperimenten konnte gezeigt werden, dass die Zugabe von rekombinantem *Kl*Gal1 zu einem *Kl*Gal4-*Kl*Gal80-Komplex, der ebenfalls rekombinant in *E. coli* exprimiert und gereinigt wurde, zur Dissoziation des Repressor-Aktivator-Komplexes führt. Die Bildung eines trimären Komplexes entsprechend dem allosterischen Modell wurde nicht beobachtet. Der Zusatz von Galaktose und ADP zu *Kl*Gal1 erhöhte die Menge des dissoziierten *Kl*Gal80. Die in den Konkurrenzexperimenten eingesetzten *Kl*Gal4 und *Kl*Gal80 Proteine wurden als GST- beziehungsweise His_6-getaggte Proteine koexprimiert und mit Hilfe eines zweistufigen Affinitätschromatographie-Protokolls gereinigt. Die Koexpression mit dem Repressor erhöhte die Stabilität des *Kl*Gal4-Proteins und ermöglichte so erst dessen Aufreinigung. Eine Analyse des *Kl*Gal4-*Kl*Gal80-Komplexes mithilfe nativer Gelektrophorese zeigte, dass der Komplex eine Reihe verschiedener Oligomere bildet. Dies ist möglicherweise auf eine Tetramerisierung von

*Kl*Gal80 oder die GST-Fusion von *Kl*Gal4 zurückzuführen. Im electrophoretic mobility shift assay (EMSA) konnte gezeigt werden, dass der *Kl*Gal4-*Kl*Gal80-Komplex an eine Gal4-spezifische DNA-Bindungsstelle binden kann. Es konnte außerdem gezeigt werden, das die Zugabe des aus den 22 C-terminalen Aminosäuren von *Kl*Gal4 bestehenden AD-22 Peptids zu dem Komplex zur Dissoziation des Repressors vom Aktivator führt. Dieser Befund bestätigt, dass die C-terminale Aktivierungsdomäne und einzig bekannte Gal80-Bindungsstelle in der Lage ist, *Kl*Gal80 zu binden. Mit hoher Wahrscheinlichkeit stellt sie daher die Hauptinteraktionsstelle für den Repressor dar.

7 Bibliography

Anders A., 2006. *Molekularer Mechanismus eines Transkriptionsschalters : Experimentelle Analyse und mathematische Modellierung des Gal4-Gal80-Gal1-Regulationsmoduls aus Kluyveromyces lactis*.

Anders A., Lilie H., Franke K., Kapp L., Stellin J., Gilles E. D., Breunig K. D., 2006. The galactose switch in Kluyveromyces lactis depends on nuclear competition between Gal4 and Gal1 for Gal80 binding. *The Journal of biological chemistry*, 281(39), pp. 29337–48.

Ansari A. Z., Koh S. S., Zaman Z., Bongards C., Lehming N., Young R. A., Ptashne M., 2002. Transcriptional activating regions target a cyclin-dependent kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 99(23), pp. 14706–9.

Archer C.T., Delahodde A., Gonzales F., Johnston S. A., Kodadek T., 2008. Activation domain-dependent monoubiquitylation of Gal4 protein is essential for promoter binding in vivo. *The Journal of biological chemistry*, 283(18), pp.12614–23.

Archer C.T., Burdine L., Liu B., Ferdous A., Johnston S. A., Kodadek T., 2008. Physical and functional interactions of monoubiquitylated transactivators with the proteasome. *The Journal of biological chemistry*, 283(31), pp.21789–98.

Badger J., Sauder J. M., Adams J. M., Antonysamy S., Bain K., Bergseid M. G., Buchanan S. G., Buchanan M. D., Batiyenko Y., Christopher J. A., Emtage S., Eroshkina A., Feil I., Furlong E. B., Gajiwala K. S., Gao X., He D., Hendle J., Huber A., Hoda K., Kearins P., Kissinger C., Laubert B., Lewis H. A., Lin J., Loomis K., Lorimer D., Louie G., Maletic M., Marsh C. D., Miller I., Molinari J., Muller-Dieckmann H. J., Newman J. M., Noland B. W., Pagarigan B., Park F., Peat T. S., Post K. W., Radojicic S., Ramos A., Romero R., Rutter M. E., Sanderson W. E., Schwinn K. D., Tresser J., Winhoven J., Wright T. A., Wu L., Xu J., Harris T. J. R., 2005. Structural analysis of a set of proteins resulting from a bacterial genomics project. *Proteins*, 60(4), pp.787–796.

Baleja J. D., Marmorstein R., Harrison S. C., Wagner G., 1992. Solution structure of the DNA-binding domain of Cd2-GAL4 from S. cerevisiae. *Nature*, 356(6368), pp.450–453.

Bhaumik, S.R., Raha T., Aiello D. P., Green M. R., 2004. In vivo target of a transcriptional activator revealed by fluorescence resonance energy transfer. *Genes & development*, 18(3), pp.333–43.

Bork P., Sander C., Valencia, A., 1993. Convergent evolution of similar enzymatic function on different protein folds: The hexokinase, ribokinase, and galactokinase families of sugar kinases. *Protein Science*, 2(1), pp.31–40.

Bram R.J., Lue N.F., Kornberg R.D., 1986. A GAL family of upstream activating sequences in yeast: roles in both induction and repression of transcription. *the The European Molecular Biology Organization Journal*, 5(3), pp.603–608.

Breunig K. D., 1989. Multicopy plasmids containing the gene for the transcriptional activator LAC9 are not tolerated by K. lactis cells. *Current genetics*, 15(2), pp.143–8.

Breunig K. D., Kuger P., 1987. Functional homology between the yeast regulatory proteins GAL4 and LAC9: LAC9-mediated transcriptional activation in Kluyveromyces lactis involves protein binding to a regulatory sequence homologous to the GAL4 protein-binding site. *Molecular and cellular biology*, 7(12), pp.4400–6.

Brzovic P.S., Heikaus C. C., Kisselev L., Vernon R., Herbig E., Pacheco D., Warfield L., Littlefield P., Baker D., Klevit R. E., Hahn S., 2011. The acidic transcription activator Gcn4 binds the mediator subunit Gal11/Med15 using a simple protein interface forming a fuzzy complex. *Molecular cell*, 44(6), pp.942–53.

Chasman D.I., Kornberg R.D., 1990. GAL4 protein: purification, association with GAL80 protein, and conserved domain structure. *Molecular and cellular biology*, 10(6), pp.2916–23.

Cheng J., Randall A. Z., Sweredoski M. J., Baldi P., 2005. SCRATCH: a protein structure and structural feature prediction server. *Nucleic acids research*, 33(Web Server issue), pp.W72–6.

Cole C., Barber J.D., Barton, G.J., 2008. The Jpred 3 secondary structure prediction server. *Nucleic acids research*, 36(Web Server issue), pp.W197–201.

Conaway R.C., Brower C.S., Conaway, J.W., 2002. Emerging roles of ubiquitin in transcription regulation. *Science (New York, N.Y.)*, 296(5571), pp.1254–8.

Davis G.D., Elisee C., Newham D. M., Harrison R. G., 1999. New fusion protein systems designed to give soluble expression in Escherichia coli. *Biotechnology and Bioengineering*, 65(4), pp.382–388.

Dent S.Y.R., Malave T.M., 2006. MINIREVIEW / MINISYNTHE Transcriptional repression by Tup1 – Ssn6 1. *Cell*, 443(4), pp.437–443.

Ding, W.V., Johnston, S. A., 1997a. The DNA binding and activation domains of Gal4p are sufficient for conveying its regulatory signals. *Molecular and cellular biology*, 17(5), pp.2538–49.

Ding, W.V. & Johnston, S. A., 1997b. The DNA binding and activation domains of Gal4p are sufficient for conveying its regulatory signals. *Molecular and cellular biology*, 17(5), pp.2538–49.

Dyson, H. J., Wright P. E., 2005. Intrinsically unstructured proteins and their functions. *Nature reviews. Molecular cell biology*, 6(3), pp.197–208.

Egriboz, O., Jiang, F., Hopper, J. E., 2011. Rapid GAL Gene Switch of Saccharomyces cerevisiae Depends on Nuclear Gal3, Not Nucleocytoplasmic Trafficking of Gal3 and Gal80. *Genetics*, 189(3), pp.825–36.

Febbraio F., Andolfo A., Tanfani F., Briante R., Gentile F., Formisano S., Vaccaro C., Scirè A., Bertoli E., Pucci P., Nucci R., 2004. Thermal stability and aggregation of sulfolobus solfataricus beta-glycosidase are dependent upon the N-epsilon-methylation of specific lysyl residues: critical role of in vivo post-translational modifications. *The Journal of Biological Chemistry*, 279(11), pp.10185–10194.

Ferdous A., O'Neal M., Nalley K., Sikder D., Kodadek T., Johnston S. A., 2008. Phosphorylation of the Gal4 DNA-binding domain is essential for activator mono-ubiquitylation and efficient promoter occupancy. *Molecular bioSystems*, 4(11), pp.1116–25.

Frey P.A., 1996. The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *The FASEB journal official publication of the Federation of American Societies for Experimental Biology*, 10(4), pp.461–470. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8647345.

Friden P., Reynolds C., Schimmel P., 1989. A large internal deletion converts yeast LEU3 to a constitutive transcriptional activator. *Molecular and Cellular Biology*, 9(9), pp.4056–4060.

Gardner K.H., Anderson S.F., Coleman J.E., 1995. Solution structure of the Kluyveromyces lactis LAC9 Cd2 Cys6 DNA-binding domain. *Nature Structural Biology*, 2(10), pp.898–905.

Garnier J., Gibrat J.F., Robson B., 1996. GOR method for predicting protein secondary structure from amino acid sequence. *Methods*, 266(1995), pp.540–53.

Geourjon C., Deléage G., 1995. SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Computer applications in the biosciences : CABIOS*, 11(6), pp.681–4.

Ghaemmaghami S., Huh W., Bower K., Howson R. W., Belle A., Dephoure N., O'Shea E. K., Weissman J. S., 2003. Global analysis of protein expression in yeast. *Nature*, 425(6959), pp.737–741.

Giles N.H., Geever R. F., Asch D. K., Avalos J., Case M. E., 1991. The Wilhelmine E. Key 1989 invitational lecture. Organization and regulation of the qa (quinic acid) genes in Neurospora crassa and other fungi. *The Journal of heredity*, 82(1), pp.1–7.

Giniger E., Varnum S.M., Ptashne M., 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell*, 40(4), pp.767–74.

Griggs D.W., Johnston M., 1991. Regulated expression of the GAL4 activator gene in yeast provides a sensitive genetic switch for glucose repression. *Proceedings of the National Academy of Sciences of the United States of America*, 88(19), pp.8597–601.

Gsponer J., Babu M.M., 2009. The rules of disorder or why disorder rules. *Progress in biophysics and molecular biology*, 99(2-3), pp.94–103.

Halvorsen Y.C., Nandabalan K., Dickson R.C., 1990. LAC9 DNA-binding domain coordinates two zinc atoms per monomer and contacts DNA as a dimer. *The Journal of biological chemistry*, 265(22), pp.13283–9.

Hidalgo P., Ansari A. Z., Schmidt P., Hare B., Simkovich N., Farrell S., Shin E. J., Ptashne M., Wagner G., 2001. Recruitment of the transcriptional machinery through GAL11P : structure and interactions of the GAL4 dimerization domain. *Genes & Development*, pp.1007–1020.

Hiebert S.W., Chellappan S. P., Horowitz J. M., Nevins J. R., 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes & Development*, 6(2), pp.177–185.

Hirst M., Kobor M. S., Kuriakose N., Greenblatt J., Sadowski I., 1999. GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8. *Molecular Cell*, 3(5), pp.673–678.

Hong M., Fitzgerald M. X., Harper S., Luo C., Speicher D. W., Marmorstein R., 2008. Structural basis for dimerization in DNA recognition by Gal4. *Structure (London, England : 1993)*, 16(7), pp.1019–26.

Horak J., Wolf D.H., 1997. Catabolite inactivation of the galactose transporter in the yeast Saccharomyces cerevisiae : ubiquitination, endocytosis and catabolite inactivation. *Journal of Bacteriology*, 179(5), pp. 1541-49.

Jablonowski D., Fichtner L., Martin V. J., Klassen R., Meinhardt F, Stark M. J., Schaffrath R., 2001. Saccharomyces cerevisiae cell wall chitin, the Kluyveromyces lactis zymocin receptor. *Yeast Chichester England*, 18(14), pp.1285–1299.

Jiang F., Frey B. R., Evans M. L., Friel J. C., Hopper J. E., 2009. Gene activation by dissociation of an inhibitor from a transcriptional activation domain. *Molecular and cellular biology*, 29(20), pp.5604–10.

Johnston M., 1987. A Model Fungal Gene Regulatory Mechanism: the GAL Genes of Saccharomyces Cerevisiae. *Mircobiological Reviews*, 51(4), pp.458–476.

Johnston M., Dover J., 1988. Mutational Analysis of the GAL4-Encoded Transcriptional Activator Protein of Saccharomyces cerevisiae. *Genetics,* 120, pp. 63-74.

Johnston S. A., Salmeron J. M., Dincher S.S., 1987. Interaction of positive and negative regulatory proteins in the galactose regulon of yeast. *Cell*, 50(1), pp.143–6.

Kaplan W., Hüsler P., Klump H., Erhardt J., Sluis-Cremer N., Dirr H., 1997. Conformational stability of pGEX-expressed Schistosoma japonicum glutathione S-transferase: a detoxification enzyme and fusion-protein affinity tag. *Protein science : a publication of the Protein Society*, 6(2), pp.399–406.

Keegan L., Gill G., Ptashne M., 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science*, 231(4739), pp.699–704.

Kelley L. A., Sternberg M. J. E., 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nature protocols*, 4(3), pp.363–71.

Kraulis P. J., Raine A. R., Gadhavi P. L., Laue E. D., 1992. Structure of the DNA-binding domain of zinc GAL4. *Nature*, 356(6368), pp.448–450.

Kuger P., Gödecke A., Breunig K. D., 1990. A mutation in the Zn-finger of the GAL4 homolog LAC9 results in glucose repression of its target genes. *Nucleic acids research*, 18(4), pp.745–51.

Kumar P. R., Yu Y., Sternglanz R., Johnston S. A., Joshua-Tor L., 2008. NADP regulates the yeast GAL induction system. *Science (New York, N.Y.)*, 319(5866), pp.1090–2.

Kussie P.H., Gorina S., Marechal V., Elenbaas B., Moreau J., Levine A. J., Pavletich N. P., 1996. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science (New York, N.Y.)*, 274(5289), pp.948–53.

Kuzhandaivelu N., Jones W. K., Martin A. K., Dickson R. C., 1992. The signal for glucose repression of the lactose-galactose regulon is amplified through subtle modulation of transcription of the Kluyveromyces lactis Kl-GAL4 activator gene. *Molecular and cellular biology*, 12(5), pp.1924–31.

Kuznetsov I. B., Rackovsky S., 2003. On the properties and sequence context of structurally ambivalent fragments in proteins. *Protein Science* 12(11), pp.2420–2433.

Lamphier M.S., Ptashne M., 1992. Multiple mechanisms mediate glucose repression of the yeast GAL1 gene. *Proceedings of the National Academy of Sciences of the United States of America*, 89(13), pp.5922–5926.

Laughon A., Driscoll R., Wills N., Gesteland R. F., 1984. Identification of two proteins encoded by the Saccharomyces cerevisiae GAL4 gene. *Molecular and Cellular Biology*, 4(2), pp.268–275.

Lavy T., Kumar P. R., He H., Joshua-Tor L., 2012. The Gal3p transducer of the GAL regulon interacts with the Gal80p repressor in its ligand-induced closed conformation. *Genes & development*, 26(3), pp.294–303.

Lee C., Chang J. H., Lee H. S., Cho Y., 2002. Structural basis for the recognition of the E2F transactivation domain by the retinoblastoma tumor suppressor. *Genes & Development*, pp.3199–3212.

Leuther K. K., Johnston S. A., 1992. Nondissociation of GAL4 and GAL80 in vivo after galactose induction. *Science*, 256(5061), pp.1333–1335.

Leuther K. K., Salmeron J. M., Johnston S. A., 1993. Genetic evidence that an activation domain of GAL4 does not require acidity and may form a beta sheet. *Cell*, 72(4), pp.575–85.

Leverentz M. K., Reece R. J., 2006. Phosphorylation of Zn(II)2Cys6 proteins: a cause or effect of transcriptional activation? *Biochemical Society transactions*, 34(Pt 5), pp.794–7.

Ma J., Ptashne M., 1987a. Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell*, 48(5), pp.847–53.

Ma J., Ptashne M., 1987b. The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. *Cell*, 50(1), pp.137–42.

Mamane Y., Hellauer K., Rochon M. H., Turcotte B., 1998. A linker region of the yeast zinc cluster protein leu3p specifies binding to everted repeat DNA. *The Journal of Biological Chemistry*, 273(29), pp.18556–18561.

Marmorstein R., Carey M., Ptashne M., Harrison S. C., 1992. DNA recoginition by GAL4: structure of a protein-DNA complex. *Nature*, 356, pp.408–414.

McEwan I. J., Dahlman-Wright K., Ford J., Wright A. P., 1996. Functional interaction of the c-Myc transactivation domain with the TATA binding protein: evidence for an induced fit model of transactivation domain folding. *Biochemistry*, 35(29), pp.9584–93.

Melcher K., 2000. The strength of acidic activation domains correlates with their affinity for both transcriptional and non-transcriptional proteins. *Journal of molecular biology*, 301(5), pp.1097–112.

Melcher K., Xu H. E., 2001. Gal80-Gal80 interaction on adjacent Gal4p binding sites is required for complete GAL gene repression. *The EMBO journal*, 20(4), pp.841–51.

Menezes R., Amuel A. C., Engels R., Gengenbacher U., Labahn J., Hollenberg C.P., 2003. Sites for Interaction between Gal80p and Gal1p in Kluyveromyces lactis: Structural Model of Galactokinase based on Homology to the GHMP Protein Family. *Journal of Molecular Biology*, 333(3), pp.479–492.

Meyer J., Walker-Jonah A., Hollenberg C. P., 1991. Galactokinase encoded by GAL1 is a bifunctional protein required for induction of the GAL genes in Kluyveromyces lactis and is able to suppress the gal3 phenotype in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, 11(11), pp.5454–5461.

Muratani M., Kung C., Shokat K. M., Tansey W. P., 2005. The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell*, 120(6), pp.887–99.

Nehlin J.O., Carlberg M., Ronne H., 1991. Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response. *the The European Molecular Biology Organization Journal*, 10(11), pp.3373–3377.

Nehlin J.O., Ronne, H., 1990. Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *The EMBO journal*, 9(9), pp.2891–8.

Nygren P. A, Ståhl S., Uhlén M., 1994. Engineering proteins to facilitate bioprocessing. *Trends in biotechnology*, 12(5), pp.184–8.

Ouali M., King R.D., 2000. Cascaded multiple classifiers for secondary structure prediction. *Protein science : a publication of the Protein Society*, 9(6), pp.1162–76.

Parker D., Jhala U. S., Radhakrishnan I., Yaffe M. B., Reyes C., Shulman A. I., Cantley L. C., Wright P. E., Montminy M., 2004. Analysis of an activator:coactivator complex reveals an essential role for secondary structure in transcriptional activation. *Molecular Cell*, 2(3), pp.353–359.

Parthun M. R., Jaehning J. A., 1992. A transcriptionally active form of GAL4 is phosphorylated and associated with GAL80. *Molecular and cellular biology*, 12(11), pp.4981–7.

Parthun M. R., Jaehning J. A, 1990. Purification and characterization of the yeast transcriptional activator GAL4. *The Journal of biological chemistry*, 265(1), pp.209–13.

Peng G., Hopper J. E., 2000. Evidence for Gal3p's cytoplasmic location and Gal80p's dual cytoplasmic-nuclear location implicates new mechanisms for controlling Gal4p activity in Saccharomyces cerevisiae. *Molecular and cellular biology*, 20(14), pp.5140–8.

Peng G., Hopper J. E., 2002. Gene activation by interaction of an inhibitor with a cytoplasmic signaling protein. *Proceedings of the National Academy of Sciences of the United States of America*, 99(13), pp.8548–53.

Petersen B., Petersen N. T., Andersen P., Nielsen M., Lundegaard C., 2009. A generic method for assignment of reliability scores applied to solvent accessibility predictions. *BMC structural biology*, 9, p.51.

Pfeifer K., Kim K. S., Kogan S., Guarente L., 1989. Functional dissection and sequence of yeast HAP1 activator. *Cell*, 56(2), pp.291–301.

Pilauri V., Bewley M., Diep C., Hopper J. E., 2005. Gal80 dimerization and the yeast GAL gene switch. *Genetics*, 169(4), pp.1903–14.

Platt A., Reece R. J., 1998. The yeast galactose genetic switch is mediated by the formation of a Gal4p-Gal80p-Gal3p complex. *The EMBO journal*, 17(14), pp.4086–91.

Pollastri G., McLysaght A., 2005. Porter: a new, accurate server for protein secondary structure prediction. *Bioinformatics (Oxford, England)*, 21(8), pp.1719–20.

Radhakrishnan I., Pérez-Alvarado G. C., Parker D., Dyson H. J., Montminy M. R., Wright P. E., 1997. Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions. *Cell*, 91(6), pp.741–752.

Raghava G. P. S., 2002. APSSP2: A combination method for protein secondary structure prediction based on neural network and example based learning. *CASP5*, A-132, p.A–132.

Rao S. T., Rossmann M. G., 1973. Comparison of super-secondary structures in proteins. *Journal of Molecular Biology*, 76(2), pp.241–256.

Reece R. J., Ptashne M., 1993. Determinants of binding-site specificity among yeast C6 zinc cluster proteins. *Science*, 261(5123), pp.909–911.

Riley M.I., Hopper J. E., Johnston S. A., Dickson R. C., 1987. GAL4 of Saccharomyces cerevisiae activates the lactose-galactose regulon of Kluyveromyces lactis and creates a new

phenotype: glucose repression of the regulon. *Molecular and Cellular Biology*, 7(2), pp.780–786.

Sadowski I., Niedbala D., Wood K., Ptashne M., 1991. GAL4 is phosphorylated as a consequence of transcriptional activation. *Proceedings of the National Academy of Sciences of the United States of America*, 88(23), pp.10510–4.

Sadowski I., Costa C., Dhanawansa R., 1996. Phosphorylation of Ga14p at a single C-terminal residue is necessary for galactose-inducible transcription. *Molecular and cellular biology*, 16(9), pp.4879–87.

Salmeron J. M., Langdon S. D., Johnston S. A., 1989. Interaction between transcriptional activator protein LAC9 and negative regulatory protein GAL80. *Molecular and cellular biology*, 9(7), pp.2950–6.

Salmeron J. M., Johnston S. A., 1986. Analysis of the Kluyveromyces lactis positive regulatory gene LAC9 reveals functional homology to, but sequence divergence from, the Saccharomyces cerevisiae GALA gene. *Nucleic Acids Research*, 14(19), pp.7767–7781.

Schjerling P., Holmberg S., 1996. Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. *Nucleic acids research*, 24(23), pp.4599–607.

Schmidt, D. 2010. *Biophysikalische und molekulargenetische Untersuchung der Dinukleotid-Bindung von Gal80 aus Saccharomyces cerevisiae und Kluyveromyces lactis*. Martin-Luther-Universität Halle-Wittenberg.

Schönfeld H.J., Schmidt D., Schröder H., Bukau B., 1995. The DnaK chaperone system of Escherichia coli: quaternary structures and interactions of the DnaK and GrpE components. *The Journal of Biological Chemistry*, 270(5), pp.2183–2189.

Schöninger T., 2000. *Konstruktion eines Selektionssystems für Deletionsmutanten in Saccharomyces cerevisiae*. Martin-Luther-University Halle-Wittenberg.

Sellick C. A, Jowitt T. A., Reece R. J., 2009. The effect of ligand binding on the galactokinase activity of yeast Gal1p and its ability to activate transcription. *The Journal of biological chemistry*, 284(1), pp.229–36.

Sil A. K., Alam S., Xin P., Ma L., Morgan M., Lebo C. M., Woods M. P., Hopper J. E., 1999. The Gal3p-Gal80p-Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p-Gal4p complex in response to galactose and ATP. *Molecular and cellular biology*, 19(11), pp.7828–40.

Smith D.B., Johnson K.S., 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene*, 67(1), pp.31–40.

Stone G., Sadowski I., 1993. GAL4 is regulated by a glucose-responsive functional domain. *EMBO Journal*, 12(4), pp.1375–1385.

Suzuki-Fujimoto T., Fukuma M., Yano K. I., Sakurai H., Vonika A., Johnston S. A., Fukasawa T., 1996. Analysis of the galactose signal transduction pathway in Saccharomyces cerevisiae: interaction between Gal3p and Gal80p. *Molecular and Cellular Biology*, 16(5), pp.2504–2508.

Thoden J. B., Sellick C. A., Timson D. J., Reece R. J., Holden H. M., 2005. Molecular structure of Saccharomyces cerevisiae Gal1p, a bifunctional galactokinase and transcriptional inducer. *The Journal of biological chemistry*, 280(44), pp.36905–11.

Thoden J. B., Ryan L. A., Reece R. J., Holden H. M., 2008. The interaction between an acidic transcriptional activator and its inhibitor. The molecular basis of Gal4p recognition by Gal80p. *The Journal of biological chemistry*, 283(44), pp.30266–72.

Thoden J. B., Sellick C. A., Reece R. J., Holden H. M., 2007. Understanding a transcriptional paradigm at the molecular level. The structure of yeast Gal80p. *The Journal of biological chemistry*, 282(3), pp.1534–8.

Treitel M. A., Carlson M., 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proceedings of the National Academy of Sciences of the United States of America*, 92(8), pp.3132–3136.

Treitel M. A., Kuchin S., Carlson M., 1998. Snf1 Protein Kinase Regulates Phosphorylation of the Mig1 Repressor in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, 18(11), pp.6273–6280.

Vashee S., Xu H., Johnston S. A., Kodadek T., 1993. How do “Zn2 cys6” proteins distinguish between similar upstream activation sites? Comparison of the DNA-binding specificity of the GAL4 protein in vitro and in vivo. *The Journal of Biological Chemistry*, 268(33), pp.24699–24706.

Vidal M., Brachmann R. K., Fattaey A., Harlow E., Boeke J. D., 1996. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 93(19), pp.10315–20.

De Vit M.J., Waddle J. A., Johnston M., 1997. Regulated nuclear translocation of the Mig1 glucose repressor. *Molecular Biology of the Cell*, 8(8), pp.1603–1618.

Wang D., Hu Y., Zheng F., Zhou K., Kohlhaw G. B., 1997. Evidence that intramolecular interactions are involved in masking the activation domain of transcriptional activator Leu3p. *The Journal of biological chemistry*, 272(31), pp.19383–92.

Wang D., Zheng F., Holmberg S., Kohlhaw G. B., 1999. Yeast transcriptional regulator Leu3p. Self-masking, specificity of masking, and evidence for regulation by the intracellular level of Leu3p. *The Journal of Biological Chemistry*, 274(27), pp.19017–19024.

Weirich J., Goffrini P., Kuger P., Ferrero I., Breunig K. D., 1997. Influence of mutations in hexose-transporter genes on glucose repression in Kluyveromyces lactis. *European journal of biochemistry / FEBS*, 249(1), pp.248–57.

Whitmarsh A. J., Davis R. J., 2000. Regulation of transcription factor function by phosphorylation. *Cellular and molecular life sciences : CMLS*, 57(8-9), pp.1172–83.

Wightman R., Bell R., Reece R. J., 2008. Localization and interaction of the proteins constituting the GAL genetic switch in Saccharomyces cerevisiae. *Eukaryotic cell*, 7(12), pp.2061–8.

Wray L. V., Witte M. M., Dickson R. C., Riley M. I., 1987. Characterization of a positive regulatory gene, LAC9, that controls induction of the lactose-galactose regulon of Kluyveromyces lactis: structural and functional relationships to GAL4 of Saccharomyces cerevisiae. *Molecular and Cellular Biology*, 7(3), pp.1111–1121.

Wu Y., Reece R. J., Ptashne M., 1996. Quantitation of putative activator-target affinities predicts transcriptional activating potentials. *The EMBO journal*, 15(15), pp.3951–63.

Yang D., Shipman L. W., Roessner C. A., Scott A. I., Sacchettini J. C., 2002. Structure of the Methanococcus jannaschii mevalonate kinase, a member of the GHMP kinase superfamily. *The Journal of Biological Chemistry*, 277(11), pp.9462–9467.

Zachariae W., Breunig K. D., 1993. Expression of the transcriptional activator LAC9 (KlGAL4) in Kluyveromyces lactis is controlled by autoregulation. *Molecular and cellular biology*, 13(5), pp.3058–66.

Zachariae W., Kuger P., Breunig K. D., 1993. Glucose repression of lactose/galactose metabolism in Kluyveromyces lactis is determined by the concentration of the transcriptional activator LA1C9 (K1GAL4). *Nucleic Acids Research*, 21(1), pp.69–77.

Zenke F.T., Engles R., Vollenbroich V., Meyer J., Hollenberg C. P., Breunig K. D., 1996. Activation of Gal4p by galactose-dependent interaction of galactokinase and Gal80p. *Science*, 272(5268), pp.1662–1665.

Zenke F.T., Kapp L., Breunig K. D., 1999. Regulated phosphorylation of the Gal4p inhibitor Gal80p of Kluyveromyces lactis revealed by mutational analysis. *Biological chemistry*, 380(4), pp.419–30.

Zhou K. M., Bai Y. L., Kohlhaw G. B., 1990. Yeast regulatory protein LEU3: a structure-function analysis. *Nucleic Acids Research*, 18(2), pp.291–298.

Zhou T., Daugherty M., Grishin N. V., Osterman A. L., Zhang H., 2000. Structure and mechanism of homoserine kinase: prototype for the GHMP kinase superfamily. *Structure London England 1993*, 8(12), pp.1247–1257.

Declaration of Academic Honesty

Eidesstattliche Erklärung

I hereby declare that this dissertation is my own work and has not been submitted in any form for another degree at any university or other institute. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the bibliography.

Hiermit versichere ich, die vorliegende Dissertation ohne Hilfe Dritter und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben. Alle Stellen, die aus Quellen entnommen wurden, sind als solche kenntlich gemacht worden.
Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegen.

Halle (Saale), 28.03.2013

Lebenslauf

Name	Constance Zugowski
Adresse	Bahnhofstraße 14a 06905 Bad Schmiedeberg
Geburtsdatum	03.07.1982
Geburtsort	Lichtenstein/Sachsen

Schulische Ausbildung

08/1989 – 07/1993	Grundschule (Pretzsch/Elbe)
08/1993 – 03/2002	Heidegymnasium Pretzsch (Pretzsch/Elbe)

Akademische Ausbildung

10/2002 – 05/2008	Studium Biochemie/Molekularbiologie, Friedrich-Schiller-Universität Jena
06/2008 – 09/2012	Dissertation, Institut für Biologie – Genetik, Martin-Luther-Universität Halle-Wittenberg 06/2008 – 09/2011 Graduierte im Gradiuertenkolleg 1026 10/2011 – 09/2012 Assoziiertes Mitglied im Gradiuertenkolleg 1026 Projekt B2: „Structural analysis of the Gal4-Gal80-Gal3/1 transcriptional switch”