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STUDIES OF TRANSCRIPTION FACTOR DOMAINS AND THEIR INTERACTIONS WITH OTHER TRANSCRIPTION FACTORS

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In loving memory of my parents – Britt and Arne

ABSTRACT

The studies in this thesis deal with different questions concerning interactions of functional domains of factors involved in transcriptional regulation. The first study of this thesis is focused on the target factor binding mechanism of transcriptional activators. Many activators in evolutionary distant species are classified as acidic based on a high content of acidic residues in the activation domain and intrinsically unstructured in solution. Our results indicate that such activation domains interact with target factors through coupled binding and folding of the activation domain after an initial ionic interaction, and demonstrate the generality of this binding mechanism. We propose that target interaction through coupled binding and folding of the recruiting domain is important for the role of activators as regulators of transcription. In the following study we show that deletion of two regions that mediate interaction with activators *in vitro* prevents promoter recruitment of the SWI/SNF chromatin-remodeling complex *in vivo*, and causes strongly reduced transcriptional activity of the corresponding genes. This study validates direct interaction between the Swi1- and Snf5 activator binding domains of the *S. cerevisiae* SWI/SNF complex and activators previously demonstrated *in vitro*, and importantly indicates that the activator binding domains are essential for the ability of SWI/SNF to function as co-activator. In the last study we investigate which domains are involved in distinct *in vivo* function of the paralogous co-repressors Tup11 and Tup12 of the Ssn6/Tup complex in *S. pombe*. Tup11 and Tup12 have been shown to differ in importance in context of a common complex for subsets of Ssn6/Tup target genes, and it was proposed that this might depend on divergence in the histone-interaction domain. Here we show that distinct *in vivo* roles of Tup12 do not depend on differences in the highly diverged histone-interaction domain, but mainly on differences in the overall highly conserved WD40 repeat domain, which putatively mediates interaction with repressors and target factors such as histone modifying complexes and components of the transcriptional machinery. We propose that clusters of amino acids, putatively located in blade 3 of the WD40 repeat domain, could be important for interaction with distinct target factors of Tup11 and Tup12. Furthermore, we show that the stoichiometry of the Ssn6/Tup complex is likely to change under $CaCl_2$ stress, by a mechanism involving changes in the relative cellular levels of the complex components.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

DBD	DNA-binding domain
GR	Glucocorticoid receptor
GTF	General transcription factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ISWI	Imitation switch
NR	Nuclear receptor
RNA PolII	RNA polymerase II
SWI/SNF	Switch/Sucrose non-fermenting
TBP	TATA-binding protein
TAD	Trans-activation domain
TF	Transcription factor
KRAB	Krüppel-associated box
Tup1	Thymidin uptake protein
Ssn6	Suppressor of *snf1* protein
TPR	Tetratricopeptide repeat
WD	Tryptophan (W) and Aspartic acid (D)
IGR	Intergenic region
ORF	Open reading frame
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
MAPK	Mitogen-activated protein kinase
KAP	KRAB associated protein
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
ATP	Adenosine triphosphate
PIC	Pre-initiation complex
bHLH	Basic helix-loop-helix
bZIP	Basic region leucin zipper
bHLH-LZ	Basic helix-loop-helix leucin zipper

1 GENERAL INTRODUCTION

Proteins are involved in all cellular processes, and it is important that the production of various proteins is appropriately regulated in a timely manner. The major mechanism for regulation of protein levels occurs through regulation of transcription, the first step in the process of gene expression. Transcription is the process in which DNA is used as a template for making a transcript in the form of an RNA molecule. There are three different RNA polymerases (RNA Pol) in eukaryotic cells (Cramer, Armache et al. 2008). RNA PolII is the polymerase that transcribes the protein-coding genes, resulting in messenger RNA (mRNA), which contains the coding sequence that determines the amino acid sequence of the protein. RNA PolII also produces small nuclear RNAs that are involved in processing the mRNA. RNAs that are required for the protein synthesis are made by RNA PolI, responsible for transcribing genes encoding ribosomal RNA (rRNA), and RNA PolIII, which produces transfer RNA (tRNA) and other small RNAs. The mRNA molecule is used as a template by the translational machinery to synthesize the encoded protein.

1.1 FACTORS INVOLVED IN TRANSCRIPTION

The term transcription factor is used loosely in the title of this thesis. By convention, “transcription factors” (TFs) refers to regulatory factors (activators and repressors) that bind DNA with sequence specificity and recruit other factors whose functions are required to the repress or activate transcription of a particular gene. Transcriptional co-factors (co-repressors and co-activators) do not have intrinsic (sequence specific) DNA binding activity but are recruited by sequence specific transcription factors. Co-factors may be required as adapters or to catalyze enzymatic reactions, and the particular set of co-factors required for transcriptional regulation may vary between genes, even for genes that are regulated by the same transcription factor. General transcription factors (GTFs) are required for RNA PolII promoter binding and transcriptional initiation and were originally identified as a set of factors present in cell extracts that were required for RNA Pol II promoter binding and transcription initiation from the correct start site on a DNA template *in vitro* (Matsui, Segall et al. 1980; Lue and Kornberg 1987; Lee and Young 2000).

1.2 CHROMATIN

The DNA in the nuclei of eukaryotic cells is packed into highly organized chromatin. The first order of organization is the nucleosome, constituted by 146 base pairs of DNA wrapped around an octamer of histones, two copies each of histones H3, H4, H2A and H2B (Fig. 1). The resulting “beads on a string”-like structure formed by nucleosomal DNA is further organized into higher order of packing into denser fibers. The level of chromatin condensation is an important factor in regard to transcriptional activity, since it affects how accessible the DNA is to the factors involved in transcription. Two major mechanisms for regulating the level of chromatin condensation involve the enzymatic activities of histone modifiers and chromatin remodelers.

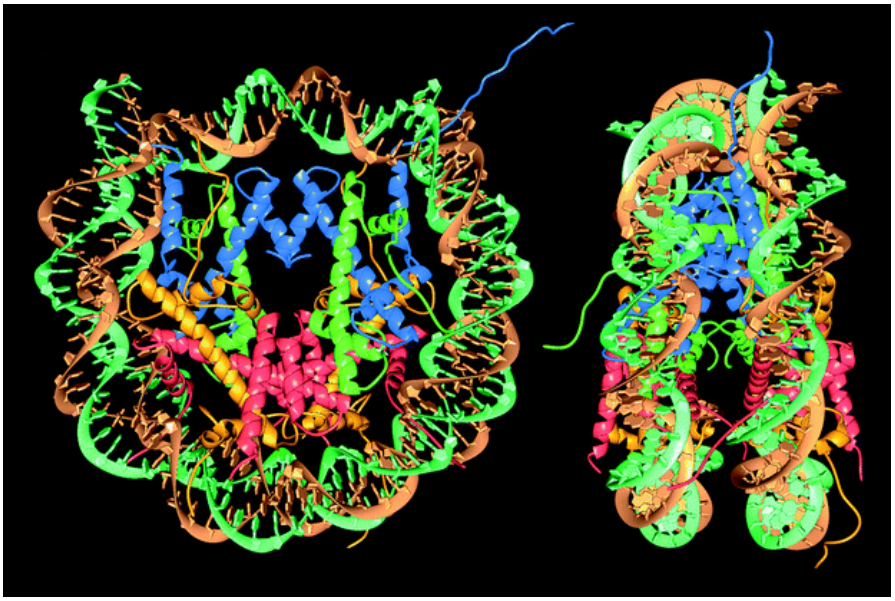

Figure 1. The crystal structure of the nucleosome core particle.
146 base-pairs of DNA (shown in brown and turquoise) is wrapped around two copies each of the four histones (blue: H3; green: H4; yellow: H2A; red: H2B) forming the histone octamer. The left image shows the nucleosome from above, the right image shows the nucleosome from a side-view rotated 90° relative to the left image. Reprinted with permission from Nature Publishing Group (Luger, Mader et al. 1997).

Histone modifying enzymes attach or remove different functional groups and particular proteins in predominantly but not exclusively the N-terminal tails of histones. Histone modifications have been proposed to affect chromatin by affecting interaction between DNA and the histone octamer (Freitas, Sklenar et al. 2004), and affect chromatin interaction of various factors. The presence of particular modifications in intergenic regions (IGRs) and different parts of the coding region correlates with the level of transcriptional activity (Li, Carey et al. 2007), and different modifications and combinations thereof have been proposed to function as a "histone-code" that is read by chromatin binding factors whose functions determine the outcome (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001). Histone acetyltransferases (HATs) acetylate lysine residues, whereas histone deacetylases (HDACs) catalyze the opposite reaction. HATs and HDACs are generally more promiscuous with regard to the position of the modified residue compared to enzymes responsible for other histone modifications. Acetylation generally correlates with transcriptional activity and the highest level of acetylation is found at the promoter of transcriptionally active genes (Workman and Kingston 1998; Pokholok, Harbison et al. 2005). Some non-histone proteins are also substrates of HATs and HDACs, for example the transcription factor p53, whose DNA binding is activated by acetylation (Gu and Roeder 1997; Vaziri, Dessain et al. 2001). Other histone modifications are methylation, phosphorylation, ubiquitinylation and sumolyation. Methylation is a more complex modification than acetylation because lysine residues can be mono-, di- or trimethylated, and arginine residues can be mono- or dimethylated, asymmetrically or symmetrically. Examples of protein domains that bind modified histones are bromodomains, which bind acetylated lysine (Hassan, Prochasson et al. 2002; Carey, Li et al. 2006), chromodomains and PHD domains, which bind methylated lysine (Lachner, O'Carroll et al. 2001; Pray-Grant, Daniel et al. 2005) and Tudor domains, which bind methylated lysine and arginine (Côté and Richard 2005; Kim, Daniel et al. 2006), and a particular histone modifying factor can contain several different types of histone binding domains (Lee and Workman 2007).

Chromatin remodelers are protein complexes with helicase-like properties that use the energy of ATP hydrolysis to disrupt interactions between DNA and histones, and remodeling may cause histone octamer sliding along the DNA molecule, loops in the DNA, eviction of the entire octamer or H2A/H2B dimers and exchange of histone variants. The fate of histones that are evicted upon remodeling depends on histone

chaperones. Histone chaperones have been shown to be involved in exchange of histone variants, reloading of nucleosomes on promoters and behind elongating RNA PolII, and to be important for histone eviction (Adkins, Howar et al. 2004; Mizuguchi, Shen et al. 2004; Adkins and Tyler 2006; Schwabish and Struhl 2006). Chromatin remodeling complexes are divided into four families based on conservation of the ATPase subunit – the SWI/SNF-, ISWI-, Ino80- and CHD family (Gangaraju and Bartholomew 2007; Hogan and Varga-Weisz 2007). The product of remodeling depends on the type of chromatin remodeling complex, and the functional differences of chromatin remodeling complexes are believed to be relevant with regard to their biological roles (Narlikar, Fan et al. 2002). For example, SWI/SNF complexes can induce super-helical torsion and stable loops in the DNA within the nucleosome without requiring sliding, and can move histone octamers along the DNA molecule, but sliding results in relatively disorderly spaced nucleosomes and remodelling appears to generally result in histone eviction rather than translational movement of nucleosomes (Lee, Sif et al. 1999; Whitehouse, Flaus et al. 1999; Narlikar, Fan et al. 2002; Boeger, Griesenbeck et al. 2004; Lorch, Maier-Davis et al. 2006; Gutiérrez, Chandy et al. 2007). Consistently, SWI/SNF complexes are involved mainly in transcriptional regulation, and commonly referred to as co-activators. By contrast, ISWI complexes have not been reported to induce stable loops in the DNA, and remodeling is translational and results in evenly spaced nucleosomes (Längst, Bonte et al. 1999; Längst and Becker 2001; Narlikar, Fan et al. 2002). Consistently, ISWI complexes have been shown to be involved in direct transcriptional repression and to be required for higher order chromatin organization (Deuring, Fanti et al. 2000; Goldmark, Fazzio et al. 2000). ACF complexes, which belong to the ISWI family and are associated mainly with transcriptional repression, have been proposed to remodel in pairs, i.e. two complexes per nucleosome, and this is thought to be important for remodeling by translational movement instead of histone eviction (Racki and Narlikar 2008).

1.3 TRANSCRIPTIONAL REGULATION

The intergenic region located upstream of the protein coding sequence of a gene contains transcription control elements. The proximal promoter region contains one or several core promoter elements (Smale and Kadonaga 2003), for example a TATA box, and the transcription start site. The core promoter is where the transcriptional

machinery assembles to form the pre-initiation complex (PIC). The transcriptional machinery consists of RNA Pol II, GTFs and the RNA Pol II associated factor Mediator, which is generally important for Pol II transcription (Thompson and Young 1995; Holstege, Jennings et al. 1998). Further upstream on the promoter region are regulatory elements that are recognized by transcription factors (TFs). Transcription factors can also act from regulatory sites far upstream, called enhancers, through loops in the DNA that brings the enhancer bound transcription factor into proximity of the core promoter. Activators may stimulate transcription through multiple mechanisms (Green 2005), such as recruitment of co-activators like HATs and chromatin remodelers that act on nucleosomes and cause a more open chromatin conformation that exposes the core promoter, and recruitment of components of the transcriptional machinery such as GTFs and Mediator (Fig. 3A). Repressors may act through multiple active repression mechanisms (Gaston and Jayaraman 2003), such as inhibitory interaction with GTFs and recruitment of co-repressors that in turn may form inhibitory interaction with Mediator and recruit histone-modifying factors, such as HDACs, that act on nucleosomes and cause a repressive state of chromatin (Fig. 3B). Repressors may also inhibit transcription by a passive mechanism of posing a sterical hindrance for binding of activators and GTFs.

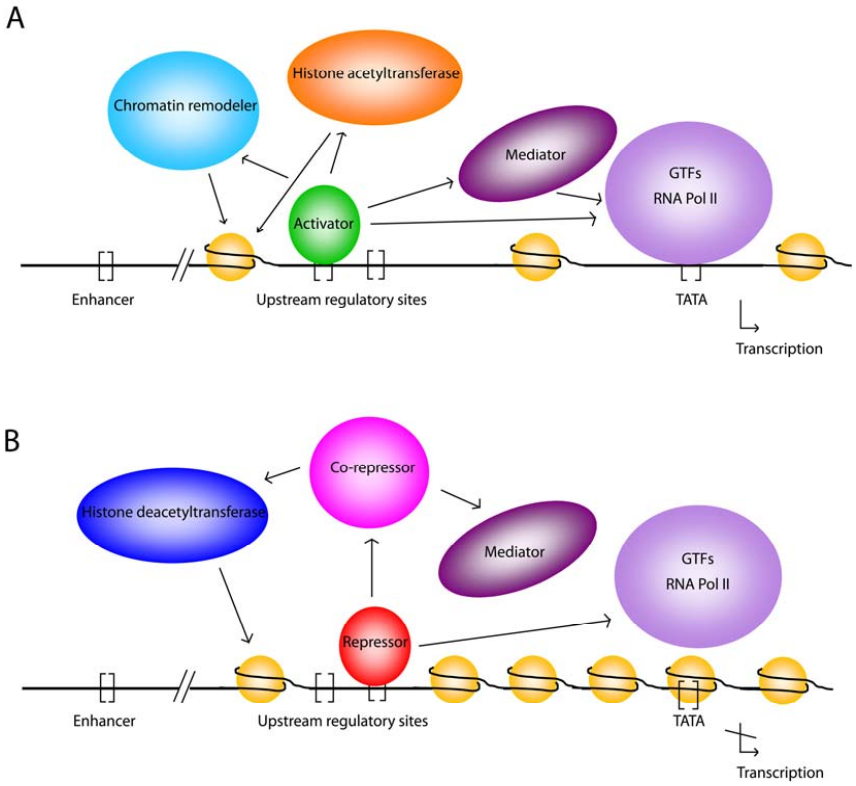

Figure 3. Mechanisms of transcription factor function. A) An activator bound to its target site in the promoter region may stimulate transcription through recruitment several different target factors, such as HATs and chromatin remodelers, resulting in acetylation of histones in nucleosomes and histone eviction or sliding, yielding a more accessible core promoter, and by recruitment of Mediator and GTFs. B) A repressor bound to its target site may inhibit transcription through inhibitory interaction with GTFs and recruitment of co-repressors that in turn may form inhibitory interaction with Mediator and recruit HDACs, which deacetylates histones in nucleosomes and may promote a repressive chromatin state directly, and indirectly by affecting chromatin association of other chromatin binding factors (not shown).

2 TRANSCRIPTION FACTOR DOMAINS

The regulatory role of transcription factors requires both DNA- and protein binding function. Many transcription factors furthermore bind to DNA as dimers and some transcription factors also bind ligands, small molecules that regulate their activity. The different functions are mediated by functional domains, which may correlate with relatively large structural domains connected by linker regions or with small regions in the size order of peptides.

2.1 DNA BINDING- AND DIMERIZATION DOMAINS

Transcription factors are divided into families based on conservation in the DNA binding- and dimerization domains. Figure 3 shows crystal structures of DNA binding- and dimerization domains of a selection of transcription factors bound to DNA as dimers in which subunits are differently colored. The basic helix-loop-helix (bHLH) family is represented by the yeast activator Pho4 (Fig. 3A), in which the basic region mediates DNA interaction, and dimerization depends on interactions between the HLH regions of the intertwined monomers. The structure of the yeast activator Gcn4 (Fig. 3B) represents the basic region leucin zipper (bZIP) family. As for the bHLH transcription factors, the basic region of bZIP TFs mediates DNA binding, and dimerization depends on a leucin rich region facing towards the other monomer, hence the name leucin zipper. In the bHLH-LZ family (not shown here), to which for example the proto-onco protein c-Myc and its obligatory dimerization partner Max belong, the structure of bHLH transcription factors is extended with a leucin zipper. This has been proposed to relevant with regard to formation of c-Myc dependent DNA loops because in addition to mediating interaction between the monomers, the extended helices of the c-Myc/Max heterodimer enable formation of an anti-parallel four-helix bundle between two DNA bound c-Myc/Max heterodimers (Nair and Burley 2003). Another common DNA binding motif is the homeobox, here represented by the yeast heterodimeric repressor Mat a1/Mat α2 (Fig. 3C), which are bound in a head-to tail orientation. Mat α2 dependent transcriptional repression requires heterodimerization, which for dimerization with Mat1a depends on the α-helix of Mat α2 (green in Fig. 3C) that extends over Mat a1 and is amphipatic in character (hydrophobic and hydrophilic on opposite sides) (Mak and Johnson 1993).

Many transcription factors contain zinc finger motifs, the most common of which is found in the ZnF-C_2H_2 family (not shown here) (Wolfe, Greisman et al. 1999). The nuclear receptor (NR) family contains ZnF-C_4 motifs (Schwabe and Rhodes 1991). In this zinc finger motif, four cystein residues coordinate each of the zinc ions, which are required to maintain the structure and DNA binding activity of NRs (Freedman, Luisi et al. 1988). The NR family is represented by the (rat) glucocorticoid receptor (GR) shown in Figure 3D, with zinc ions shown in red. The monomers of the homodimeric glucocorticoid receptor are oriented head-to-head and dimerization is mediated by interaction between the zinc fingers at the center of the dimer. Certain types of nuclear receptors heterodimerize, and they orient head-to-tail (Bain, Heneghan et al. 2007). The yeast activator Gal4 (Fig. 3E) represents a family with a different type of zinc binding motif – the $Zn(II)_2Cys_6$ binuclear cluster, which is found in many yeast transcription factors (Todd and Andrianopoulos 1997). The zinc ions are shown in yellow in the structure in Figure 3E. The linker arm connecting the binuclear cluster with the dimerization domain of Gal4 explains the relatively large size of its target sites and the lack of a consensus sequence in the DNA separating the so called half-sites (red DNA regions in Fig. 3E) (Traven, Jelicic et al. 2006), compared to for example binding sites of the bZIP (see Fig. 3B) transcription factor Gcn4 (Oliphant, Brandl et al. 1989).

Important aspects of dimerization are that it enables higher DNA sequence selectivity and higher affinity compared to DNA binding by a monomeric TF, since a dimeric TF has more contact points with the DNA molecule. The synergistic effect of dimerization on DNA affinity may be exemplified by the importance of the dimerization domain of Gal4, shown in more detail in Figure 4, where the subunits are differently colored. Deletion of the region shown in Figure 4 reduces Gal4 affinity for DNA by a factor of at least 16 (Hong, Fitzgerald et al. 2008). Dimerization is furthermore relevant from an evolutionary point of view because many transcription factors within expanded families are known to form different heterodimers, and the different heterodimers may differ with regard to affinity for a particular target site, target sequence or effect on transcriptional activity (Amoutzias, Robertson et al. 2008). Thus, dimerization promotes evolution of diversity in transcriptional regulation involving a particular transcription factor.

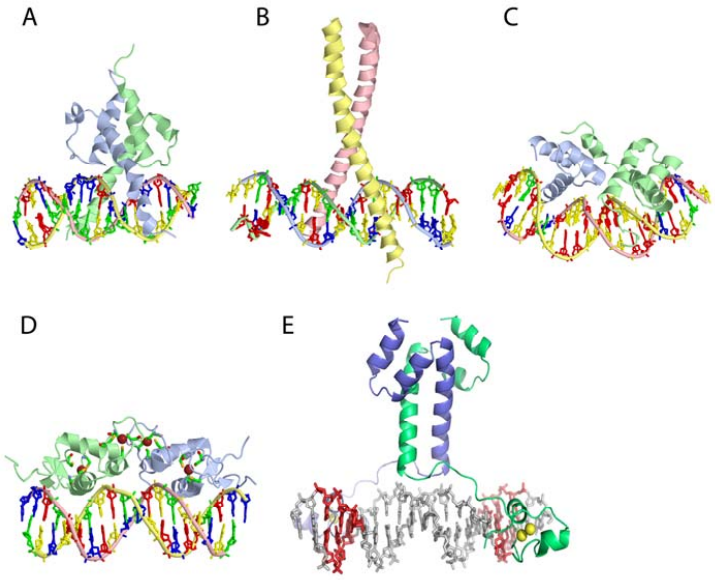

Figure 3. Crystal structures of DNA binding- and dimerization domains of different families of transcription factors bound to DNA. A) Homodimer of the bHLH TF Pho4 (Shimizu, Toumoto et al. 1997) (PDB: 1AOA), B) Homodimer of the bZIP TF Gcn4 (Ellenberger, Brandl et al. 1992) (PDB: 1YSA), C) Heterodimer of the homeodomain TFs Mat a1 (blue) and Mat α2 (green) (Li, Stark et al. 1995) (PDB: 1YRN), D) Homodimer of the glucocorticoid receptor (Luisi, Xu et al. 1991) (PDB: 1GLU), which belongs to the NR family of TFs. Zinc ions in the GR structure are shown in red. E) Homodimer of the $Zn(II)_2Cys_6$ binuclear cluster TF Gal4 (Hong, Fitzgerald et al. 2008) (PDB: 3COQ). Zinc ions in the Gal4 structure are shown in yellow. The dimer subunits are shown in different colors. Images 3A-D provided by Jena Library. Image 3E adapted with permission from Elsevier Ltd (Hong, Fitzgerald et al. 2008).

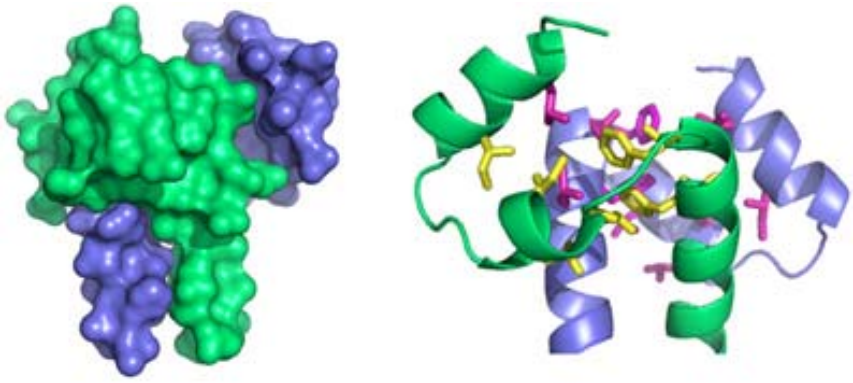

Figure 4. The dimerization domain of Gal4.
The left image shows a surface representation of the Gal4 dimerization interface, with the two subunits of the Gal4 dimer colored in green and blue. The right image shows details of the Gal4 dimerization interface with side chains of the two subunits colored in yellow and purple. Adapted with permission of Elsevier Ltd (Hong, Fitzgerald et al. 2008).

2.2 LIGAND BINDING DOMAINS

The nuclear receptor family (NRs) differs from the other transcription factors shown in Figure 4 in that they have a ligand-binding domain, and within this domain there is a ligand dependent activation domain (Webster, Green et al. 1988). The ligands (Smirnov 2002) are small fat-soluble molecules that regulate the activity of the transcription factor by inducing conformational changes that affect protein-protein interactions of the ligand-binding domain. The overall structure of the ligand-binding domain in the different classes of NRs is relatively similar – a globular domain composed of 12 α-helices with a hydrophobic pocket at the center constituting the ligand-binding site (Bain, Heneghan et al. 2007). Some NRs are largely sequestered in the cytoplasm in absence of activating signal through complex formation with heat shock proteins, whereas others are nuclear and chromatin bound independent of ligand activation and in absence of agonist (activating ligand) function as repressors (Pratt 1992; Bain, Heneghan et al. 2007).

2.3 REPRESSION- AND ACTIVATION DOMAINS

The activation- or repression domain is the functional module that is responsible for mediating direct interaction with the required co-factors and other target factors that the DNA bound transcription factor need to interact with in order to activate or repress its target gene(s). These domains tend to be small and the boundaries of these domains can be hard to define. The physical definition of a given activation- or repression domain may not be very precise but can be based on deletion analysis using restriction sites available in the coding sequence. Mapping domains that interact with target factors by deletion- and transcription analysis is complicated, since regulatory regions also affect the transcriptional activity. Furthermore, other regions that in the full-length transcription factor do not function as a target factor recruitment domain may do so to some extent when its context is altered as in deletion analysis. An example of this is the proposed second activation domain of the yeast transcriptional activator Gal4, an internal acidic region that activates a low level of transcription when in the C-terminal of a truncated version of the activator (Ma and Ptashne 1987).

There is no common denominator in the sequence of amino acids of activation domains, and for lack of better system, activators have been classified based on

enrichment of particular amino acids within the activation domain into acidic-, glutamine-rich- and proline-rich activation domains (Mitchell and Tjian 1989). Many activation domains are acidic and have been shown to be intrinsically unstructured in solution (Donaldson and Capone 1992; O'Hare and Williams 1992; Van Hoy, Leuther et al. 1993; Dahlman-Wright, Baumann et al. 1995; McEwan, Dahlman-Wright et al. 1996; Wärnmark, Wikström et al. 2001). An early model proposed that such activation domains interact with target factors based on complementary charge, in the form of “acid blobs” or “negative noodles” without adopting defined structure (Sigler 1988). However, several studies have shown that structure can be induced in such activation domains *in vitro* using different solvent conditions (Donaldson and Capone 1992; Van Hoy, Leuther et al. 1993; Dahlman-Wright, Baumann et al. 1995; McEwan, Dahlman-Wright et al. 1996), and that intrinsically unstructured activation domains are structured in complex with target factors (McEwan, Dahlman-Wright et al. 1996; Wärnmark, Wikström et al. 2001; Kumar, Betney et al. 2004 a; Kumar, Volk et al. 2004b; Jonker, Wechselberger et al. 2005). These observations are furthermore supported by genetic evidence that folding of the activation domain might be important for interaction with target factors *in vivo* (Cress and Triezenberg 1991; Hardwick, Tse et al. 1992; Regier, Shen et al. 1993; Dahlman-Wright and McEwan 1996; Almlöf, Gustafsson et al. 1997). It was proposed that target factor binding by intrinsically unstructured activation domains occurs by a coupled binding and folding mechanism, and studies from our group have demonstrated that the thermodynamic properties of activator-target binding are generally consistent with protein folding (Hermann, Berndt et al. 2001; Ferreira, Hermann et al. 2005). Importantly, a binding mechanism that is coupled with folding can explain how an acidic activation domain may interact specifically with target factors in spite of lacking defined intrinsic structure, rather than forming non-productive interactions with any positively charged protein that it may come in contact with, for example histones.

Transcription factors is one of the functional groups of proteins that occur frequently among proteins that are known or predicted to contain intrinsically disordered regions of at least 50 consecutive residues, and such proteins are more common in eukaryotes than in bacteria and archaea (Dunker, Lawson et al. 2001; Uversky 2002; Dyson and Wright 2005). This implies a link between intrinsic disorder and more complex transcriptional regulation. One aspect discussed in relation to intrinsic disorder is that

coupled binding and folding tend to yield interactions that are highly specific and of relatively low affinity (Dyson and Wright 2005). Consistently, co-activator promoter association and dissociation has been shown to be highly dynamic *in vivo* (Nagaich, Walker et al. 2004; Johnson, Elbi et al. 2008). It is possible that relatively weak but frequent interactions with target factors might apply generally to activators *in vivo*, since it appears that many activation domains interact with target factors by a coupled binding and folding mechanism.

Lack of intrinsic structure is however not limited to activation domains. A recent study has shown that the intrinsically unstructured KRAB repression domain is structured in complex with the KIP1 co-repressor (Peng, Gibson et al. 2007). The KRAB repression domain is conserved and found in many mammalian repressors within the expanded Zn-C_2H_2 zinc finger family of transcription factors, which have diverged through changes in the DNA binding domain (Emerson and Thomas 2009). Taken together this suggests that coupled binding and folding, and consistently low affinity interactions, might apply to numerous repressors as well, and further that repression domain interactions might also be highly dynamic. Such a mode of interaction might be advantageous for both activators and repressors, particularly during transition form an activated to repressed state, and *vice versa*.

3 TRANSCRIPTION FACTOR TARGETS IN THE CONTEXT OF MULTI-SUBUNIT CO-FACTORS

Co-factors of transcriptional regulators are generally composed of a few or many different proteins in a complex, thus complicating studies of transcription factor interaction with a particular target co-factor and relative importance of a particular interaction for transcriptional regulation, since several components of the complex may be targeted. Furthermore, a putative target subunit may also have an important structural role within the complex, and in such cases the importance of subunit for interaction with the co-factor in question cannot be investigated by simply disrupting the encoding gene. Different approaches have been used to identify direct transcription factor target subunits in context of intact complexes. *In vitro* approaches have been used that are based on photochemical cross-linking to and labeling of activator-interacting subunits (Brown, Howe et al. 2001; Neely, Hassan et al. 2002; Fishburn, Mohibullah et al. 2005). An *in vivo* approach that has been used is based on fluorescence resonance transfer (FRET) between variants of Green Fluorescent Protein (GFP) (Bhaumik, Raha et al. 2004).

The two co-factors investigated in Paper II and Paper III of this thesis represent co-factors where multiple domains within the complex are putative targets of transcription factors. A general description of these co-factors will follow below.

3.1 THE SWI/SNF CHROMATIN REMODELING COMPLEX

Budding yeast SWI/SNF is a 12-subunit complex that belongs to a conserved family of ATP-dependent chromatin-remodeling complexes that are classified based on conservation of the ATPase subunit. Approximately 5 % of all yeast genes are SWI/SNF dependent (Holstege, Jennings et al. 1998; Sudarsanam, Iyer et al. 2000) and defects are manifested by failure to grow under certain conditions (Neigeborn and Carlson 1984; Peterson and Herskowitz 1992; Jia, Larossa et al. 2000). SWI/SNF defects in more complex organisms, like mouse and human, can lead to developmental defects and diseases such as cancer (Versteege, Sevenet et al. 1998; Wu, Lessard et al. 2007; Huang, Gao et al. 2008).

SWI/SNF inherently interacts non-specifically with DNA through multiple surfaces, and the bromodomain of the ATPase subunit contributes to chromatin association

through interaction with hyperacetylated histone tails (Quinn, Fyrberg et al. 1996; Hassan, Prochasson et al. 2002; Dechassa, Zhang et al. 2008). SWI/SNF remodels chromatin using the energy of ATP hydrolysis to disrupt interactions between DNA, causing eviction of histone H2A/H2B dimers, histone octamers or sliding of histone octamers along the DNA molecule (Bazett-Jones, Cote et al. 1999; Whitehouse, Flaus et al. 1999; Bruno, Flaus et al. 2003; Boeger, Griesenbeck et al. 2004; Gutiérrez, Chandy et al. 2007; Dechassa, Zhang et al. 2008). SWI/SNF remodeling *in vivo* results predominantly in histone eviction (Boeger, Griesenbeck et al. 2004; Gutiérrez, Chandy et al. 2007). SWI/SNF functions as a co-activator that facilitates promoter binding of transcription factors and components of the transcription machinery (Côté, Quinn et al. 1994; Imbalzano, Kwon et al. 1994; Kwon, Imbalzano et al. 1994; Burns and Peterson 1997; Dhasarathy and Kladde 2005). Two of the SWI/SNF subunits, Swi1 and Snf5, mediate interaction with activators and these subunits are also important for complex stability (Peterson and Herskowitz 1992; Peterson, Dingwall et al. 1994; Prochasson, Neely et al. 2003). SWI/SNF has furthermore been shown to function as a transcriptional elongation factor (Schwabish and Struhl 2007), and be required for DNA repair (Chai, Huang et al. 2005).

Although SWI/SNF is commonly referred to as a co-activator, genome-wide expression studies indicate that SWI/SNF functions both as a co-activator and co-repressor (Holstege, Jennings et al. 1998; Sudarsanam, Iyer et al. 2000). However, it is not known to what extent SWI/SNF function as a true co-repressor *vs.* a co-activator of transcription generating non-coding transcripts that might be functionally relevant, whether by an antisense mechanism or transcriptional interference, as is the case with the serine metabolic gene *SER3*, previously proposed to be directly repressed by SWI/SNF (Sudarsanam, Iyer et al. 2000; Martens and Winston 2002). It was subsequently shown that SWI/SNF is required for *SER3* repression by functioning as partially redundant co-activator of *SRG1* (Martens, Wu et al. 2005), a regulatory gene that is activated by the serine dependent activator Cha4 and generates a non-coding transcript whose expression interferes with *SER3* transcriptional activation (Martens, Laprade et al. 2004). Non-coding transcripts are prevalent in both yeast and human (Cawley, Bekiranov et al. 2004; David, Huber et al. 2006; Xu, Wei et al. 2009). Although it is not know what proportion of non-coding transcripts are functionally relevant, the prevalence of such transcripts nevertheless raises the possibility that more

genes that appear to be directly repressed by SWI/SNF might rather be regulated through SWI/SNF involvement in generation of non-coding transcripts.

3.2 THE SSN6/TUP CO-REPRESSOR COMPLEX

The Ssn6/Tup11/12 co-repressor complex in the fission yeast is related to the budding yeast Ssn6/Tup1 co-repressor complex, which has served as the model for the majority of studies leading to the current knowledge about the different mechanisms by which Ssn6/Tup complexes function in transcriptional regulation. Ssn6/Tup1 consists of one Ssn6 subunit and four copies of Tup1 that can oligomerize independently of Ssn6 (Tzamarias and Struhl 1995; Varanasi, Klis et al. 1996). The yeast Ssn6/Tup complexes are required for regulation of a variety of genes, involved in for example metabolic pathways, ion transport, mating and different types of cellular stress (Keleher, Goutte et al. 1988; Proft and Struhl 2002; Green and Johnson 2005; Fagerström-Billai, Durand-Dubief et al. 2007). The Tup co-repressors furthermore have functional homologs in metazoans, for example Grg proteins in mouse and TLE proteins in human, that are required for regulation of for example developmental genes and furthermore has been linked to adult human disease (Paroush, Finley et al. 1994; Dang, Inukai et al. 2001; Swingler, Bess et al. 2004).

Although it has been shown that Ssn6/Tup can be converted to a co-activator of a gene where it functions as a co-repressor under non-inducing conditions (Proft and Struhl 2002), its function as a co-repressor is well established and has been more extensively studied. The Ssn6/Tup complex has no intrinsic DNA binding- or enzymatic activity and requires interaction with specific repressors to associate with regulatory chromatin regions, where it is has proposed to repress transcription by several mechanisms. An important property of Ssn6/Tup as a co-repressor is that it interacts with HDACs, whose enzymatic activity leads to a hypoacetylated repressive state of chromatin (Watson, Edmondson et al. 2000; Davie, Trumbly et al. 2002). By recruiting HDACs, Ssn6/Tup furthermore promotes its own association to chromatin, because the Tup1 co-repressor binds to histone tails and has highest affinity for hypoacetylated histone tails (Edmondson, Smith et al. 1996; Davie, Trumbly et al. 2002). A study indicates that lysine residues 15-19 are more important for Tup1 interaction and repression than other lysine residues in the tail of histone H4 (Edmondson, Smith et al. 1996). The apparent importance of particular lysine

residues in histone H4 observed for Tup1, together with the high divergence in the histone-interaction domain of the fission yeast homologues Tup11 and Tup12, suggest that Tup11 and Tup12 might have diverged functionally due to evolution of differential affinity for particular modification states (Fagerström-Billai and Wright 2005). However, we found that differences between the histone-interaction domains of Tup11 and Tup12 are not generally relevant for distinct function (unpublished data, Paper III). Ssn6/Tup may repress transcription through inhibitory interactions with components of the transcriptional machinery and maintenance of an organized chromatin structure through its association to histone tails (Ducker and Simpson 2000; Gromöller and Lehming 2000; Papamichos-Chronakis, Conlan et al. 2000). Interaction with Mediator might explain the finding that fission yeast Ssn6/Tup localizes also to coding regions (Fagerström-Billai, Durand-Dubief et al. 2007), because fission yeast homologues of Mediator components involved in Ssn6/Tup dependent repression in budding yeast have also been found to localize to coding regions (Kuchin and Carlson 1998; Andrau, van de Pasch et al. 2006; Zhu, Wiren et al. 2006).

Ssn6 and Tup1 both contain domains that interact with sequence specific repressors, and with HDACs and other putative targets downstream of promoter recruitment. The repressor-binding region of Ssn6, the tetratricopeptide (TPR) domain, also mediates interaction with the Tup1 tetramer (Tzamarias and Struhl 1995). The C-terminal region of Tup1 has been crystallized (Sprague, Redd et al. 2000), and contains a conserved protein-protein interaction module called WD40 repeat domain that has a seven-bladed β-sheet propeller-like structure (Fig. 5). Overall, repression of different genes requires distinct regions of Ssn6/Tup1 (Tzamarias and Struhl 1995). Mutational analysis of the Tup1 WD40 repeat domain has shown that the majority of genes that are de-repressed in Tup1 mutants are affected by Tup1 defects downstream of promoter recruitment, rather than defect Tup1 chromatin association (Green and Johnson 2005). This implies that Ssn6 generally is more important than Tup1 for Ssn6/Tup1 interaction with repressors, otherwise a higher proportion of the Tup1 dependent genes would be expected to display defects in promoter recruitment.

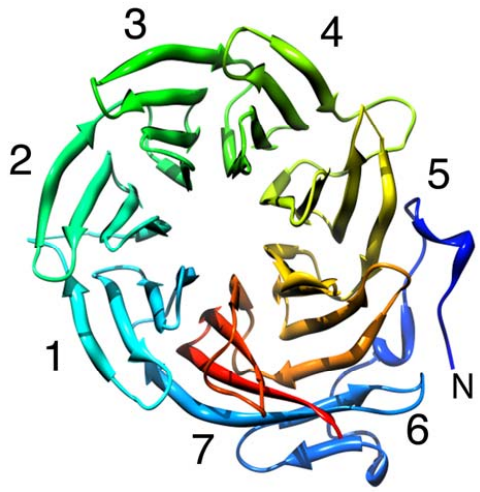

Figure 5. Crystal structure of the C-terminal domain of *S. cerevisiae* Tup1.
The C-terminally located WD40 repeat domain of *S. cerevisiae* Tup1 seen from the bottom. The N-terminal end of the fragment is labeled "N". The structure image represents chain C in the crystal structure (PDB: 1ERJ) (Sprague, Redd et al. 2000) and was prepared using Chimera (Pettersen, Goddard et al. 2004).

Genome-wide studies in fission yeast have shown that the different components of the Ssn6/Tup11/12 complex have partially distinct target genes (Fagerström-Billai and Wright 2005; Fagerström-Billai, Durand-Dubief et al. 2007). General co-localization of the three complex components, also on genes that depend differentially on the different subunits, demonstrates stable interaction between the Ssn6/Tup11/12 complex subunits (Fagerström-Billai, Durand-Dubief et al. 2007). Our recent study of Tup11 and Tup12 shows that functional differences between Tup11 and Tup12 depend mainly on divergence in the WD40 repeat domain, and significant differences that are believed to be located in distinct patterns on the surface of blade 3 of the structure are likely relevant for regulation of Ssn6/Tup target genes with a requirement for specifically Tup11 or Tup12 (unpublished data, Paper III).

In $CaCl_2$ stressed fission yeast there is a rapid and dramatic decrease in Tup11 levels (unpublished data, Paper III). A likely consequence would be a stoichiometric change in the Ssn6/Tup11/12 complex. This might involve the stress-induced Sty1 MAP kinase, which is activated under a number of different environmental stress conditions (Chen, Toone et al. 2003; Wang, Shimada et al. 2005), and recruited to

stress induced genes (Reiter, Watt et al. 2008). It is possible that differential regulation of Tup11 and Tup12 might apply to other conditions involving Sty1 dependent transcriptional regulation, and result in an Ssn6/Tup complex where the Tup components consist predominantly of Tup12. This would present another possible explanation for the previous observation that Tup12 specific target genes are over-represented among genes regulated by environmental stress (Fagerström-Billai and Wright 2005), in addition to the proposed general importance of the Tup12 WD40 repeat domain for mediating interaction with target factors involved in regulation of Tup12 dependent genes (unpublished data, Paper III).

4 PROTEIN FUNCTION REQUIRES STRUCTURE

Individually weak non-covalent interaction like hydrogen bonds, van der Waals interactions, hydrophobic effects and ionic interactions are important for structural stability of proteins, and provide specificity and stability in interaction between proteins. It is generally accepted that proteins require a unique, three-dimensional structure to function specifically. Yet, an early model, based largely on results with artificial activator- and promoter constructs, postulated that unstructured activation domains with a high content of acidic residues interact with target factors based entirely on complementary charge (Sigler 1988). This would imply that mutating hydrophobic resides within such a domain should have little effect on transcription potential. In contrast, more recent studies together indicate that hydrophobic residues in particular positions of natural acidic activation domains are functionally important, whereas individual acidic residues are relatively unimportant (Cress and Triezenberg 1991; Hardwick, Tse et al. 1992; Leuther, Salmeron et al. 1993; Regier, Shen et al. 1993; Almlöf, Gustafsson et al. 1997; Almlöf, Wallberg et al. 1998). This led to a new model for target factor binding by acidic activation domains (Fig 6), postulating coupled binding and folding, such that initial interaction is based on complementary charge, which is followed by a slower interaction phase during which the activation domain folds, resulting in a specific and more stable interaction (Hermann, Berndt et al. 2001; Ferreira, Hermann et al. 2005).

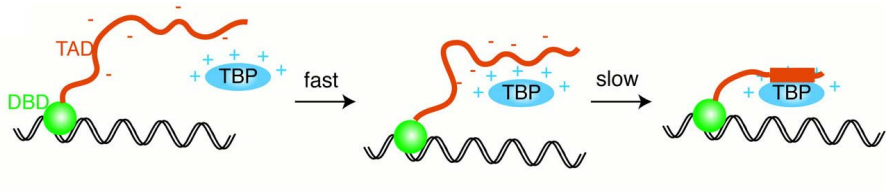

Figure 6. Model for mechanism of target factor binding by acidic activators.
DBD: DNA binding domain; TAD: trans-activation domain. Interaction between the acidic and intrinsically unstructured activation domain of the promoter bound activator and its target factor is initiated by a short phase of ionic interaction, followed by a slower phase where the activation domain folds driven by hydrophobic effects upon interaction with the target factor, resulting in a more stable interaction. Adapted with permission from the American Society for Biochemistry and Molecular Biology (Hermann, Berndt et al. 2001).

It is nowadays accepted that many proteins are intrinsically disordered and interact with target factors by a coupled binding and folding mechanism, and efforts are being made towards understanding what makes a protein natively unfolded (Dunker, Lawson et al. 2001; Uversky 2002; Dyson and Wright 2005). It has been found that the primary sequences are uninformative in prediction of unfolded proteins, and that it rather depends on the overall of content of hydrophobic and charged residues (Uversky 2002b). Suggestive of intrinsic disorder is the combination of low sequence complexity with a bias towards certain amino acids, relatively low content of bulky hydrophobic resides and high content of particular polar and charged amino acids, predominantly glutamine, proline, lysine, serine and glutamate (Dyson and Wright 2005).

4.1 PROTEIN CONFORMATION AND ENTROPY

Each molecule has motional energy (vibrational, rotational and translational). As molecules within a system move and collide, energy is transferred between molecules, such that translational movement energy of one molecule may become vibrational, rotational or translational movement of another. While the total energy of the system remains the same, a new combination of the individual energy levels arises when one molecule looses energy to another, and each distinct combination of energy levels constitutes a microstate. The higher the number of microstates that are accessible to a system at a given time, the larger the entropy is. Entropy is a measure of the spontaneous dispersal of energy. That is to say, how much energy is spread out in a process, or how widely spread out it becomes, at a specific temperature.

The second law of thermodynamics states that for any process that occurs spontaneously there is an increase in entropy of the universe. Protein structure is an interesting lesson in entropy. Because there are more conformations available to an unfolded protein, the polypeptide chain has higher entropy in an unfolded state. From the point of view of the protein, folding reduces entropy. However, one must consider the entire system, which in the case of protein folding includes the solvent – water. The presence of non-covalent interactions and the order and disorder (entropy) of solvent water lay at the heart of understanding the apparent paradox of spontaneous folding despite decreased entropy of the polypeptide chain. Globular proteins require water to maintain their three-dimensional structure. It is thought that the reason for this

spontaneous globular structure is the release of solvent water as the hydrophobic core is formed (Dill 1990). This so-called hydrophobic effect occurs because the increased entropy of the polar solvent upon protein folding yields higher entropy for the entire system when hydrophobic residues of the protein are contained largely within the core of the folded structure, despite the local entropy decrease of the polypeptide chain. It has further been proposed that increased translational entropy of volume excluded water molecules upon folding makes an important contribution to the solvent entropy increase (Harano and Kinoshita 2005).

As mentioned earlier, a relatively low content of hydrophobic residues and a high content of polar and charged residues is typical for proteins that do not fold spontaneously in aqueous solution (Uversky 2002b). Folding of such proteins may also be dependent on the hydrophobic effect, but here the balance must be supplied by interaction with a target, which provides specific interaction between hydrophobic residues in the two proteins. Studies using mutant variants of the intrinsically unstructured activation domain of the glucocorticoid receptor indicate correlation between potential for a hydrophobic patch in the secondary structure and *in vitro* target factor affinity and *in vivo* transcription potential (Almlöf, Gustafsson et al. 1997; Almlöf, Wallberg et al. 1998). This correlation suggests that the extent of localized hydrophobicity in the secondary structure of this acidic activation domain might affect the kinetics of folding, such that potential for a larger hydrophobic effect yields more rapid folding.

Different *in vitro* approaches can be used to investigate the mechanism of target factor binding. One method that could be used is stopped-flow circular dichroism, which monitors the increase in secondary structure over time. Another approach, which we have used, is to measure how temperature affects the binding equilibrium – called van't Hoff analysis. This is an indirect approach to investigate coupled binding and folding and is based on the anticipation that an interaction for which protein folding is induced by hydrophobic interaction between the two interacting proteins should yield an entropy increase of the system. Since the universe strives towards the lowest possible energy, a reaction that occurs spontaneously at constant temperature and pressure yields a decrease in free energy (a negative value of ΔG, where G symbolizes free energy). Further, $\Delta G = -RT\ln K$, where R is the gas constant, T is temperature in Kelvin and lnK is the natural logarithm of the equilibrium constant. This relationship can be used to

determine the thermodynamic properties of a protein-protein interaction. Substitution of ΔG in the Gibbs' function ($\Delta G = \Delta H - T\Delta S$, where H is the enthalpy and S is the entropy) with $-RT\ln K$ and division with -RT yields the van't Hoff equation:
$\ln K = -\Delta H/RT + \Delta S/R$. By determining the equilibrium constant (affinity) of a protein-protein interaction at a range of temperatures, the enthalpy change may subsequently be calculated from a van't Hoff plot, where lnK is plotted on the y-axis and the inverse temperature (1/T) plotted on the x-axis. Since the change in free energy (ΔG) is negative for spontaneous reactions, a positive value of the enthalpy change (ΔH) would indicate that the interaction is entropy driven and would be evidence of the hydrophobic effect inducing protein folding.

5 COMMENTS ON METHODOLOGY

5.1 MODEL ORGANISMS

Two model systems, the budding yeast *Saccharomyces cerevisiae* (baker's yeast) and the fission yeast *Schizosaccharomyces pombe*, were used for the *in vivo* studies in this thesis to address different questions regarding function of distinct regions in components of complex co-factors. Both species present the advantage of being good genetic tools since they are relatively easy to manipulate genetically and have a short generation time, compared to for example mammalian cells. *S. cerevisiae* has been used as a model for a longer time than *S. pombe* but both species are established model systems for studies of transcriptional regulation in more complex eukaryotic organisms. Much of the knowledge about the function of SWI/SNF chromatin remodeling complexes comes from studies on *S. cerevisiae* SWI/SNF, and the *S. cerevisiae* transcriptional activator Gal4 is a commonly used model transcriptional activator. Similarly, the functional homologues of yeast Tup co-repressors in higher eukaryotes appears to function through conserved mechanisms.

5.2 THE *GAL* REGULON

The advantage of the *GAL* regulon as a model is that it represents a defined system for investigating recruitment of the SWI/SNF chromatin remodeling complex *via* its two activator-binding domains in a set of different promoter contexts, regulated by the same activator, Gal4. The Gal4 regulated genes are not all alike in regard to number of activator binding sites, basal (derepressed) expression levels *vs.* induced expression levels (inducibility) and nucleosome positioning. This collection of genes might therefore be useful for correlating general SWI/SNF dependence for transcriptional activation to differences in promoter context, most importantly to presence of nucleosomes positioned over core promoter elements, and furthermore to the relative change in transcriptional activity upon induction of expression. Furthermore, investigating requirement for two activator-binding domains relative to one another on a set of different genes might reveal differences between the genes in regard to other factors. Different sets of co-factors can be required for activation of different genes regulated by the same transcription factor (Hertel, Langst et al. 2005), and different factors may interact with- and depend on one another for recruitment (Bhaumik, Raha et al. 2004). If SWI/SNF were to simultaneously interact with the activator and another

factor also required for transcriptional activation, *via* another region of the complex, this might impose positional constraints that could make recruitment *via* a particular activator-binding domain necessary to enable such interaction.

The *GAL* regulon consists of regulatory genes, genes required for transporting and metabolizing galactose and a few genes of less clear importance for galactose metabolism. *GAL3* and *GAL80* encode positive and negative regulators, respectively. *GAL2* encodes the galactose transporter. *GAL1*, *GAL7*, *GAL10* and *PGM2* (also called *GAL5*) constitute the galactose metabolic genes. *FUR4*, *MTH1* and *PCL10* have been proposed to indirectly promote galactose metabolism by different mechanisms (Ren et al., 2000), whereas *GCY1* does not have a clear role in galactose metabolism. The *GAL* genes are glucose repressed, directly or indirectly. The transcriptional repressor Mig1 directly represses the *GAL4* gene (Nehlin, Carlberg et al. 1991), encoding the activator. Also the *GAL3* and *GAL1-10* promoters contain Mig1 sites to which Mig1 binds (Nehlin, Carlberg et al. 1991; Lundin, Nehlin et al. 1994). *GAL4* is not activated by its own gene product but expressed at low levels also in galactose.

An advantage of using Gal4 regulated genes to investigate importance of the SWI/SNF activator-binding domains is that the function of the activation domain is negatively regulated in absence of the inducer (galactose). Therefore, it is not necessary to use an activator lacking the activation domain as a negative control to support the notion that co-factor recruitment depends on interaction with the activation domain, because the full-length activator cannot interact with- and recruit target factor until the inducer (galactose) is added and the inhibition of the activation domain is alleviated. In growth media that does not contain either or glucose or galactose for a carbon source (non-repressive, non-inducing media), such as raffinose or glycerol containing media, glucose repression is relieved and the *GAL* system is poised for activation. At this point, Gal4 still does not activate transcription, due to inhibitory interaction between its activation domain and the inhibitor, Gal80. Expression levels under those conditions are generally very low, with the exception of *GAL80*, *GCY1* and *PGM2*, which have relatively high basal expression levels. When galactose subsequently is added to the media, the positive regulator, Gal3, binds galactose and ATP and this induces its interaction with Gal80. The Gal3-Gal80 interaction alleviates inhibition of the Gal4 activation domain and transcriptional activation occurs.

5.3 A SURFACE PLASMON RESONANCE (SPR) ANALYSIS APPROACH TO INVESTIGATE COUPLED BINDING AND PROTEIN FOLDING

For the first study in this thesis we used the BIAcore 2000 system to investigate contribution of ionic interactions and as an indirect approach to investigate whether protein folding is concurrent with target factor binding by intrinsically unstructured activation domains. The technique is based on the physical phenomenon surface plasmon resonance (SPR), described by Piliarik et al. (Piliarik, Vaisocherova et al. 2009). Briefly, interaction is monitored as follows. One of the proteins is immobilized onto a sensor chip surface and a sample of the other protein is injected into the serially connected flow cells (analogous to test tubes), one of which contains a negative control surface. As the injected protein binds to the immobilized protein, the increasing mass causes a proportional change in refractive index at the interface of the sensor chip surface and the solution flowing over it. This causes a proportional change in the reflection angles of a wedge of polarized light aimed at the glass face of the sensor chip. This change in reflection angles is detected within the system and converted to a response relative to the baseline with only buffer solution, and plotted in real-time in resonance units (RU). The binding response will keep increasing until equilibrium is reached or, for interactions with slow off-rate, until the sample injection is replaced by a continuous flow of buffer solution, and the dissociation phase is monitored in the same way. For each set of conditions tested, a dilution series of protein is injected in replicates to generate a data set for determining binding constants using computer software. By determining equilibrium constants at a range of temperatures one can subsequently use van't Hoff analysis to investigate whether the thermodynamic properties of the interaction are consistent with protein folding.

A disadvantage of this approach to investigate putative folding upon binding is that it is indirect, as opposed to for example stopped-flow circular dichroism, which measures the level of secondary structure over time. Furthermore, some proteins may be difficult to study using the van't Hoff analysis approach because of poor solubility at the relatively low temperatures required for this approach. Such problems may however be possible to solve by modifying the sample buffer.

Advantages of this *in vitro* system is that it is label-free, highly sensitive, requires small sample volumes, and can be used to for example obtain rate- and equilibrium constants

for interaction between various biomolecules, since interactions are monitored in real-time. There are however limitations to the time resolution. It is not possible to obtain reliable kinetic constants for a response as rapid as the initial ionic phase of the interaction between the interaction partners investigated here.

A range of different immobilization approaches can be used for proteins, for example amine coupling *via* lysine side chains or thiol coupling *via* cystein side chains, or different capture approaches such as by antibodies or Streptavidin (of protein subjected to a low level of biotinylation *via* lysine side chains). Optimal immobilization approach has to be determined empirically to ensure that immobilization does not interfere with the interaction. For the intrinsically unstructured acidic activation domains investigated in our study, we used capture by antibody against an affinity tag, because covalent coupling would interfere with interaction for this type of protein. Experimental parameters have to be determined empirically, such as which interaction partner to immobilize, how much to immobilize, choice of sample buffer, solution for regenerating the surface between injection cycles, flow rate and appropriate time for monitoring association and dissociation.

5.4 CHROMATIN IMMUNOPRECIPITATION

This method can be used to investigate chromatin binding of a protein of interest, and is commonly used also to investigate the presence of different chromatin modifications. Living cells are treated with a formaldehyde solution, which cross-links proteins to DNA. Because the cells are rapidly inactivated by the formaldehyde treatment, this method enables obtaining a snapshot of *in vivo* chromatin binding at a given time. After the cross-linking procedure, the cells are mechanically disrupted and the cross-linked chromatin sonicated to obtain fragments of an average size of approximately 500 base pairs. The chromatin extract is subsequently immunoprecipitated and washed, resulting in enrichment of fragments that are bound by the protein of interest. The protein-DNA cross-link is then reversed by heating and the DNA fragments purified. Enrichment of a particular genomic region relative to a control region, where the protein of interest is not expected to bind, can then be quantified by quantitative PCR, which is a highly sensitive method and was suitable for the study in this thesis because a relatively small collection of genes was under investigation. The DNA can also be amplified and

labeled for hybridization to DNA microarrays for obtaining genome-wide data. Achieving sufficient enrichment while keeping background low requires specific antibodies and may require optimization of the immunoprecipitation step and subsequent washing steps. Background binding to beads that are used in the immunoprecipitation step can be significantly reduced by pre-clearing the cross-linked chromatin using beads alone in a step prior to immunoprecipitation, and further minimized by use of beads that are pre-incubated with salmon sperm DNA and bovine serum albumin. The DNA quantification step may also require optimization of conditions and/or re-design of primers due to problems with primer-dimer formation.

5.5 QUANTITATIVE RT-PCR

The hot phenol protocol is a powerful method for extracting RNA for subsequent quantification of expression levels at a given time, because it lyses the cells immediately, which is particularly useful when studying rapid changes in expression. After further purification of total RNA and control reactions to ensure that samples are DNA-free, mRNA levels can be quantified using one-step quantitative RT-PCR with transcript specific primers. Certain transcripts can be difficult to quantify using one-step quantitative RT-PCR, and it has been shown that the efficiency of the reverse transcriptase reaction depends on the priming strategy and that optimal priming strategy differs between transcripts (Ståhlberg, Håkansson et al. 2004). A two-step approach, using a mixture of random primers and poly-dT primers for separate reverse transcriptase reaction prior to quantitative PCR using sequence specific primers can significantly facilitate quantification of transcript levels. Transcription levels are usually quantified relative to expression of a housekeeping gene that is unaffected by genetic defects and experimental conditions investigated.

5.6 *IN VITRO VS. IN VIVO* APPROACHES

Generally, a deeper understanding requires a combination of alternating *in vitro* and *in vivo* approaches. Although *in vitro* studies of protein function may be hampered by problems such as poor solubility or loss of activity, they can be necessary to address certain questions. For example, to my knowledge, there is no method that enables investigating target-induced protein folding or protein-folding kinetics *in vivo*.

Furthermore, an *in vitro* approach can be a useful intermediate step to facilitate further *in vivo* studies, and may be useful for addressing putative functional properties more directly in a simplified system.

6 RESULTS AND DISCUSSION

6.1 PAPER I

The aim of this study was to test the generality of the target-induced folding model for activation domain interactions. This binding model was motivated by studies suggesting coupled target binding and folding of intrinsically unstructured activation domains (Dahlman-Wright and McEwan 1996; McEwan, Dahlman-Wright et al. 1996; Almlöf, Gustafsson et al. 1997), classified as acidic based on the relatively content of acidic residues. Importantly, contrary to the earlier "acid blob" model (Sigler 1988), postulating that acidic activation domains interact with target factors through opposite charge and without adopting a defined structure, a binding mechanism involving induced folding is reconcilable with specificity in spite of intrinsic disorder. Consistent with the notion of target-induced folding, a study by our group showed that the activation domain of the human transcriptional activator c-Myc binds the general transcription factor TATA binding protein (TBP) by a two-step mechanism involving a short ionic phase, followed by a slow phase dependent on hydrophobic effects, resulting in a stable complex (Hermann, Berndt et al. 2001).

It was however possible that the binding mechanism of c-Myc might not be representative for other unstructured activation domains, because it is not very acidic and requires a relatively large region for target binding compared to many other activators, and the activation domain is interspersed with short sequences that *in vivo* are involved in targeted degradation of c-Myc (Flinn, Busch et al. 1998; Flinn, Wallberg et al. 2002). In this study we therefore investigated binding mechanisms of a broader selection of activator-target interactions, using an *in vitro* approach based on surface plasmon resonance (SPR) technology.

The activation domains of the viral co-activator VP16 and the Gal4 activator, regulator of galactose metabolic genes in budding yeast, are both highly acidic and intrinsically unstructured (O'Hare and Williams 1992; Van Hoy, Leuther et al. 1993), and structure in complex with target factors has been demonstrated for VP16 (Jonker, Wechselberger et al. 2005). The target factors used in this study are yeast TBP and the activator binding domains of Swi1 and Snf5 of the yeast SWI/SNF chromatin remodeling complex. Deletion of these domains (the N-terminal third of Snf5 and the second

quarter of Swi1) does not compromise complex integrity or remodeling activity but severely reduces interaction with transcriptional activators and causes growth defects on galactose and other conditions that require SWI/SNF (Prochasson, Neely et al. 2003).

We show that all of the investigated interactions display bi-phasic kinetics in association and dissociation, as previously seen for c-Myc-TBP interaction, and subsequently investigated the contribution of ionic interaction and hydrophobic effects for a selection of activator-target combinations. In all cases, the affinity of the initial interaction correlates inversely with salt concentration whereas the slow phase affinity is favored by increasing temperature, and by increasing salt concentration. The relative importance of hydrophic effects for these activator-target interactions are apparent from the observation that overall affinity increases with temperature and, in most cases, salt concentration. We used van't Hoff analysis to investigate the thermodynamic properties of activator-target binding, and our result indicates that binding is coupled with protein folding, for the whole collection of interactions that were characterized. Our results show that a broader range of interactions between activation domains and target factors are consistent with coupled binding and folding, and thus support the generality of the proposed binding model.

6.2 PAPER II

In this study we have investigated the relative importance of the Swi1- and Snf5 activator binding domains of the yeast SWI/SNF chromatin-remodeling complex for its recruitment to a set of target genes and transcriptional activation *in vivo*. Deletion of both of these regions (the N-terminal third of Snf5 and the second quarter of Swi1) do not affect SWI/SNF complex stability or the inherent remodeling activity but severely impairs SWI/SNF interaction with activation domains *in vitro*, and causes growth phenotypes under conditions that require SWI/SNF (Prochasson, Neely et al. 2003). Several studies indicate that recruitment of SWI/SNF co-activator function is activation domain dependent (Ryan, Jones et al. 1998; Natarajan, Jackson et al. 1999; Neely, Hassan et al. 1999; Yudkovsky, Logie et al. 1999; Wallberg, Flinn et al. 2000; Gutiérrez, Chandy et al. 2007). Taken together this suggests that the identified activator binding domains are important for SWI/SNF promoter recruitment. However, *in vivo*

other regions of SWI/SNF might contribute to chromatin association, such as the bromo-domain of Swi2/Snf2, which binds acetylated histone tails, regions that might interact with Mediator and regions that contribute to the non-specific DNA binding (Quinn, Fyrberg et al. 1996; Hassan, Prochasson et al. 2002; Lemieux and Gaudreau 2004; Dechassa, Zhang et al. 2008). Furthermore, the Swi1- and Snf5 activator-binding domains might differ in importance, suggested by previous *in vitro* interaction assay (Prochasson, Neely et al. 2003). We have previously demonstrated interaction between the isolated Swi1 activator binding domain and the activation domain of Gal4 *in vitro* (Ferreira, Hermann et al. 2005). We did however not investigate interaction between Gal4 and the Snf5 activator-binding domain in the previous study. Here we have investigated the importance of the Swi1- and Snf5 activator binding domains, relative to one another and to other regions of SWI/SNF, for SWI/SNF promoter recruitment to Gal4 target genes *in vivo*.

Out of the Gal4 regulated genes that were induced in wild-type cells within 30 min after addition of galactose, we found that five genes (*GAL2*, *GAL1*, *GAL7*, *GAL10* and *GCY1*) are SWI/SNF dependent at this time-point. With the exception of *GCY1*, the SWI/SNF dependent genes correspond to the most highly inducible out of the Gal4 regulated genes. This suggests that the relative change in expression level generally is an important factor in regard to requirement for SWI/SNF.

We screened the SWI/SNF dependent genes for galactose induced SWI/SNF recruitment in wild-type cells by performing chromatin immunoprecipitation assays, using antibodies that target the ATPase subunit of the SWI/SNF complex, and show that SWI/SNF is recruited to the *GAL2*, *GAL1-10* and *GAL7* promoters within 30 min of induction. We subsequently performed chromatin immunoprecipitation assays on extracts from cells deleted for one or both activation binding domains, to investigate their relative importance for SWI/SNF recruitment to *GAL2* and *GAL1-10*. Our results show that deletion of either the Swi1- or Snf5 activator-binding domain on both investigated promoters reduces SWI/SNF recruitment to approximately half relative to SWI/SNF recruitment in wild-type cells, and that no significant level of SWI/SNF recruitment occurs when both activator-binding domains are deleted. We conclude that interactions of the Swi1- and Snf5 activator binding domains are equally important in context of these genes and collectively necessary for SWI/SNF recruitment *in vivo*.

This result furthermore suggests that Gal4 *in vivo* has similar affinity for both activator-binding domains.

We further show that the reduced level of SWI/SNF recruitment achieved with either one the activator binding domains alone is sufficient for transcriptional activation under these conditions. In contrast, deletion of both activator-binding domains reduces expression of the direct target genes to approximately the same levels as in the control strain with disrupted SWI/SNF. Taken together, our results therefore indicate that the activator binding domains of SWI/SNF collectively are required for SWI/SNF to function as a co-activator since no other regions of SWI/SNF can mediate promoter recruitment. Taken together with previous evidence of direct interaction *in vitro*, this result strongly supports the notion of direct interaction with the activator as the mechanism of SWI/SNF promoter recruitment.

6.3 PAPER III

In this study we have focused on functional domains of the Tup components of the *Schizosaccharomyces pombe* Ssn6/Tup co-repressor complex. Due to a gene duplication event, there are two Tup co-repressor genes in *S. pombe* – *tup11*$^+$and *tup12*$^+$. Gene duplication is known to be an important evolutionary mechanism behind functional diversification, and previous studies by our group have shown that *tup11*$^+$and *tup12*$^+$ are not equally important for regulation of all Ssn6/Tup target genes (Fagerström-Billai and Wright 2005). The three complex components have been shown to co-localize on genes that differ in requirement on *tup11*$^+$and *tup12*$^+$ for transcriptional regulation (Fagerström-Billai and Wright 2005; Fagerström-Billai, Durand-Dubief et al. 2007). Thus, Tup11 and Tup12 appear to perform partly distinct functions in context of a common complex. It is however not known which of the functional properties of the Tup proteins are important on genes that do not depend equally on *tup11*$^+$and *tup12*$^+$. A functional property that is important for the co-repressor function is the interaction with hypoacetylated histone tails, mediated by the middle region of the Tup proteins (Edmondson, Smith et al. 1996; Mukai, Matsuo et al. 1999; Davie, Trumbly et al. 2002). The histone-interacting repression domain is highly diverged between Tup11 and Tup12, and differs more between the two than compared to Tup1 homologues in other fungi (Fagerström-Billai and Wright 2005). It was

therefore proposed that their differential requirement on subsets of Ssn6/Tup target genes might be due evolution of distinct preferences for particular histone tails and/or histone tail modification patterns.

In this study we have combined phylogenetic and structural analysis with phenotypic assays under conditions that require specifically Tup12 to investigate which functional domain is important for the functional differences between Tup11 and Tup12. In contrast to our hypothesis, our results indicate that divergence in the histone-interaction domain is not functionally relevant. Instead, our results indicate that the C-terminal region of Tup12, containing the WD40 repeat domain, is key to its distinct role *in vivo*. This indicates that Tup11 and Tup12 differ in regard to interaction with repressors and/or factors downstream of Ssn6/Tup recruitment to genes, such HDACs, HMTs and components of the transcriptional machinery. We found that the top surface of the structure is conserved between Tup11 and Tup12, whereas residues that might be relevant for functional differences between Tup11 and Tup12 are predominantly located in distinct elongated patterns in propeller blade 3 of the WD40 repeat domain. One of these patterns of residues is conserved between Tup1 and Tup11, consistent with previous demonstration that the Tup11 C-terminal domain is functional in *S. cerevisiae* (Mukai, Matsuo et al. 1999), whereas the other pattern consists of residues that differ between Tup1, Tup11 and Tup12. Since the top surface is highly conserved among fungi and known to be generally important for Tup1 dependent repression (Green and Johnson 2005), our result suggests that Tup11 and Tup12 might interact similarly with target factors *via* the top surface, whereas distinct surface properties on the side of the structure that might affect overall affinity for distinct target factors differ between the two.

Interestingly, we found that Tup11 is rapidly down regulated under $CaCl_2$ stress, indicating that the requirement for Tup12 under these conditions could be due the very low levels of Tup11. However, since both Tup11 and Tup12 levels are unaffected under KCl stress, a difference in relative protein levels is not a general explanation for Tup12 requirement, and we propose that the putative differences in β-sheet 3 in the WD40 repeat domain could be important for interaction with distinct target factors of Tup11 and Tup12.

6.4 CONCLUDING REMARKS

In Paper I we show that a broader range of acidic activators interact with different target factors through coupled binding and folding of the activation domain after the initial ionic interaction. This study importantly demonstrates how intrinsic disorder in general can be reconciled with specific interactions of the intrinsically unstructured activation domains found in many transcription factors in various species, as opposed to interaction based merely on complementary charge as was previously proposed. Intrinsic disorder has been proposed to facilitate interacting specifically with many structurally different target factors (Wright and Dyson 1999). The advantage might however be more in terms of kinetics rather than ability to fit, i.e. that coupled binding and folding might enable interacting with several different factors more rapidly compared to interaction using a predefined three-dimensional structure. A combination of rapid association kinetics and a relatively low affinity in interaction with target factors is likely important for the potency of a transcriptional activator, since the time spent on each individual interaction must set the limit for the dynamics in recruitment of target factors to promoters. It is noteworthy that unpublished data of ours is consistent with the anticipated correlation between potential for localized hydrophobicity, association kinetics and transcription potential that would be expected for a transcription factor that interacts with target factors through coupled binding and folding. Another aspect of intrinsic disorder is that it inherently makes a protein more susceptible to degradation compared to a more compact protein. This could be advantageous from a regulatory point of view and may be enhanced by degradation signals within the activation domain, targeting the transcription factor for proteosomal degradation, exemplified by the Myc-boxes within the activation domain of transcriptional activator c-Myc (Flinn, Busch et al. 1998). Furthermore it is possible that coupled binding and folding could be advantageous from an evolutionary point of view, i.e. that folding in contact with the target factor might increase the tolerance for changes in the primary sequence and therefore confer evolutionary flexibility. In support of this possible advantage of coupled binding and folding, it has been found that putatively intrinsically disordered regions are subject to a relatively high frequency of positive selection (Dr. Johan Nilsson, Södertörn University, personal communication).

Two of the activator targets in the first study (Paper I) were the activator binding domains of SWI/SNF – the N-terminal third of Snf5 and the second quarter of Swi1. In Paper II we investigated the relative importance of the activator binding domains in context of the SWI/SNF complex for promoter recruitment and transcriptional activation *in vivo*. The most important conclusion from this study is that the activator binding domains are necessary for SWI/SNF to function as a co-activator, since there is no significant level of promoter recruitment of the SWI/SNF complex lacking both activator-binding domains and, consistently, this correlates with a strong transcriptional defect. From the observation that either activator-binding domain mediates approximately the same reduced level of SWI/SNF promoter recruitment in context of induced Gal4 regulated genes, may further be concluded that the Gal4 activation domain appears to have similar affinity for Swi1 and Snf5 *in vivo*. Although deletion of either one of the activator binding domains have been shown to generally have little effect on growth under several conditions that require SWI/SNF (Prochasson, Neely et al. 2003), it is possible that other activators might differ somewhat from Gal4 in regard to the importance of the activator-binding domains relative to one another. Together with previous *in vitro* evidence of direct interaction mediated by the activator binding domains (Prochasson, Neely et al. 2003; Ferreira, Hermann et al. 2005), this study (Paper II) strongly supports the view that SWI/SNF promoter recruitment *in vivo* occurs by a mechanism of direct interaction with the activation domain of transcriptional activators.

In Paper II we furthermore demonstrated SWI/SNF dependence for a group of *GAL* genes induced from a poised state, i.e. de-repressed and ready for rapid activation upon addition of galactose. Taken together with our unpublished data, indicating that the demonstrated SWI/SNF dependence is only seen early after addition of the inducer, it may be inferred that nucleosome remodeling by SWI/SNF is required for the rapid on-switch of *GAL2*, *GAL1*, *GAL10* and *GAL7* expression. This conclusion would be consistent with a recent study (Bryant, Prabhu et al. 2008) published shortly after submission of Paper II. Bryant et al. investigated the chromatin state of the *GAL1-10* promoter under different conditions, and showed that nucleosome removal from the *GAL1-10* promoter and transcriptional activation of *GAL1* and *GAL10* upon addition of galactose is delayed in a strain lacking the ATPase subunit of the SWI/SNF complex (Bryant, Prabhu et al. 2008). It was proposed that during induction in absence of SWI/SNF, the recruited transcriptional machinery eventually accomplishes

nucleosomal removal from the promoter by competing with nucleosomes for core promoter elements (Bryant, Prabhu et al. 2008). It is noteworthy that prior studies of SWI/SNF in context of *GAL* genes have stated that SWI/SNF does not affect activation of *GAL* genes when they are induced from a poised (de-repressed) state (Lemieux and Gaudreau 2004; Kundu, Horn et al. 2007). A likely explanation for the different conclusion regarding SWI/SNF dependence in those studies could be differences in methodology for quantification of transcripts.

In Paper III we focused on the functional domains of the Tup11 and Tup12 co-repressors in *S. pombe*, towards understanding by what mechanism they affect genes differentially. Tup11 and Tup12 have previously been shown to generally co-localize with one another and Ssn6 on promoters and ORFs of genes that are differentially affected by deletion of $tup11^{+}$ and $tup12^{+}$ (Fagerström-Billai and Wright 2005; Fagerström-Billai, Durand-Dubief et al. 2007), investigated under normal growth conditions. One of the conclusions from our study (Paper III) is that the stoichiometry of the co-repressor complex may be conditionally regulated, since we found that Tup11 is rapidly down regulated under $CaCl_2$ stress. However, Tup11 and Tup12 protein levels are unaffected by KCl stress, and the requirement for specifically Tup12 under these conditions cannot be explained by absence of Tup11. We conclude that the requirement for specifically Tup11 or Tup12 is not due to evolution of distinct specificities for particular histone modification patterns that could be associated with distinct target genes, since the Tup11 histone interaction domain can replace that of Tup12 *in vivo*. Furthermore, there is no consistent conservation between the fission yeast Tup11 proteins or Tup12 proteins in the histone-interaction domain that would suggest such a mechanism underlying distinct roles on particular genes. Instead, we conclude that a specific role for Tup12 under the investigated conditions depend mainly on distinct properties that have evolved in the overall highly conserved WD40 repeat domain. This implies that Tup11 and Tup12 differ in interaction with one or more of the target factors that are important for regulating genes that depend more on one than the other of the two, which would be consistent with differential effect on gene expression also while in context of a common complex. More specifically our results suggest that this is likely to involve interaction with the third blade of the WD40 repeat structure, where Tup11 and Tup12 are predicted to differ significantly in surface properties.

Studies of chromatin localization and transcriptional regulation in the two single deleted strains *vs.* the double deleted strain would be required to normalize for contribution by Ssn6, and determine whether Tup11 and Tup12 differ in importance for regulation of distinct target genes by a mechanism of interaction with repressors or with putative target factors downstream of Ssn6/Tup chromatin association, such as HDACs and HMTs. Since it has previously been shown that Tup12 dependent genes are in vast majority under both normal growth conditions and under KCl stress (Fagerström-Billai and Wright 2005), it is possible that Tup12 could be a superior interaction partner of one or more target factors generally associated with repression of Tup11/Tup12 target genes, whereas Tup11 might be superior for interaction with particular repressors. A possible candidate factor that might have a preference for the Tup12 WD40 repeat domain could for example be the Clr6 class I HDAC, since Ssn6/Tup target genes have been shown to generally correlate with genes that are affected by *clr6*$^{+}$ deletion (Fagerström-Billai, Durand-Dubief et al. 2007). It is however possible that this association could depend largely on Ssn6. One approach to identify distinct target factors of Tup11 and Tup12 for further investigation *in vivo* could be to screen whole cell extracts from the double deleted strain for *in vitro* interaction with the isolated Tup11 and Tup12 WD40 repeat domains, coupled with mass-spectrometry of bound factors.

Further investigation would be required to address how the N-terminal domain influences Tup12 specific function under KCl stress, since a hybrid protein containing the N-terminal from Tup12 was superior to Tup11 but not comparable to Tup12 or the fully functional hybrid protein with only the C-terminal domain from Tup12. The same question applies to *S. octosporus* Tup12, which was fully functional in the *S. pombe* deletion strain under $CaCl_2$ stress but not under KCl stress. The influence of the N-terminal domain is likely related to its role in complex formation, and putative involvement and possible posttranslational regulation of Ssn6 under these conditions would have to be investigated.

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