Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

Functional Studies of RNA Polymerase II Recruitment to Promoter DNA and Impact of *BRF1* Mutations on RNA Polymerase III-dependent Transcription

Friederike Hög

aus Berlin

2013

ERKLÄRUNG

Diese Dissertation wurde im Sinne von §7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. Dr. Patrick Cramer betreut.

EIDESSTATTLICHE VERSICHERUNG

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, 6.12.2013

_____

Friederike Hög

Dissertation eingereicht am	06.12.2013
1. Gutachter:	Prof. Dr. Patrick Cramer
2. Gutachter:	PD Dr. Dietmar Martin
Mündliche Prüfung am	20.01.2014

ACKNOWLEDGEMENTS

Working at the Gene Center in Munich and in the group of Prof. Patrick Cramer was a great experience influencing my way of thinking and working in a positive way. We always wondered and wanted to fill our gap of knowledge. I am very thankful to Patrick, who was a great supervisor. I am very happy that he accepted me as a PhD student and offered me a place in his lab with that extra-ordinary inspiring, open and warm atmosphere. He did not only allow to 'wondering' and thinking outside the box but also gave me a lot of freedom to work independently and constantly supported me with good advice. His motivation, enthusiasm and knowledge affect everyone around.

To write that thesis would not have been possible without the people around me, so I have to thank Martin and Laurent, who helped me a lot to start my experimental work and taught me how to never forget a control and design conclusive experiments. Like Larissa, Clemens and Kayo they belonged to the Mediator Bay, which was a very nice and enjoyable place to work at. Beside my bay colleagues I have to thank Kerstin K., Christoph, Tobi, Simon and Carrie from the other side of the lab and Wolfgang, Steffi, Amelie and Kerstin M. for loads of fun, advice and help also outside the scientific life. I am very happy that I met you and wish you all the best. Although I cannot mention everyone, I could since the whole research group was really nice and created a comfortable working atmosphere.

I also have to thank Holger Hartmann, Johannes Söding, Barbara Treutlein, Nadine Sowada and Guntram Borck for fruitful and successful collaborations.

My life as a PhD student became even better when I joined the international graduate program 'Protein Dynamics in Health and Disease' of the elite network of Bavaria. I met a lot of inspiring and nice people there. Being part of that program opened a lot of doors and broadened my horizon in a scientific and personal manner. I will always think of the people when ordering a 'Kaiserschmarrn'!

Since life is not only science I also want to thank Jette, Tina, Julia G., Anja and especially Linus who enhanced enormously the quality of Munich by exploring the city with me and supporting me for the last four years with girls evenings, watching TV series after a hard day or showing me the cultural, creative, urban side of Munich. Of course I won't forget my non-Munich friends Pauline, Fabi, Michaela and Friedi. I missed you here and it always feels like yesterday when we meet after a long time again.

Last but not least, I want to thank my family who supported me my whole life and made me to the person I am. Without them I would not be where I am right now. Vielen Dank liebe Antonia, liebe Mutti und lieber Papi!

SUMMARY

Transcription by RNA polymerase (Pol) II begins with binding of general transcription factors to the promoter DNA. It is believed that binding of the TATA box-binding protein (TBP) to the TATA box nucleates pre-initiation complex (PIC) assembly. However, genome-wide analysis revealed that a classical TATA box occurs in only 15% of all *Sacchoromyces cerevisiae* (*Sc*) promoters. Since TBP is absolutely required for PIC assembly this raises the question of how the PIC is assembled at the other 85% TATA-less promoter. In this study, a novel conserved DNA element, the GA element (GAE), is characterized. This element is present in one third of all yeast promoters and is mutually exclusive to the TATA box. The GAE – characterized by one guanine followed by at least four adenines – can occur in multiple copies in the core promoter region. Its distance to the transcription start site (TSS) is similar to the distance between the TATA box and the TSS. The TATA-less model promoter *TMT1* contains a GAE, binds TBP and supports formation of a TBP-TFIIB-DNA complex. Moreover, mutation of the GAE and its surrounding nucleotides decreases transcription *in vivo* and *in vitro*. Additionally, a 32-nucleotide promoter region containing the GAE can functionally substitute for the TATA box in a TATA box-containing promoter, both *in vivo* and *in vitro*. This demonstrates a direct role of the GAE-containing region in transcription and identifies the GAE as a conserved core promoter element in TATA-less Pol II promoters.

Transcription by Pol III begins with binding of TFIIIC to the promoter. Next, TFIIIB (composed of TBP, Brf1 and Bdp1) is recruited, followed by Pol III and the initiation complex is formed. Pol III synthesizes tRNAs and other small non-coding RNAs such as 5S rRNA and thereby regulates protein synthesis on different levels. Dysregulation of transcription by Pol III has been linked to cancer and mutations in genes encoding Pol III subunits and tRNA processing factors cause different forms of neurogenetic diseases. Here, an autosomal-recessive disease is described characterized by cerebellar, dental and skeletal anomalies possibly caused by identified biallelic missense alterations of *BRF1* in the patients. These mutations impair cell growth, reduce Brf1 occupancy at tRNA gene promoters and decrease Pol III-dependent *in vitro* transcription in the model organism *Sc*. These results indicate that hypomorphic *BRF1* mutations cause a neurodevelopmental syndrome and that transcription by Pol III might be required for normal cerebellar and cognitive development.

PUBLICATIONS

Parts of this work have been published or are in the process of publication.

1. Seizl M*, Hartmann H*, **Hoeg F***, Kurth F, Martin DE, Söding J, Cramer P. *'A Conserved GA Element in TATA-Less RNA Polymerase II Promoters.'* PLoS One. 2011;6(11)

* These authors contributed equally.
MS conceived and designed the experiments, analyzed the data, contributed reagents and materials and wrote the paper. HH generated and analyzed the bioinformatic data, provided analysis tools and wrote the paper. FH, conceived, designed and performed all shown yeast experiments, analyzed the data, contributed reagents and materials and wrote the paper. FK performed pre-experiments. DM contributed reagents and materials. JS and PC conceived and designed the experiments and wrote the paper.

2. Treutlein B, Muschielok A, Andrecka J, Jawhari A, Buchen C, Kostrewa D, **Hög F**, Cramer P, Michaelis J. *'Dynamic architecture of a minimal RNA polymerase II open promoter complex.'* Mol Cell. 2012 Apr 27;46(2):136-46.

BT, conceived, designed and performed all FRET measurements, analyzed the data, contributed reagents and materials and wrote the paper. AM and JA analyzed the data and wrote the paper. AJ and CB performed protein purification. DK analyzed the data. FH performed the *in vitro* transcription assay. PC and JM conceived and designed the experiments and wrote the paper.

3. Nadine Sowada*, **Friederike Hög***, Maria Lisa Dentici,Perciliz Tan,Ana Medeira, Holger Thiele, Larissa Wenzeck, Francesca Lepri, Janine Altmüller, Peter Nürnberg, Bruno Dallapiccola, Christian Kubisch,Nicholas Katsanis, Patrick Cramer,Guntram Borck. *'Biallelic BRF1 mutations alter RNA polymerase III-dependent transcription and potentially cause a neurodevelopmental syndrome'*. Manuscript *in preparation*.

* These authors contributed equally.

NS conceived, designed and performed the clinical dissection and genetic experiments for identification of the mutations. FH conceived, designed and performed all experiments regarding the yeast *Saccharomyces cerevisiae* and wrote the manuscript. LW helped with yeast experiments. PC and GB conceived and designed the experiments and wrote the manuscript. Contributions of the other co-authors are not complete yet and remain to be specified.

TABLE OF CONTENTS

1 GENERAL INTRODUCTION

1.1 Transcription by RNA Polymerases

Transcription is one of the key events in the central dogma of molecular biology. This dogma was firstly described by Francis Crick in 1970 [1]. He proposed a basic scheme for the general transfer of information from deoxyribonucleic acid (DNA) via ribonucleic acid (RNA) to protein conserved throughout the three domains of life (Figure 1).

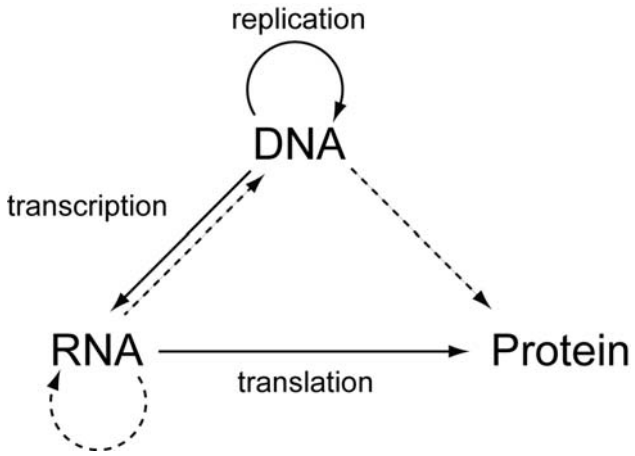

Figure 1 Central dogma of molecular biology proposed by Francis Crick. Adapted from Crick, 1970, Nature [1].
Solid arrows represent general transfer of information, dotted arrows special transfer. General transfers occur in all cells whereas special transfers occur only under certain circumstances. General transfers include replication of DNA, transcription from DNA into RNA and translation of messenger RNA into protein amino acid sequence. Special transfers like RNA to DNA transcription can occur in virus-infected cells.

The information encoded in the DNA is inherited by DNA duplication (replication) and read out by RNA polymerases which transcribe the DNA into RNA (transcription). Protein-coding messenger RNA (mRNA) is translated by ribosomes into an amino acid sequence (translation), which folds into proteins with a 3D structure. All three steps are highly regulated and depend on each other. Francis Crick also suggested rare special transfers, e.g. transcription from RNA to DNA in virus-infected cells [1].

1.1.1 RNA polymerases

RNA polymerases can be either single- or multi-subunit enzymes. Both groups do neither show sequence conservation nor structural homology [2].

Single-subunit Polymerases are usually found in bacteriophages, chloroplasts or mitochondria. Recently, the structure of the human mitochondrial RNA polymerase was solved revealing besides a lot of similarities differences to the T7 RNA polymerase structure [3].

Multi-subunit polymerases are present in archaea, prokaryotes and eukaryotes. In archaea and prokaryotes, all cellular RNA is synthesized by one RNA polymerase whereas in eukaryotes, this process is divided between at least three different polymerases: RNA polymerase I, II and III (Pol I, II and III). Interestingly, although they share a conserved catalytic core [4] they differ in the number of subunits and sensitivity to the inhibitor α-amanitin [5]. Pol I (14 subunits) is sensitive to high concentrations of α-amanitin whereas Pol II consists of 12 subunits and is inhibited by low concentrations of α-amanitin. Pol III is the largest polymerase composed of 17 subunits and is not affected by α-amanitin at all [5]. The nucleolar Pol I transcribes a locus encoding for the 5.8S, 18S and 28S ribosomal RNA (rRNA). Pol II and Pol III are both located in the nucleoplasm. The former synthesizes mRNAs and some small nuclear RNAs whereas the latter is responsible for transfer RNA (tRNA), 5S rRNA and other small RNA (snRNA) transcription. In plants, there were recently discovered Pol IV and V synthesizing RNAs involved in non-coding RNA-mediated gene silencing processes [6].

The transcription mechanism shows different level of complexity between the three domains of life. In all cases, additional regulatory proteins are necessary. Archaea require TFB and the TATA box-binding protein (TBP) for proper RNA polymerase recruitment and transcription initiation [7]. Prokaryotic RNA polymerases need the sigma factor for binding to the promoter sequence and subsequent initiation [8]. In eukaryotes, diverse factors are needed to distinguish between the different polymerases and to assure specific regulation of transcription. This can be achieved through distinct DNA elements and a lot of more factors involved in transcription than in archaeal and prokaryotic transcription [9]. These factors are specific for Pol I, II and III but have the same function. Interestingly, there is only one protein – TBP – being shared and essential for transcription by all three RNA polymerases (Table 1) [10].

Table 1 Yeast RNA polymerase subunits and initiation factor homologies. Adapted from Vannini and Cramer, 2012, Molecular Cell [11].

Pol I	Pol II	Pol III	Function
A49 (N-terminal domain) and A34.5	TFIIF	C37 and C53	Initiation complex stabilization, start site selection
A49 (C-terminal domain)	TFIIE	C82 and C34	Open complex stabilization
Rrn7	TFIIB	Brf1	TBP/polymerase binding, DNA opening, start site selection
TBP	TBP	TBP	DNA binding, nucleation of-PIC assembly

1.1.2 Transcription by RNA polymerase I

RNA polymerase I-dependent transcription is responsible for synthesizing up to 80% of the total cellular RNA but transcribing only a single gene in a transcription foci of the nucleus, the nucleolus [12]. This gene encodes for up to 200 – 300 copies of one precursor rRNA, which is spliced into the 5.8S, 18S and 28S rRNA. The Pol I promoter sequence is not well conserved but contains two distinct functional elements: the core promoter sequence (core) and the upstream promoter element (UE) [12]. In yeast, formation of the pre-initiation complex requires binding of the upstream activating factor (UAF), TBP and TBP associated factors (TAFs, called core factor (CF) in yeast) to the promoter sequence, which then recruits Pol I resulting in the initiation complex (IC) (Figure 2). RNA polymerase I transcription is highly regulated and influences or even regulates cell growth. That makes it to an interesting field for cancer research [12].

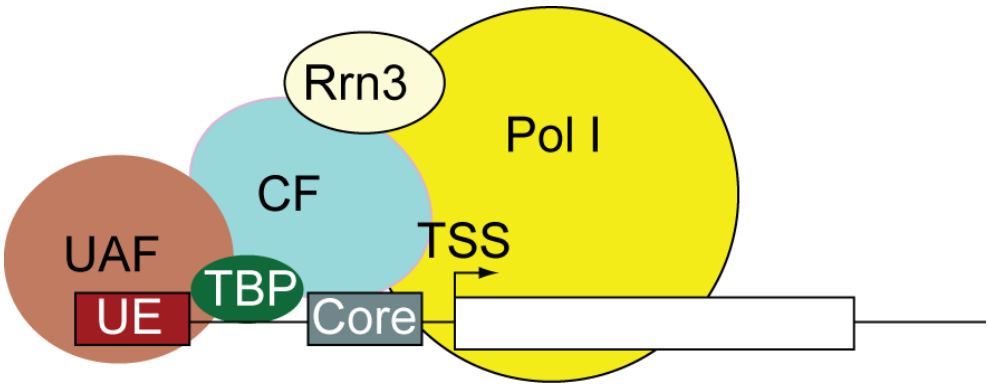

Figure 2 Scheme of RNA polymerase I IC and promoter architecture in yeast. Adapted from Moss et al, 2007, Molecular and Cellular Life Sciences [12].
In yeast, RNA Polymerase I PIC assembly begins with binding of the upstream activating factor (UAF) to the upstream element (UE). Afterwards, TATA box-binding protein (TBP) and the core factor (CF) are recruited to the core sequence (Core) and form a stable complex with the DNA. The core factor is composed of Rrn6, 7 and 11. In humans, this factor is called SL-1 and composed of at least five TATA box-associated factors (TAFs). Eventually, Pol I is recruited in an initiation-competent form through Rrn3-mediated interaction with the core factor, the IC is formed and transcription is initiated.

1.1.3 Transcription by RNA polymerase II

RNA polymerase II-dependent transcription is the first step in eukaryotic gene expression. Pol II transcribes the protein-coding genes into mRNA, which is then translated into an amino acid sequence. It is a highly regulated process and depends not only on general transcription factors (GTFs) but also on positive and negative cofactors like chromatin modifiers and coactivator complexes (e.g. Mediator or SAGA), elongation factors and RNA processing and termination factors [13].

The GTFs TFIIA, -B, -D, -E, - F, -H and -S are required for promoter recognition, melting, transcription start site (TSS) selection and primary RNA synthesis [13] (Table 2).

Table 2 The general transcription factors and their function. Adapted from Thomas and Chiang, 2006 [13].

Factor	Subunits	Function
TFIIA	3	Stabilizing TBP-DNA interaction, coactivator
TFIIB	1	Stabilizing TBP-DNA interaction, recruits Pol II with TFIIF, TSS selection
TFIID	TBP and 14 TBP associated factors (TAFS)	Core promoter binding, nucleates PIC assembly, coactivator, protein kinase, histone acetyltransferase
TFIIE	2	Recruits TFIIH, stimulates TFIIH activity, involved in promoter clearance
TFIIF	2	Associated with Pol II, recruits TFIIE and TFIIH, functions with TFIIB and Pol II in TSS selection, facilitates Pol II promoter escape, enhances elongation efficiency
TFIIH	10	ATPase/helicase activity for promoter melting and clearance, kinase activity for Pol II CTD phosphorylation, transcription-coupled nucleotide excision repair
TFIIS	1	Stimulates PIC assembly at some promoters and intrinsic cleavage activity of Pol II to release it from transcriptional arrest

The RNA polymerase II promoter region is the most complex of all RNA polymerases. It is divided into a regulatory and a core promoter region. The regulatory region is responsible for ensuring accurate response to environmental stimuli and cell-specific transcription. The core promoter is sufficient to direct transcription *in vitro* and is bound by the GTFs in a distinct order [13]. Depending on the core promoter elements and the distribution of the TSSs Pol II promoters can be classified into TATA box containing and TATA-less promoters [14] or focused and dispersed promoters[15].

Transcription can be described as a cycle with four main steps (Figure 3): (1) transcription initiation, (2) elongation, (3) termination and (4) recycling of the machinery for re-initiation [16].

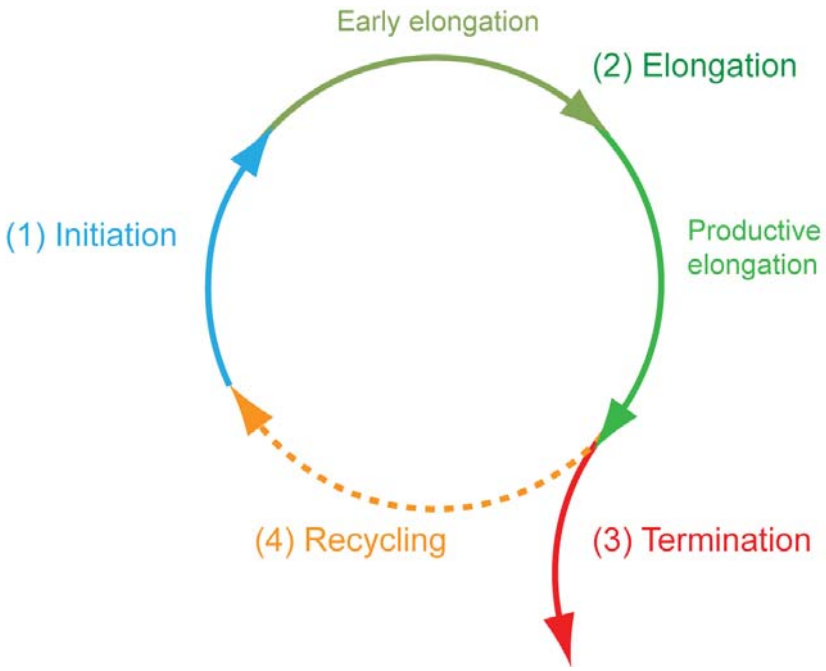

Figure 3 RNA polymerase II transcription cycle. Adapted from Nechaev and Adelman, 2011 [17].
RNA polymerase II enters the transcription cycle during PIC formation and transcription is initiated (1). The re-initiation complex consisting of GTFs and coactivators remains bound to the template. After a potential pause state during early elongation Pol II proceeds into productive elongation (2). Finally transcription is terminated (3) and the released Pol II can bind again to the re-initiation complex (4).

Before transcription initiation, the pre-initiation complex (PIC) has to assemble on RNA polymerase II promoters (Figure 4). During activation, gene-specific transcription factors recruit co-activator complexes and TFIID, thereby facilitating PIC assembly at the promoter. A subunit of TFIID, TBP, binds to the TATA box, which is located upstream of the TSS, and nucleates PIC assembly [18, 19]. TBP causes a kink in the DNA, which facilitates binding of the following GTFs [20]. Subsequent TFIIA binding leads to TBP-DNA complex stabilization enhanced through TFIIB binding. TFIIB recruits afterwards RNA polymerase II together with TFIIF to the PIC. PIC formation is completed through binding of Mediator, TFIIH and TFIIE. Finally, TFIIH opens the promoter DNA and the first nucleotides (NTPs) are incorporated and TFIIB assists in TSS selection [16]. After promoter escape and transition into productive elongation, synthesis is terminated with the help of special termination factors. Some of the GTFs and Mediator remain associated with the DNA facilitating RNA polymerase II to enter a new round of transcription after termination [16]. The synthesized pre-mRNA is capped and processed (co-transcriptionally) before export to the cytoplasm [21].

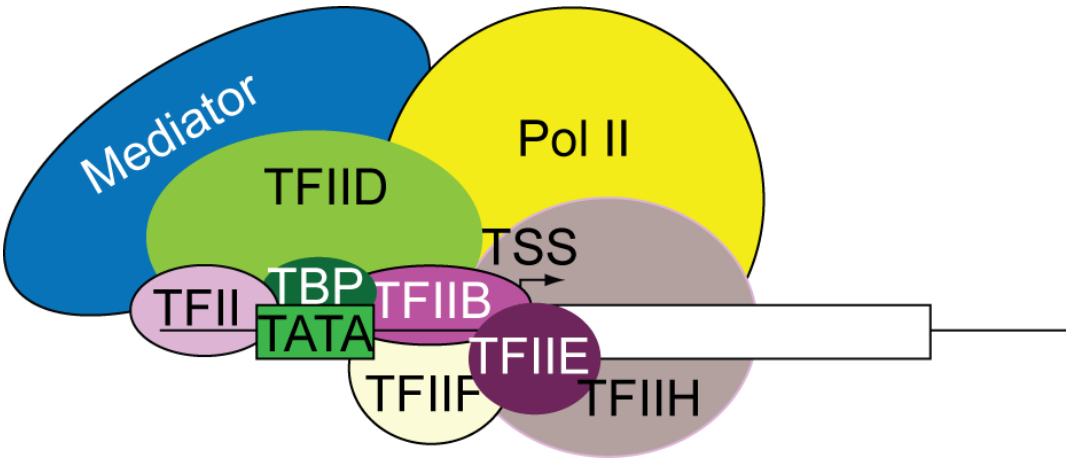

Figure 4 Scheme of RNA polymerase II PIC and promoter architecture. Adapted from Bywater et al., 2013, Nature Reviews Cancer [22].
TBP (dark green, component of TFIID, green) nucleates PIC assembly by binding to the DNA. Subsequently, TFIIA and TFIIB bind to the TBP-DNA complex and strengthen this interaction. TFIIB recruits Pol II in complex with TFIIF. Thereby, Pol II is positioned over the TSS. Finally, Mediator, TFIIH and TFIIE are recruited and complete PIC assembly. TFIIH melts the promoter DNA and transcription by Pol II is initiated.

Besides the regulation of transcription initiation through factors and DNA elements, elongation and termination can be regulated as well. Modifications, like phosphorylation, glycosylation and peptidyl-prolyl isomerization, of the Rbp1 C-terminal domain (CTD) play a major role for transitions in the transcription cycle resulting in the term 'CTD code' [23, 24]. The CTD is composed of multiple tandem hepta-peptide repeats of the sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7, which are conserved from yeast to human only differing in the number of repeats and their deletion is lethal [23].

1.1.4 Transcription by RNA polymerase III

RNA polymerase III transcribes genes encoding for short RNAs not longer than 400 base pairs (bp) having a catalytic or structural function, for example tRNAs, 5S rRNA and snRNAs functioning in the splicing machinery [25]. Depending on the promoter type TFIIIA, B and C and SNAPc (small nuclear RNA-activating protein complex) are needed for transcription [25]. In short, the promoter types differ in promoter architecture but all involve direct or indirect TFIIIB recruitment to the promoter DNA [26] (Figure 5). TFIIIB is a three protein complex composed of TBP, B-related factor 1 (Brf1) and B double prime 1 (Bdp1).

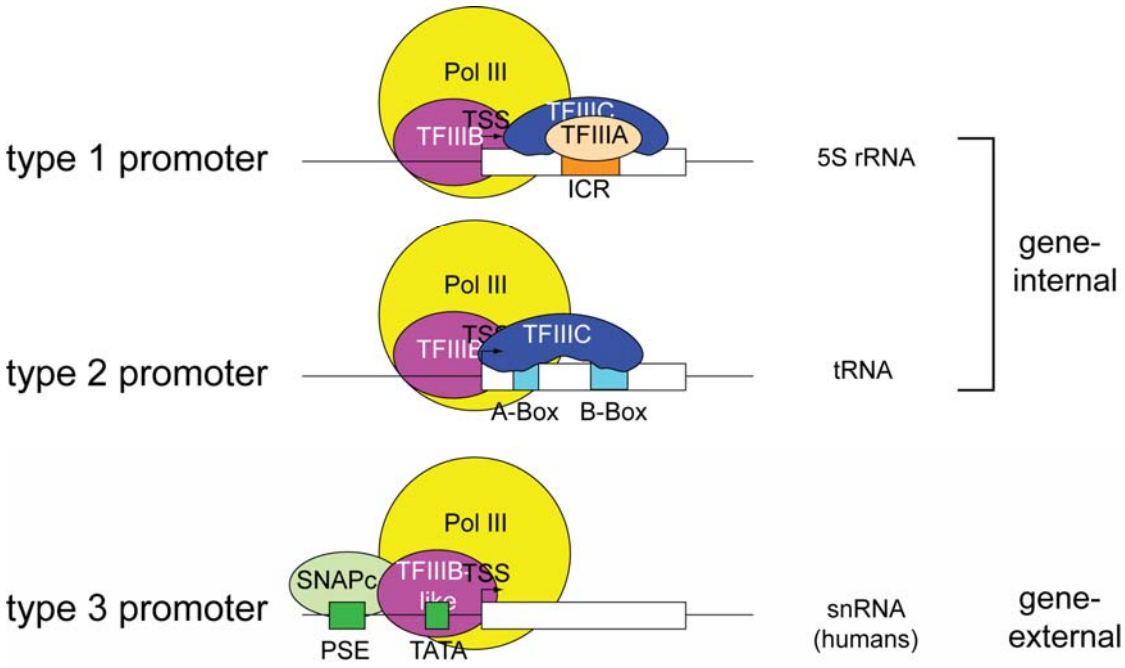

Figure 5 Scheme of RNA polymerase III ICs and promoter types. Adapted from Schramm and Hernandez, 2002, Genes and Development [25] and Bywater et al., 2013, Nature Reviews Cancer [22].
The composition of the Pol III ICs depends on the promoter type. The 5S rRNA promoter belongs to promoter type 1, where TFIIIA is recruited to the internal control region (ICR) which recruits and orientates TFIIIC and TFIIIA. Finally, Pol III is recruited by TFIIIB to the transcription start site (TSS) and transcription is initiated. TFIIIB is composed of TBP, Brf1 and Bdp1. In type 2 promoters, like tRNA genes, TFIIIC is recruited through the gene-internal A- and B-box which recruits TFIIIB. Again Pol III is recruited by TFIIIB and transcription is initiated. For a small number of Pol III genes, like snRNA genes, the promoter is located gene-external (promoter type 3) and contains a TATA box (TATA) and a proximal sequence element (PSE). In humans, a TFIIIB-like factor (Brf2 instead of Brf1) binds to the TATA box and the SNAPc binds to the PSE.

The promoter type I is characterized by an internal control region (ICR) consisting of the A-box, an intermediate element and the C-box. TFIIIA binds to the ICR and recruits TFIIIC, which in turn recruits TFIIIB [27]. Pol III is eventually recruited through interaction with TFIIIB to the promoter and transcription is initiated. The 5S rRNA promoter is the only known type I promoter.

A typical type II promoter consists of an A-Box and B-Box located 12-20 bp downstream of the TSS and 30-60bp downstream of the A-Box, respectively. They serve as binding sites for TFIIIC, which recruits TFIIIB followed by Pol III. tRNA genes and a few viral RNA genes belong to this promoter class.

In higher eukaryotes, additional type III promoters are defined by gene-external upstream elements – the TATA-box and the proximal sequence element (PSE). In this

case, TFIIIB is recruited through the upstream binding factor SNAPc and not TFIIIC and binds to the TATA box. Moreover, this TFIIIB contains Brf2 instead of Brf1, a slightly different variant of Brf1 [28]. Examples for type III promoters are the U6 snRNA gene and 7SK gene.
Termination of Pol III-dependent transcription is achieved through a stretch of Thymines and involvement of several factors resetting RNA polymerase III for re-initiation [29].

1.1.5 Regulation of initiation of transcription by RNA polymerase

RNA polymerase transcription initiation needs to be tightly regulated otherwise it can lead to diseases like cancer, autoimmunity or neurological diseases [30]. Regulation occurs on different levels but in the end it all leads to restricting RNA polymerase access to the DNA, in other words regulating transcription initiation.
Trans-acting factors, DNA binding transcription factors, bind to *cis* DNA elements thereby regulating recruitment of the transcriptional machinery. On the first level of regulation, different chromatin modifications restrict or enhance binding of gene-specific activators which in turn make the *cis* DNA elements accessible for GTFs and Polymerases [31]. On the second level, *cis* DNA elements regulate binding of these gene-specific transcription factors and the general transcription machinery. *Cis* DNA elements located in close proximity to the TSS are called core promoter elements [32]. They serve as binding platforms for the transcription factors and the RNA polymerase, hence determining the point of IC assembly. In case of transcription by Pol II combinations of different core promoter elements can influence strength and rate of transcription initiation [32].
Additionally, general transcription factors can regulate transcription by themselves as well, e.g. phosphorylation of yeast TFIIB at Serine 65 leads to initiation of transcription by Pol II [33] or recruitment of TFIIIB by TFIIIC results in Pol III recruitment followed by initiation [34].

1.2 Aims of this Study

The first part of this study concentrates on the identification and characterization of a new core promoter element in *Saccharomyces cerevisiae* (yeast). Transcription by all three RNA polymerases requires the binding of TBP, which nucleates PIC assembly [10]. Although the name – TATA box binding protein – implies binding to the consensus TATA box sequence TATAWAWR only 10 – 15% of all yeast core promoters actually encode that sequence [14, 35]. Thus, promoters can be divided into TATA box-containing and TATA-less promoters which are linked to SAGA-dominated and TFIID-dominated transcription, respectively [36]. Based on the observation that TATA-less core promoters also require TBP in its complex TFIID [10, 37, 38], it was hypothesized that TATA-less core promoters contain DNA elements that are functionally similar to the TATA box in promoting PIC assembly and transcription by Pol II. Although the GTFs are highly conserved throughout eukaryotes, alternative core promoter elements binding TBP could not be identified in yeast, so far.

To identify and functionally characterize a region in TATA-less yeast core promoters that contain a novel conserved core promoter element, is bound by TBP and required for Pol II-dependent transcription a combination of bioinformatics, *in vivo* reporter gene assays and *in vitro* biochemistry was used.

The second part of my study focuses on a general transcription factor of the RNA polymerase III machinery – Brf1, component of TFIIIB. Transcription by Pol III begins with TFIIIB recruitment through TFIIIC to a Pol III promoter. TFIIIB recruits in turn Pol III and transcription is initiated. Dysregulation of this recruitment due to overexpression of transcription factors or TFIIIB can lead to cancer [22]. Interestingly, only a few human genetic diseases are known to be caused by mutations in genes encoding components of the Pol III or tRNA processing machineries. The general principle of Pol III-dependent transcription is conserved from yeast to humans making it possible to investigate phenomena observed in humans in yeast and to draw general conclusion from findings in yeast to higher eukaryotes.

In human siblings showing all the same autosomal-recessive syndrome characterized by cerebellar, dental and skeletal anomalies, novel point mutations in *BRF1* – potentially causing this disease – were identified by whole exome sequencing.To investigate these mutations and their impact on transcription by Pol III, they were introduced and characterized with *in vivo* assays and *in vitro* transcription in yeast.

1.3 Additional Contributions

Additional experiments resulting in a co-authorship are described in more detail in section 3. Briefly, for the investigation of the Pol II open complex architecture, composed of a TATA box-containing mismatch DNA, TBP, Pol II, TFIIB and TFIIF, it was shown that the used fluorescently labeled TFIIB variant is functional in an *in vitro* transcription assay. In this study, a single-molecule approach was applied using single-molecule Förster resonance energy tranfer experiments and nano-positioning analysis [39]. The authors could determine the position of the TBP-DNA complex in relation to Pol II – it is located above the cleft – and suggested large structural changes during the initiation to elongation transition caused by a high intrinsic flexibility of TFIIB.

2 A CONSERVED GA ELEMENT IN TATA-LESS RNA POLYMERASE II PROMOTERS

2.1 Introduction

The first described and best characterized core promoter element in RNA polymerase II transcription is called TATA box referring to its prominent sequence TATA [40]. It is conserved through all domains of life – even in viruses– and bound by TBP, the TATA box-binding protein [15, 41-43]. In humans, the TATA box is located at a defined position of 30 nucleotides (nt) upstream of the TSS. In yeast, contrary, the distance between TATA box and TSS or the point of PIC assembly and initiator (INR), respectively, can vary from 40 to 120 nt upstream of the TSS. It is suggested that Pol II scans the DNA until it reaches the Initiator region before transcription is initiated [44]. As already mentioned, TBP binding is the first step in PIC assembly and essential for transcription initiation. However, recent genome-wide analyses revealed that not all but only a small fraction of 10 – 15% of all eukaryotic Pol II promoters encode a TATA box with the consensus sequence TATAWAWR (W = A/T, R = A/G) [14, 35].Thus, promoters can be divided into TATA box-containing and TATA-less promoters which are linked to SAGA-dominated and TFIID-dominated transcription, respectively [36]. That is supported by the finding that a TATA box is not strictly required for TFIID-dependent activity of a yeast model promoter [45]. Moreover, a recent study of the TATA-less yeast RPS5 promoter showed that functionally redundant AT-rich stretches within the core promoter region promote TFIID-dependent transcription [46]. Additionally, those AT-rich stretches are proposed to create nucleosome-free regions (NFRs) thereby keeping the core promoter region open for binding of the general transcription machinery [47, 48].

Since TATA-less promoters require TBP for function [10, 37, 38] and can bind TFIID [49], alternative pathways for PIC assembly were proposed [50, 51]. In metazoans, many TATA-less promoters contain a downstream promoter element (DPE) and an INR, which interact with components of TFIID to facilitate PIC assembly. The INR also determines the region of transcription initiation. Both elements are conserved but show different consensus sequences throughout species [15, 52].

Another core promoter element, called TFIIB-recognition element (BRE), directs the binding of the general transcription factor TFIIB to the core promoter. It can be

located up- or downstream of the TATA-box [53]. Several more core promoter elements have been discovered in the last years. Some of them function independent of and synergistically with the TATA box and INR, like the X core promoter element 1 (XCPE1) or the motif ten element (MTE) and the downstream core element (DCE), respectively [54, 55].

A summary of all known core promoter elements can be found in Figure 6.

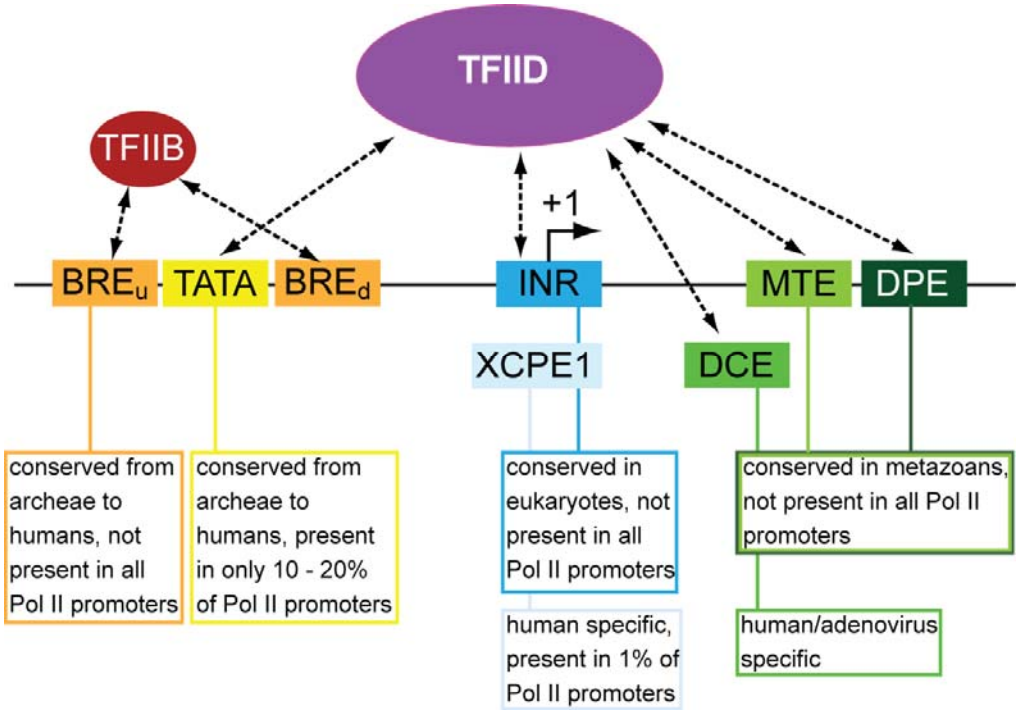

Figure 6 Core promoter elements in RNA polymerase II transcription. Adapted from Juven-Gershon et al, 2008, Current Opinion in Cell Biology [15].
Summary of all known promoter elements. $BRE_{u/d}$ – TFIIB recognition element up- or downstream of the TATA box; TATA – TATA box; INR – Initiator, XCPE1 – X core promoter element 1; MTE – motif ten element; DCE – downstream core element; DPE – downstream core promoter element. +1 and the arrow mark the transcription start site of a protein-coding gene.

Interestingly, there is no universal core promoter element, since all known core promoter elements usually occur only in a fraction of Pol II promotersand functional combinations of several core promoter elements are widespread. The most common one is probably the INR. Although the GTFs are highly conserved throughout eukaryotes, besides the TATA box and INR other core promoter elements have not been identified in yeast so far.

2.2 Results and Discussion

2.2.1 Many TATA-less yeast promoters contain a conserved GAE

In contrast to higher eukaryotes, where the distance between the TATA box and the TSS is fixed at around 30 nucleotides, a variable distance of 40−120 nucleotides is observed in the yeast *Sacchormyces cerevisia* (*Sc*), apparently due to a TSS scanning mechanism [44]. This has hampered bioinformatic discovery of core promoter elements other than the TATA box in yeast. To systematically search for a core promoter motif that could be functionally similarto the TATA box in TATA-less yeast promoters, four criteria were defined: (1) The motif should peak within a core promoter window of -110 to -50 nucleotidesrelative to the TSS. (2) The motif occurrence should be anti-correlated to the TATA box. (3) The motif should be frequent in this region in TATA-less promoters. (4) The motif should be highly conserved.

For each of the 1024 possible 5-mers, the frequency of occurrence within the core promoter window and the Matthews correlation coefficient with the TATA box (consensus TATAWAWR) were calculated. This search identified a DNA element comprising one guanine followed by four adenines (GAAAA) as being most highly anti-correlated to the TATA box (Figure 7A, criterion 1 and 2).

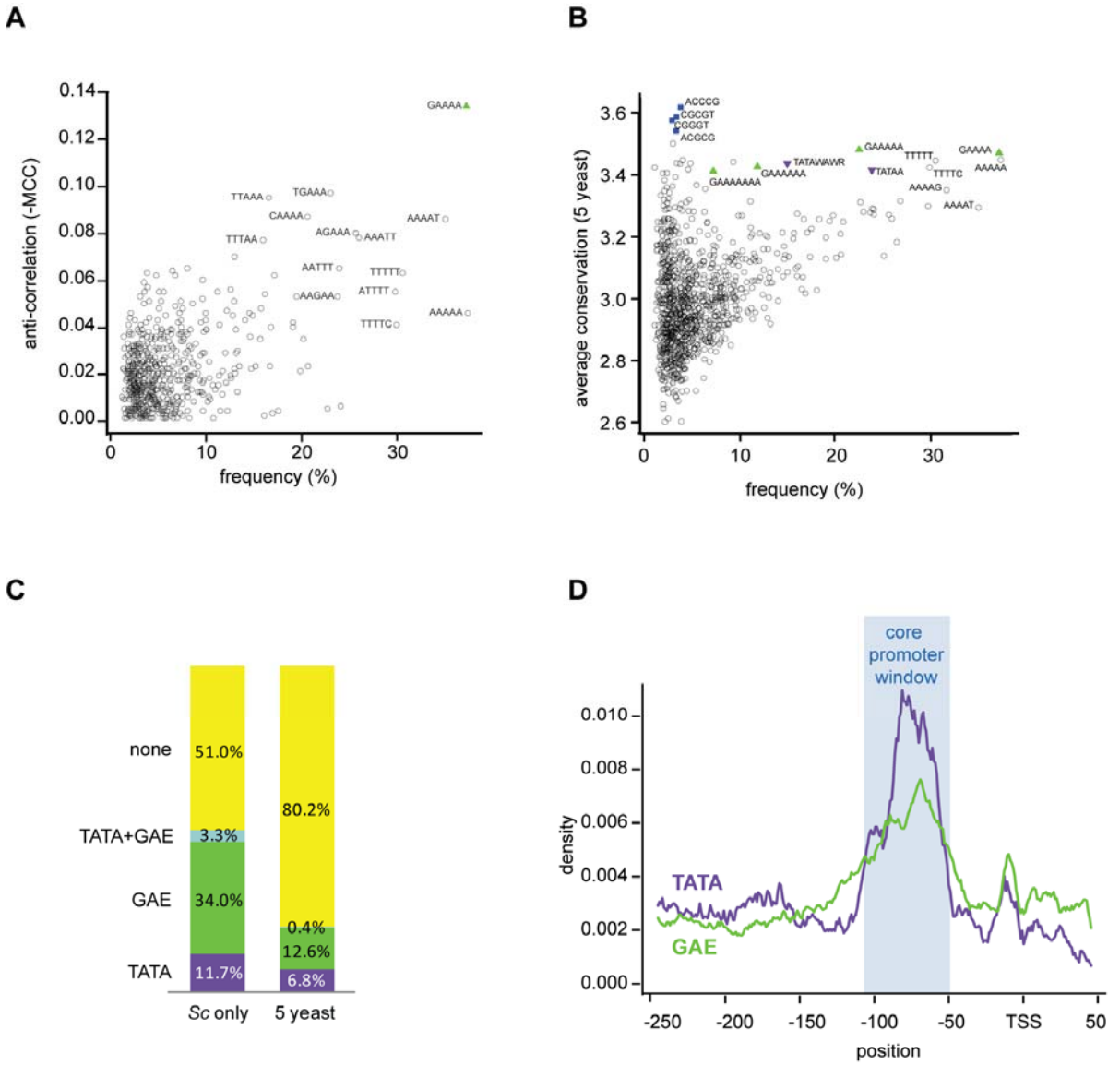

Figure 7 Many TATA-less yeast promoters contain a conserved GAE.
(A) Anti-correlation between different 5-mers and the TATA box in *Sc* core promoters. The GAE is marked by a green triangle. **(B)** Frequency of DNA 5-mers and their conservation between five closely related yeast species (*Sc, S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. bayanus)* in the core promoter window (-110 to -50 relative to TSS) of 3711 Pol II promoters. For comparison, the TATA consensus (TATAWAWR) and GAE-related sequences are shown. TATA-related sequences are marked by a purple triangle, GAE-related sequences are marked by a green triangle, transcription factor binding sites are marked by a blue square, and other 5-mers are marked by an open circle. **(C)** Percentage of *Sc* core promoters containing the TATA box, GAE, both elements or none of the elements with and without conservation between five closely related yeast species (left and right column, respectively). **(D)** Occurrence of the TATA box and GAE in 3711*Sc* Pol II promoters aligned to the TSS. The core promoter window used for bioinformatics analyses is highlighted by a blue box.

This novel element was termed GA element (GAE). It displays the second highest frequency of occurrence in the core promoter window (Figure 7B), only trumped in frequency by a subsequence of its longer version GAAAAA (criterion 3).

Next, the conservation of all 5-mers in the core promoter window among five closely related yeast species (*Sc, S. paradoxus*, *S. mikatae*, *S. kudriavzevii*,*S. bayanus*)was analyzed. In the multiple sequence alignments a shift of up to ± 3 nucleotides between motifs was allowed. Again, the GAE stands out as being the most highly conserved of all the more frequent 5-mers (Figure 7B, criterion 4). Its degree of conservation is slightly higher than that of the full and partial TATA box consensus (TATAWAWR and TATAA) and only a little lower than the rarer consensus binding sites of transcription factors Reb1 (ACCCG) and Mbp1 (ACGCG). Therefore, the GAE meets all four criteria defined above.

Sc promoters containing only a GAE are three times more abundant than promoters containing only a TATA box (34% vs. 11.7%, respectively, Figure 7C). An overview of all GAE-containing promoters is listed in the appendix. When filtered by conservation between the five closely related yeast species, 12.6% of promoters still contained the GAE, whereas 6.8% contained a TATA box (Figure 7C). The GAE is 3.4 times more frequent than expected from a mononucleotide background model calculated on the same region. This exceeds the TATA box and an A-stretch of length five, which are 3.3 times and 2.0 times more frequent, respectively. The presence of a GAE is anti-correlated with the presence of a TATA box (Figure 7C). Only 0.4% of all promoters between those five yeast species contain both elements (Figure 7C). In contrast to the TATA box, the GAE can be present in more than one copy in the core promoter region (Figure 8) and can contain additional adenines at the 3'end (Table 3). Similarly, a study of the TATA-less *RPS5* promoter demonstrated the presence of multiple, functionally redundant AT-rich sequences, which were shown to recruit TFIID [46].

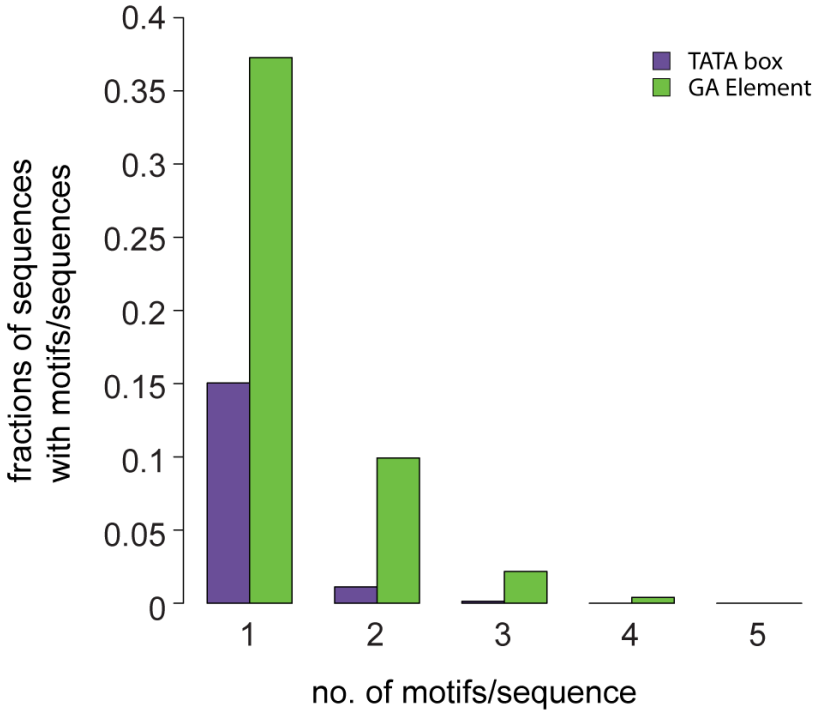

Figure 8 The GAE can occur in multiple copies.
Fraction of sequences with the TATA box (purple) and GAE (green) in at least a given number of multiple occurrences per sequences in 3711*Sc* promoters aligned at the TSS.

Table 3 Frequency of the GAE with different length of A-tracts in 3711 *Sc* core promoters.

GAE with A-tract of different length	Frequency (%)
GAAAA	37.3
GAAAAA	22.6
GAAAAAA	11.9
GAAAAAAA	7.3

When the annotated promoters are aligned to the TSS, the GAE peaks in the same region as the TATA box (Figure 7D). The similar position of the TATA box and the GAE with respect to the TSS, and the mutual exclusion of these two elements from core promoters suggested a functional similarity of both elements during PIC assembly.

2.2.2 A TATA-less yeast promoter that functions *in vitro.*

To functionally characterize the GAE *in vivo* and *in vitro*, the *ScTMT1* promoter, a TATA-less promoter with a single GAE in the core promoter region was chosen as GAE-containing model promoter. For comparison, the *ScHIS4* promoter was used, which contains a TATA box and is widely used for *in vitro* transcription assays [19, 56-58]. Both *TMT1* and *HIS4* are involved in amino acid biosynthesis and are regulated by the activator Gcn4. Both promoters were tested in a β-galactosidase reporter gene assay, which measures promoter strength *in vivo*. Both promoters were active under standard growth conditions (Figure 9A). Next, it was examined whether the promoters also supported Gcn4-activated transcription *in vitro*. Indeed, not only the TATA-containing *HIS4* promoter but also the TATA-less *TMT1* promoter was active (Figure 9B, lanes 2 and 5). Promoter activity depended on Pol II, since the Pol II-specific inhibitor α-amanitin abolished transcription (Figure 9B, lanes 3 and 6). These results establish the *TMT1* promoter as the first native TATA-less promoter in yeast that is active in an activator-dependent *in vitro* transcription assay.

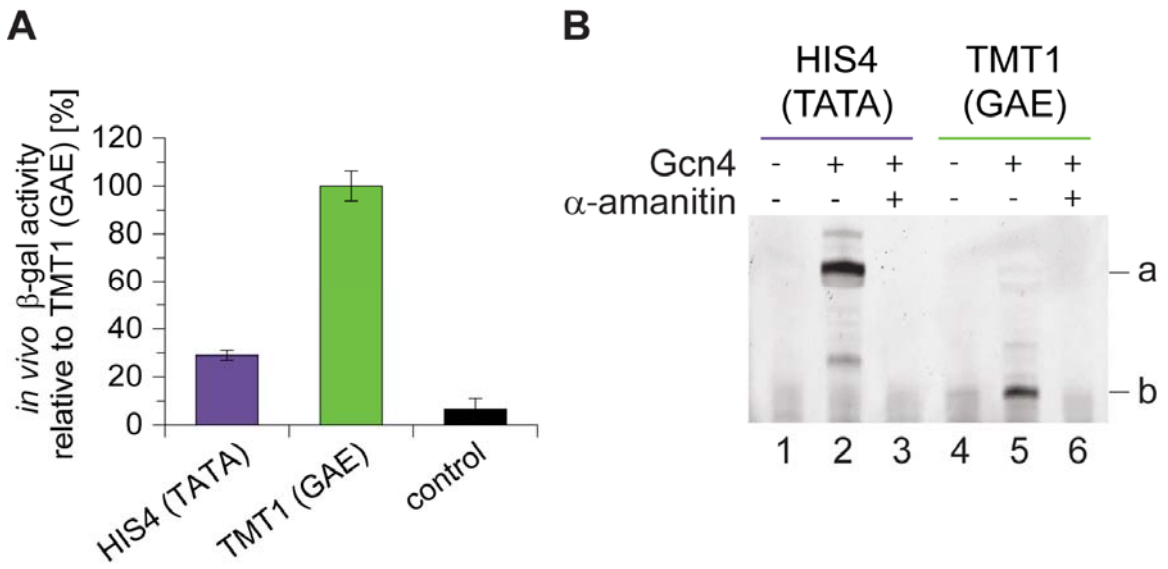

Figure 9 A TATA-less yeast promoter that functions in vitro.
(A) Promoter activity in an *in vivo* β-galactosidase reporter gene assay of *HIS4* and *TMT1 Sc* promoters. Negative control is background signal of the reporter plasmid without a promoter. **(B)** Promoter activity in an *in vitro* transcription assay of *Sc HIS4* and *ScTMT1* promoter using yeast nuclear extracts. *In vitro* transcription was carried out with and without recombinant Gcn4 activator. To test Pol II specificity of transcription, 0.04 mM α-amanitin was added. Specific transcripts from the *HIS4* TSS and *TMT1* TSS are marked by a and b, respectively.

2.2.3 The GAE-containing region is required for promoter function

To test whether the GAE is required for *TMT1* promoter function, several promoter mutants were generated (Figure 10A) and their activities were tested in *in vivo* β-galactosidase reporter gene assays. Deletion of the bioinformatically defined 5-nucleotide GAE had a moderate negative effect on promoter activity (Figure 10B, lane 2). Whereas replacing the 5-nucleotide GAE with GGCCG had no apparent defect (Figure 10B, lane 3), mutating the 12-nucleotide GAE-containing region (four and three nucleotides up- and downstream, respectively) strongly impaired promoter activity (Figure 10B, lane 4). Screening of additional promoter mutants revealed a strong dependence of promoter function on the four nucleotides directly upstream of the GAE (Figure 10B, lane 9).

Activity of the *TMT1* promoter was also strongly decreased when the guanines in the 12-nucleotide GAE-containing region were changed to adenines (Figure 10B, lane 10), generating a poly(dA) stretch. A similar defect in promoter activity was observed in activated transcription assays using a nucleosome-free DNA template and yeast nuclear extracts (Figure 10C) *in vitro*. This demonstrates a direct role of the GAE-containing region in transcription and suggests a function independent of nucleosome-dependent promoter accessibility. Thus the GAE-containing region is functionally distinct from previously described poly(dA) stretches. Several studies of poly(dA:dT) tracts in individual model promoters indicated a role in promoter function that is linked to nucleosome positioning [47, 48, 59-61]. Further, a recent theoretical genome-wide study described G/C-capped poly(dA:dT) tracts associated with the nucleosome-free region of TATA-less promoters in yeast [62]. These tracts were suggested to define the center of the nucleosome-free region. However, a distinction of a direct effect on nucleosome positioning and indirect effects, such as recruiting chromatin remodelers or the transcription machinery, could not be made. Consistent with that finding, the deletion of a poly(dA:dT) tract in the *ILV1* promoter decreased transcription significantly without affecting nucleosome organization *in vivo* [60]. Since the GAE described here appears to overlap with a part of the previously described poly(dA:dT) tracts, those tracts that fall in the described core promoter window may be redefined as GAE.

The defects of the GAE deletion, the mutations in the flanking regions, and the polyA mutation suggest that *TMT1* promoter function is strongly dependent on the sequence context in the GAE-containing region.

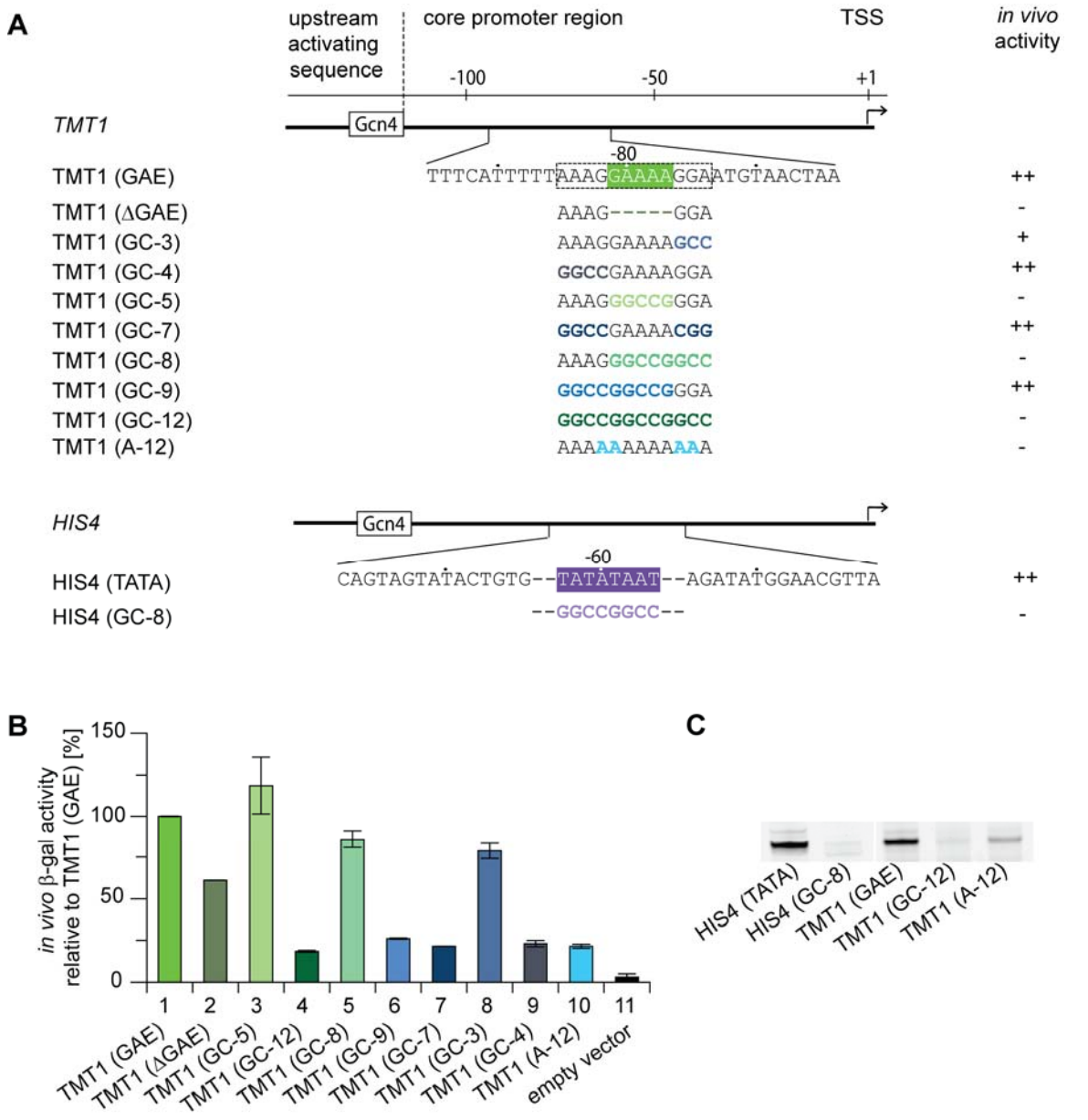

Figure 10 The GAE-containing region is required for promoter function.
(A) Schematic depiction of the TATA-less *TMT1*and the TATA-containing *HIS4* promoter. TATA box and bioinformatically defined GAE are marked by a black box. The 12-nucleotide GAE-containing region is marked by a dashed line. Promoter mutants are shown below the sequences. Activity of each variant in *in vivo* β-galactosidase reporter gene assays is shown (wild type activity (++), decreased activity (+) and severely decreased activity (-)). **(B)** Determination of the core promoter region required for *TMT1*promoter function. Activities of wild type *TMT1* and several mutants in the GAE-containing region in an *in vivo* β-galactosidase reporter gene assay are shown. Negative control is background signal of the reporter plasmid without a promoter. **(C)** Promoter activities in an *in vitro* transcription assay using a nucleosome-free DNA template and yeast nuclear extracts.

2.2.4 The GAE has no inhibitory effect on the HIS4 TATA box-containing promoter

As shown in Figure 7A – C the GAE is anti-correlated to the TATA box. The mutual exclusion of TATA box and GAE appears not to be the result of an inhibitory role of GAE. Insertion of the GAE up- or downstream of the *HIS4* TATA box had no effect on promoter function (Figure 11).

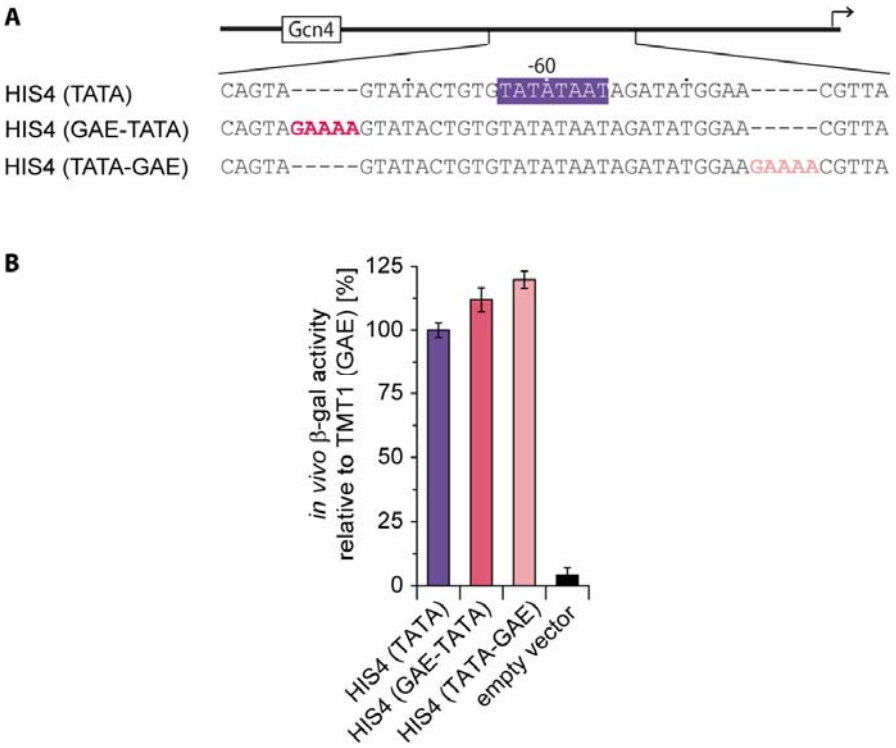

Figure 11 GAE has no inhibitory effect on the TATA-containing HIS4 promoter. Promoter activity in an *in vivo* β-galactosidase reporter gene assay of the wild type and mutated HIS4 promoter. **(A)** Schematic depiction of the *HIS4* promoter constructs with GAE insertions. **(B)** Activities of wild type *HIS4*promoter and insertions of the GAE in the proximity of the HIS4 TATA box in an *in vivo* β-galactosidase reporter gene assay are shown. Negative control is background signal of the reporter plasmid without a promoter.

2.2.5 A TATA-less promoter is bound by TBP

Since the GAE and the TATA box occur at similar distances from the TSS, it was tested whether the GAE-containing promoter region binds TBP. An electrophoretic mobility shift assay (EMSA) and fluorescently labeled DNA encompassing 40 base pairs of *HIS4* and *TMT1* promoter DNA were used. As expected TBP bound to *HIS4* DNA, but also to the TATA-less *TMT1* DNA (Figure 12A, lane 2 and 8).

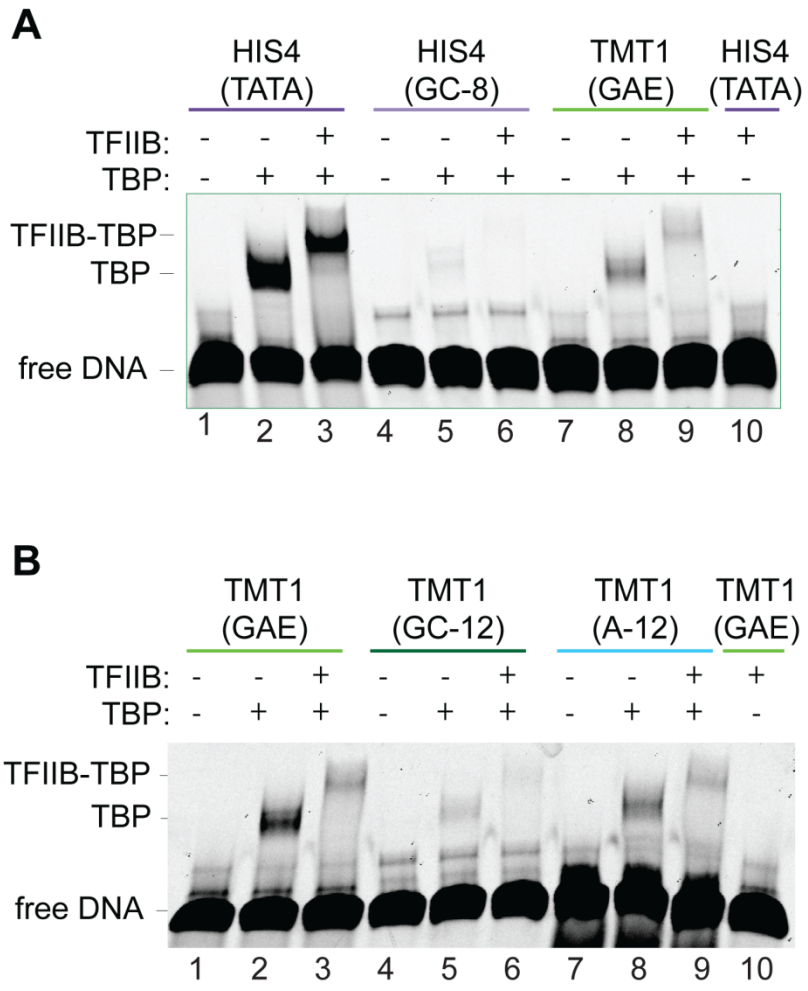

Figure 12 The TATA-less TMT1 core promoter is bound by TBP.
(A) and **(B)** Electrophoretic mobility shift assay. A 5'-Cy5-labeled 40 bp double stranded DNA of the respective promoter construct was incubated with recombinant TBP_{core} alone or TBP_{core} and TFIIB. The protein-DNA complex was separated from free DNA by native polyacrylamide gel electrophoresis. Free DNA and bound protein-DNA complexes are indicated.

Both promoter DNAs could also form a stable complex with TBP and TFIIB (Figure 12A, lane 3 and 9). The observed binding was specific to double stranded DNA (Figure 13).

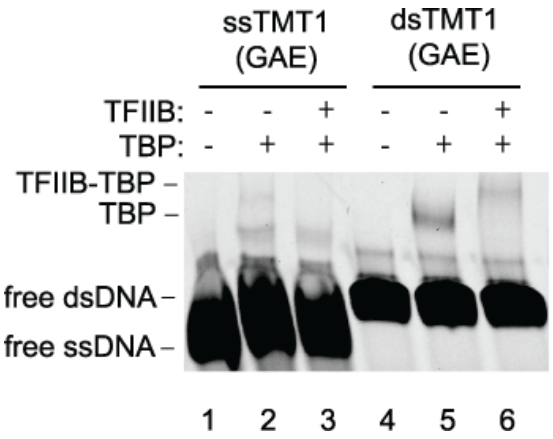

Figure 13 Only double stranded templates are bound by TBP and TFIIB.
Electrophoretic mobility shift assay. A 5'-Cy5-labeled 40 bp single (ssDNA) and double stranded (dsDNA) template was incubated with recombinant TBP_{core} alone or TBP_{core} and TFIIB. The protein-DNA complex was separated from free DNA by native polyacrylamide gel electrophoresis. Free DNA and bound protein-DNA complexes are indicated.

As expected, TBP and TFIIB binding was strongly decreased by mutating the TATA box of *HIS4* (Figure 12A, lane 5 and 6). Mutating the 12-nucleotide GAE-containing region also impaired TBP and TFIIB binding (Figure 12B, lane 5 and 6). TBP and TFIIB binding was not affected when guanines in the 12-nucleotide GAE-containing region were replaced with adenines (Figure 12B, lane 8 and 9). These results show that a stretch of adenines is sufficient for TBP binding, but not for promoter activity. The guanines in and around the GAE thus appear to have a function distinct from TBP binding. This is consistent with the observation that TBP binding does not necessarily correlate with promoter activity [63, 64].

2.2.6 The GAE-containing region functionally substitutes for the TATA box

To further investigate the potential functional similarity of the GAE-containing region and the TATA box, promoter substitution experiments *in vivo* and *in vitro* were performed. The *HIS4* TATA box can functionally replace the 12-nucleotide GAE-containing region in the *TMT1* promoter (Figure 14B, lane 3).

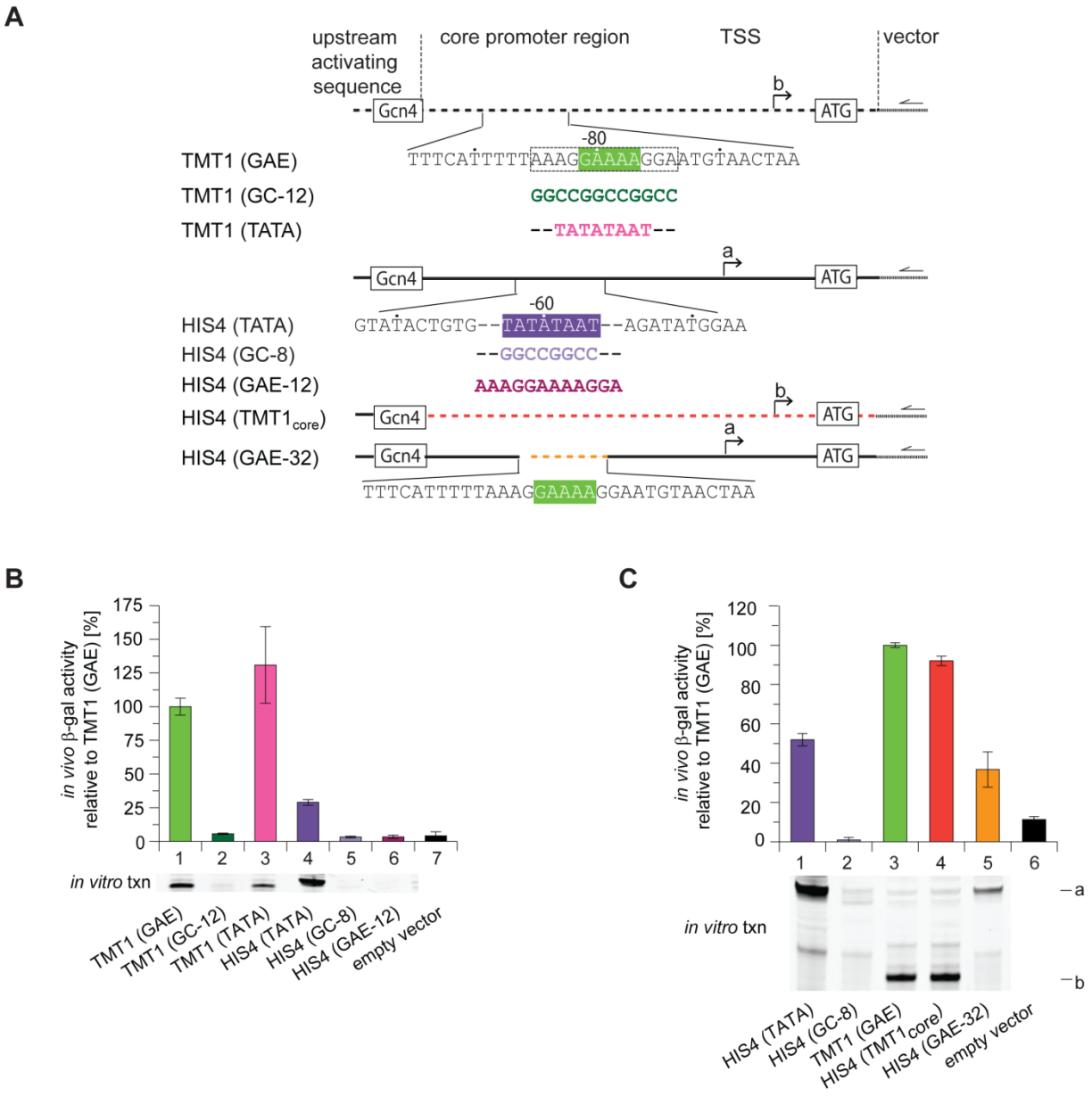

Figure 14 The GAE-containing region functionally substitutes for the TATA box.
(A) Schematic depiction of promoter substitution constructs of the TATA-less *TMT1*and the TATA-containing *HIS4* promoter. TATA box and bioinformatically defined GAE are marked by a black box. Solid and dashed lines represent *HIS4* and *TMT1* promoter sequences, respectively. TSS of *HIS4* and *TMT1* are labeled with a and b, respectively. Half-arrows mark primer annealing sites for primer extension reaction. **(B)** Promoter activity in an *in vivo* β-galactosidase reporter gene assay and *in vitro* transcription (txn) assay of the wild type (lane 1 and 4) and mutated *TMT1* and *HIS4* promoter. Mutations as indicated in Figure 5A. Negative control (lane 7) is background signal of the reporter plasmid without a promoter. **(C)** Promoter activity in an *in vivo* β-galactosidase reporter gene assay and *in vitro* transcription (txn) assay of the wild type (lane 1 and 3) and promoter substitution constructs as indicated in Figure 5A. Negative control (lane 6) is background signal of the reporter plasmid without a promoter.

This substitution does not impair binding of TBP and TFIIB (Figure 15A, lanes 5 and 6).

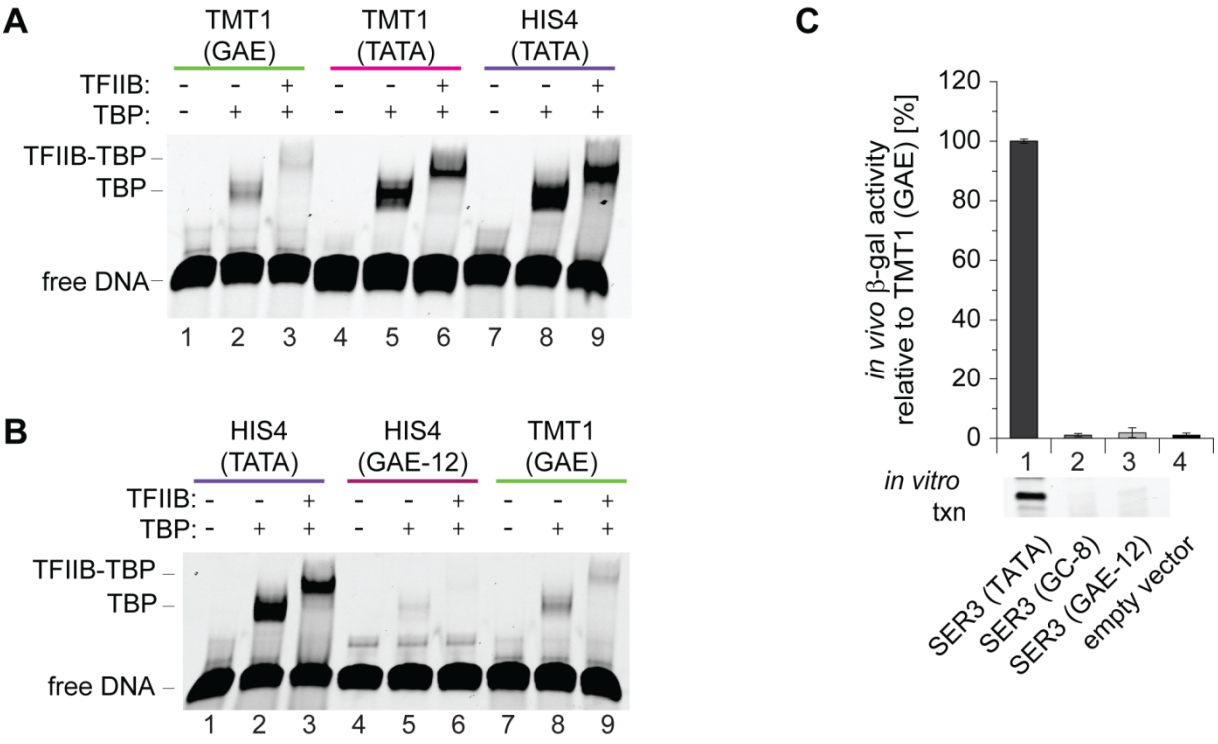

Figure 15 GAE function depends on promoter context.
(A) and **(B)** Electrophoretic mobility shift assay. A 5'-Cy5-labeled 40 bp double stranded DNA of the respective promoter construct was incubated with recombinant TBP_{core} alone or TBP_{core} and TFIIB. The protein-DNA complex was separated from free DNA by native polyacrylamide gel electrophoresis. Free DNA and bound protein-DNA complexes are indicated. **(C)** Promoter activity in an *in vivo* β-galactosidase reporter gene assay and *in vitro* transcription (txn) assay of the wild type and mutated *ScSER3* promoter. The 8-nucleotide TATA box of the *SER3* promoter was substituted by the 12-nucleotide GAE-containing region of the *TMT1* promoter.

In contrast, the 12-nucleotide GAE-containing region is neither sufficient for functional replacement of the TATA box in the *HIS4* promoter (Figure 14B, lane 6) nor for binding of TBP and TFIIB (Figure 15B, lane 5 and 6). Similar observations were made when replacing the TATA box in the *SER3* yeast promoter by the 12-nucleotide GAE-containing region (Figure 15C).

To determine a minimal region in the *TMT1* promoter that could substitute part of the *HIS4* promoter without loss of activity, several *HIS4-TMT1* fusion constructs were tested (Figure 14B). The *TMT1* core promoter fused to the *HIS4* upstream activating sequence is active both *in vivo* and *in vitro* (Figure 14C, lane 4). This suggests that regions surrounding the 12-nucleotide GAE-containing region are required for func-

tion. Indeed, inserting a 32-nucleotide GAE-containing region (14 and 13 nucleotides up- and downstream of the GAE, respectively) could maintain partial *HIS4* activity (Figure 14C, lane 5). Including even longer up- or downstream *TMT1* sequences did not increase activity (Figure 16).

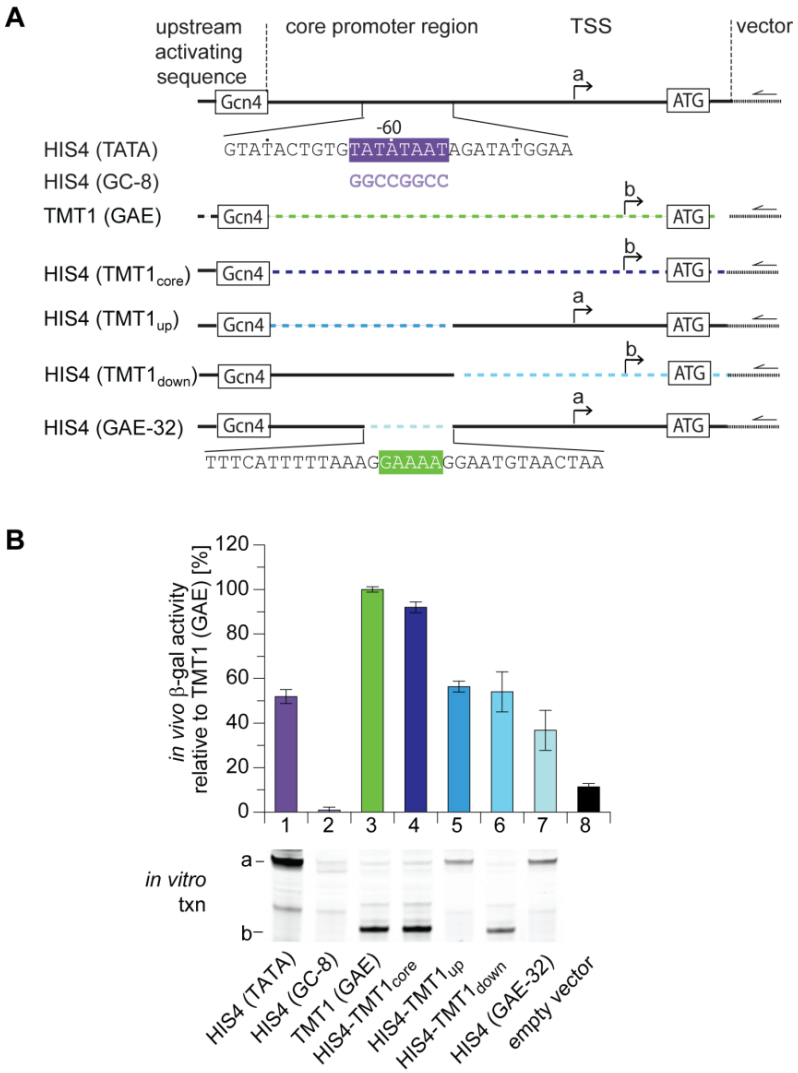

Figure 16 The GAE-containing region can functionally replace the TATA box.
(A) Schematic depiction of promoter substitution constructs of the TATA-less *TMT1*and the TATA-containing *HIS4* promoter. TATA box and bioinformatically defined GAE are marked by a black box. Solid and dashed lines represent *HIS4* and *TMT1* promoter sequences, respectively. TSS of *HIS4* and *TMT1* are labeled with a and b, respectively. Half-arrows mark primer annealing sites for primer extension reaction. **(B)** Promoter activity in an *in vivo* β-galactosidase reporter gene assay and *in vitro* transcription (txn) assay of the wild type and mutated *HIS4* and *TMT1* promoter. Negative control (lane 8) is background signal of the reporter plasmid without a promoter.Specific transcripts from the *HIS4* TSS and *TMT1* TSS are marked by a and b, respectively.

This is consistent with the observation that sequence context is critical for *TMT1* activity (Figure 10B). This indicates that the AT-rich sequences flanking the GAE in the *TMT1* promoter are also important for activity.

Thus, *TMT1* promoter function is highly dependent on sequence context. The GAE-flanking region and the guanine residues in the 12-nucleotide GAE-containing region are crucial for promoter function. Similarly, previous studies have shown that also TATA box function and TBP-TATA binding is influenced by TATA-flanking sequences [65].

2.2.7 The GAE correlates with TFIID-dominated genes

Recent genome-wide studies indicated that activation of TATA-less genes is dominated by TFIID [14, 36]. Consistently, a detailed study of the TATA-less *TUB2* promoter had demonstrated a strong TFIID dependence [66]. *TUB2* activity was severely reduced in temperature-sensitive TFIID mutant backgrounds. Insertion of a canonical TATA box at -55 relative to the TSS could alleviate this defect and restore promoter function. Intriguingly, when the *TUB2* sequence is inspected, a conserved GAE is present at the exact point of insertion. This is consistent with the finding that the GAE is mainly found in TFIID-dominated promoters (Figure 17).

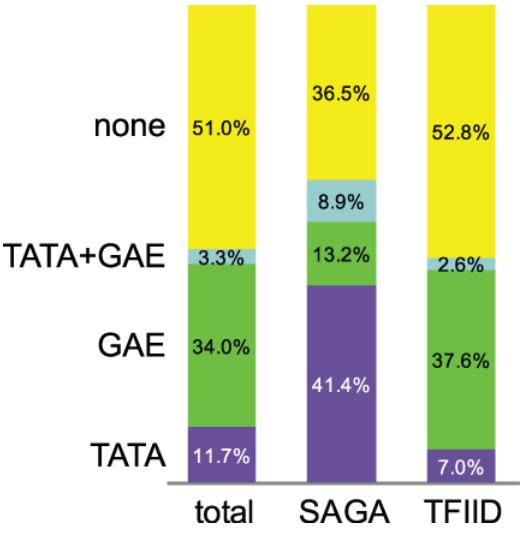

Figure 17 The GAE correlates with TFIID-dominated genes.
Percentage of *Sc* core promoters containing a TATA box, a GAE, both elements or none of the elements in SAGA- and TFIID dominated *Sc* promoter classes [14, 36].

3 BIALLELIC BRF1 MUTATIONS ALTER RNA POLYMERASE III-DEPENDENT TRANSCRIPTION AND POTENTIALLY CAUSE A NEURODEVELOPMENTAL SYNDROME

3.1 Introduction

Pol II-dependent transcription has attracted much attention because its mRNA products are protein-coding, but recent years have seen an increasing interest in the Pol III apparatus because of its implication in disease. Increased Pol III-dependent transcription has been linked to cell transformation and cancer [67, 68], but only a few human genetic diseases are known to be caused by mutations in genes encoding components of the Pol III or tRNA processing machineries. Pontocerebellar hypoplasias (PCH) are a heterogeneous group of severe progressive neurodegenerative conditions often leading to early death [69]. Nine types of PCH are recognized and their associated mutations are predicted to lead to loss of function of tRNA splicing endonuclease. However, the pathomechanism leading to PCH is poorly understood [70].

More recently, mutations in the Pol III subunits *POLR3A* and *POLR3B* have been identified in syndromic hypomyelinating leukodystrophies [71-74]. *POLR3A* mutations lead to a decrease in POLR3A levels and are predicted to interfere with the interaction with other Pol III subunits, which in turn would perturb Pol III-mediated transcription. These findings suggest that the central nervous system is vulnerable to changes in Pol III-dependent transcription [71].

The basal Pol III transcription machinery requires the orchestrated interaction of the transcription factors TFIIIB and TFIIIC with Pol III [75, 76]. These factors and the underlying mechanism of transcription initiation are conserved through eukaryotes. In short, TFIIIC recognizes and binds to internal promoters of e.g. tRNA genes and then recruits TFIIIB. TFIIIB is composed of Brf1, Bdp1 and TBP, each of which is required for TFIIIB function *in vitro* [75]. TFIIIB recruits Pol III for transcription initiation.

3.2 Results and Discussion

3.2.1 Clinical delineation of a cerebellar-dental-skeletal syndrome

Our collaborators of the University of Ulm, Guntram Borck and colleagues, identified four children (three girls and one boy) from two unrelated families with an apparently identical syndrome characterized by cerebellar, dental and skeletal anomalies. In both families, two affected children were born to unrelated and unaffected parents, suggesting autosomal-recessive inheritance. The affected children had strikingly similar dysmorphic features, e.g sparse eyebrows, apparently low-set ears, proportionate short stature, microcephaly of prenatal onset, mild-to-moderate intellectual disability with speech delay and scoliosis (data not shown). Cranial magnetic resonance imaging revealed cerebellar hypoplasia in all four children. On skull and orthopanoramic X-rays they saw dental anomalies, like taurodontism, a specific malformation of the pulp of molar teeth. Array-CGH did not detect causative copy number variants (data not shown). They refer to this syndrome as cerebellar-dental-skeletal syndrome.

3.2.2 Identification of *BRF1* mutations

Whole exome sequencing was performed in one affected individual from each of the two families and detected possibly homozygous or compound heterozygous mutations of the same gene in both affected children in a single gene, *BRF1*. Each affected child was heterozygous for two variants that predicted missense alterations, for a total of four distinct mutations (data not shown). In family 1, the affected siblings were compound heterozygous for a maternally inherited c.677C>T mutation and a paternally inherited c.776C>T mutation, predicting p.Ser226Leu and p.Thr259Met, respectively. In family 2, the affected sisters had inherited a c.667C>T mutation from their mother and carried additionally a c.875C>A mutation that was likely transmitted from their father, who did not participate in the study. These mutations predict p.Arg223Trp and p.Pro292His missense alterations, respectively. The mutations were absent from ethnically matched controls as shown by sequencing and not present or very rare in public SNP databases. The cerebellar-dental-skeletal syndrome is likely a rare disease entity with a specific phenotype. Neither *BRF1* mutations in

three individuals with clinically overlapping but distinct malformation syndromes nor in seven individuals with genetically unresolved forms of PCH were identified (data not shown).

BRF1 encodes a 677 amino acid protein that – together with TBP and Bdp1 – forms the transcription factor IIIB (TFIIIB), which recruits Pol III to its templates and is involved in promoter opening [77]. The four missense alterations invariably affect amino acids that are conserved in evolution, including orthologs from mouse, rat, and zebrafish, but that are not conserved in Brf2. In *Saccharomyces cerevisiae* Brf1, Arg223, Thr259 and Pro292 are conserved, whereas Ser226 is conservatively replaced by an alanine [78]. For easier understanding, the human amino acid numbering is used throughout the text. The corresponding numbers in yeast Brf1 can be found in the Methods section (see Chapter 6). Since Ser226 is replaced by an alanine in yeast, all experiments were also carried out with a replacement of alanine to serine to investigate position-dependent effects.

3.2.3 *BRF1* mutations affect cell growth

Consistent with a pathogenic nature of the identified missense changes, yeast lacking the chromosomal *BRF1* gene and expressing *BRF1* with mutations corresponding to Arg223Trp or Pro292His did not grow, whereas the mutation analogous to Thr259Met induced a severe growth defect (Figure 18). Yeast cells transformed with plasmids containing *BRF1* substitutions Ala226Ser (reflecting the human reference sequence) or Ser226Leu (as found in family 1) grew as wild type. Thus, two mutations are lethal and another mutation leads to a severe growth defect *in vivo*, consistent with loss of Brf1 function. To simulate the compound heterozygosity present in the families yeast cells were transformed with two distinct plasmids expressing Brf1 variants Arg223Trp/Pro292His or Ser226Leu/Thr259Met. The combination was either lethal or led to a growth defect (Figure 18).

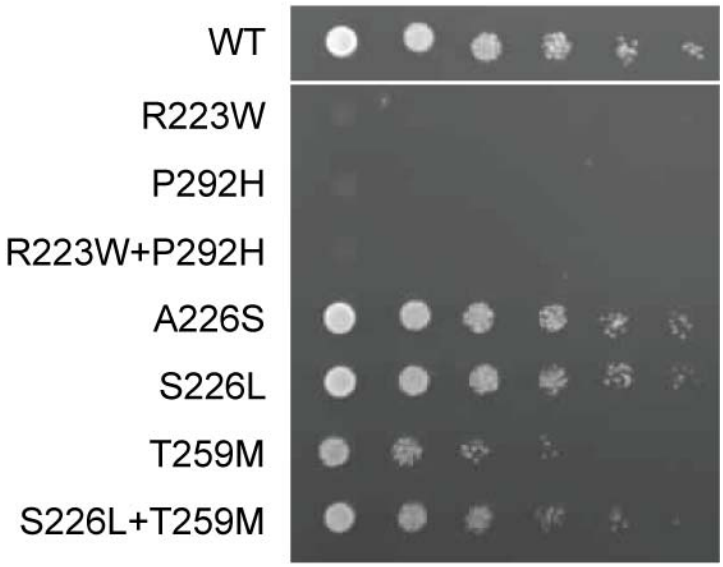

Figure 18 *BRF1* mutations affect cell growth.
Spot dilutions of the variants introduced into a Brf1 knockout strain grown at 30°C. Wild type (WT) and variant Brf1 were encoded on plasmids. For the combination of two mutations two plasmids were used each harboring one mutation and a distinct marker. Although the experiment was done in yeast, the residue numbers correspond to the numbers in the human protein for better understanding.

Whereas intellectual disability can be due to mutations in synaptic genes [79, 80], recent studies have – to some extent unexpected – highlighted impairments of basal cellular functions and pathways such as transcription and translation in the etiology of cognitive disorders and neurogenetic syndromes [81]. The growth phenotype caused by the identified *BRF1* mutations supported the implication of the human biallelic missense alterations of *BRF1* in the observed cerebellar-dental-skeletal syndrome.

3.2.4 *BRF1* mutations are predicted to disturb Brf1-DNA interaction

The N-terminal region of Brf1 has a role in DNA binding and Pol III recruitment [34], is homologous to the Pol II transcription factor TFIIB, and contains an N-terminal zinc ribbon domain and two cyclin domains. To investigate whether the altered BRF1 residues may be important for DNA binding, the mutated BRF1 residues were mapped on the known crystal structure of the homologous human TFIIB-TBP-DNA complex [82] (Figure 19).

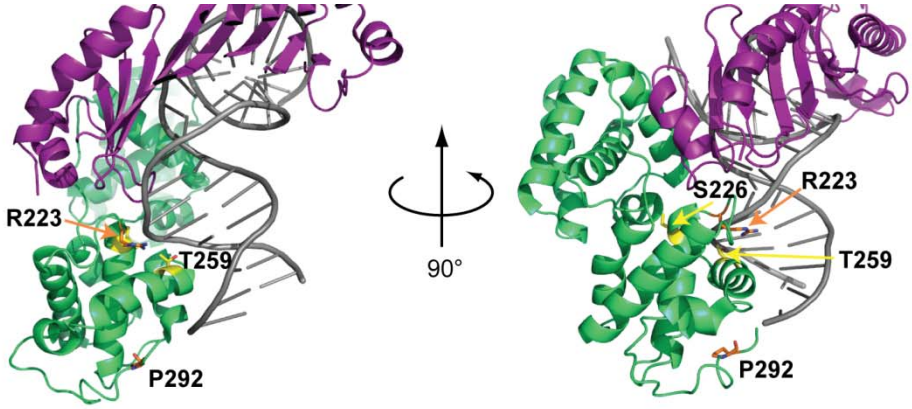

Figure 19 Structure of the human TFIIB (green) in complex with TBP (purple) and DNA (grey) solved by Tsai and Sigler [82].
pdb code 1C9B. hTFIIB is homologue to human Brf1, hence the mutations of Brf1 can be mapped on that structure (T259 and S226 in yellow and R223 and P292 in orange).

Residues Arg223 and Thr259 are conserved in human TFIIB and are predicted to contact DNA (Figure 19). Residues Ser226 and Pro292 are not conserved, and are predicted to be in the proximity of the DNA, although not in direct contact (Figure 19). These modelling results suggested that the observed *BRF1* mutations would affect Brf1 binding to DNA, impair TFIIIB-mediated Pol III recruitment, and reduce Pol III-mediated transcription.

3.2.5 *BRF1* mutations impair Brf1 recruitment to target promoters

To test whether the identified *BRF1* mutations influence Pol III recruitment to target gene promoters *in vivo*, chromatin immunoprecipitation (ChIP) with Tandem Affinity Purification (TAP) tagged versions of Brf1 variants was carried out in yeast.

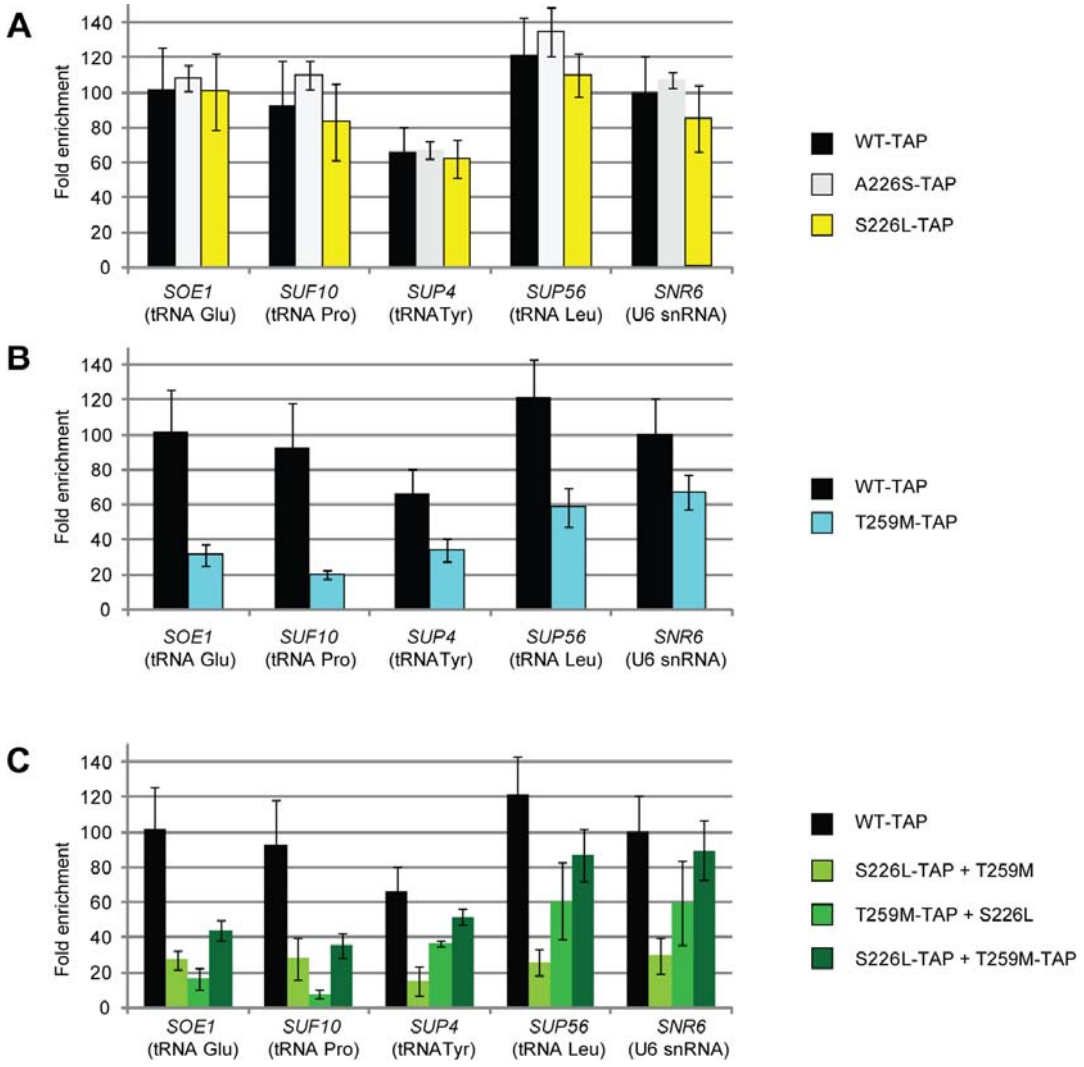

Figure 20 *BRF1* mutations impair Brf1 recruitment to target tRNA promoters.
Fold enrichments of ChIP experiments conducted with TAP-tagged Brf1 variants in yeast. Although the experiment was done in yeast, the residue numbers correspond to the numbers in the human protein for better understanding. **(A)** Mutation of the non-conserved amino acid S226 to leucine (yellow bar), which is an alanine in yeast (conversion to serine, white bar).shows no effect in fold enrichment compared to wild type (black bar). **(B)** Result for mutation of Thr259Met. That leads to a decreased fold enrichment on tRNA genes (blue bar). **(C)** Combination of the two variants resulted in a much lower occupancy of the Ser226Leu (light green bar) and Thr259Met (green bar) variant. Summing up both signals (dark green bar) still results in less occupancy of the two mutated Brf1 than the wild type Brf1 (black bar).

Consistent with the prediction, the Brf1 variant Thr259Met showed strongly decreased promoter occupancy at four tested tRNA loci and a significant decrease in occupancy of the U6 snRNA promoter (Figure 20B). The ChIP signals for TAP-tagged Ala226Ser and Ser226Leu variants were not different from wild-type Brf1 (Figure 20A), when solely present. However, the mutation Ser226Leu decreased Brf1

promoter occupancy in a Thr259Met background (Figure 20C). Although the two other Brf1 variants, Arg223Trp and Pro292His, were not tested due to their lethality, these results show that the mutations can affect Brf1 occupancy at different Pol III promoters *in vivo*.

Whether the Brf1 alterations additionally impair TFIIIB assembly through defects in Bdp1 or TBP binding remains to be investigated. Interestingly, poly-glutamine encoding trinucleotide repeat expansions in TBP cause autosomal dominant spinocerebellar ataxia, SCA17 [83, 84]. Nevertheless, the pathophysiological dissection of SCA17 is complicated since TBP is a component not only of TFIIIB, but also of the transcription factor complexes involved in Pol I and Pol II-mediated transcription.

3.2.6 *BRF1* mutations impair Pol III-dependent transcription

To test whether the *BRF1* mutations identified in the cerebellar-dental-skeletal syndrome cause a transcriptional defect, an established *in vitro* transcription assay was used [77]. This assay is based on a nuclear extract from a temperature-sensitive yeast strain carrying the Brf1 mutation Trp107Arg. Extracts from this strain are transcriptionally inactive, but addition of recombinant Brf1 protein restores activity on a template encoding the native *SUP4* promoter (Figure 21).

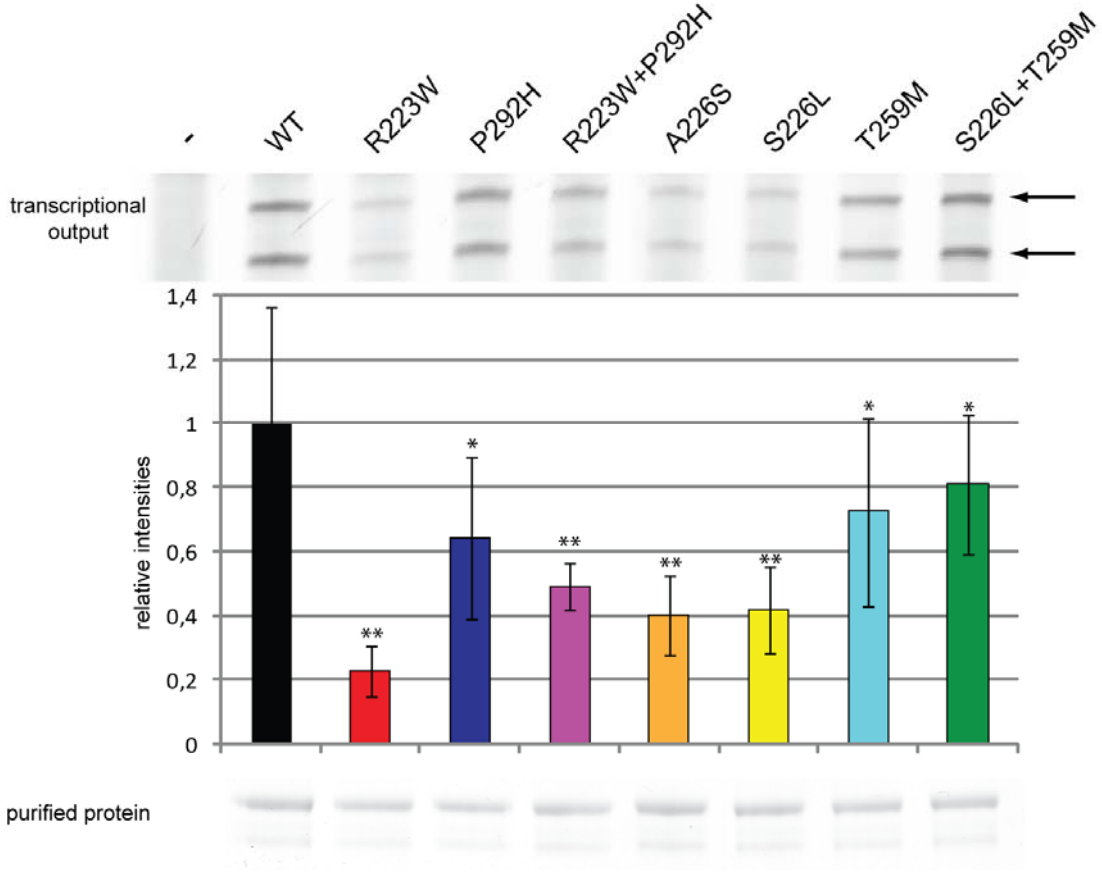

Figure 21 *BRF1* mutations impair Pol III-dependent *in vitro* transcription.
The upper panel shows a representative gel of the *in vitro* transcription reaction using a nuclear extract harboring a deficient Brf protein, hence being impaired in Pol III-dependent transcription. Addition of wild type (WT) Brf1 rescues activity whereas the different Brf variants and the combinations present in the children show a defect in transcription. The top arrow indicates the full-length product whereas the bottom arrow a processed product derived from the full-length transcript. The graph below depicts the average of eight independent, quantified experiments with standard deviations. Asterisks indicate significance determined via unpaired t-test, * significant at p≤0.05, ** significant at p≤0.001.The lower panel shows a twelve times excess of the Brf protein amount used for this assay separated on an SDS gel. Although the experiment was done in yeast the residue numbers correspond to the numbers in the human protein for better understanding.

When purified recombinant Brf1 variants were used at the same concentration as wild-type Brf1in this assay, far less transcription for the Brf1 variant Arg223Trp was observed (Figure 21). Milder, but highly reproducible defects were observed for the variants Thr259Met, Pro292His, Ala226Ser, and Ser226Leu (Figure 21). When two Brf1 variants were combined, simulating the situation present in the children, transcriptional defects were also observed in this assay (Figure 21). These results demonstrate that Brf1 mutations identified in patients can impair transcription in a well-defined *in vitro* transcription assay.

Taken together, the identified missense variants are recruited to a much lesser extent than the wild type protein to target genes and retain little transcriptional activity, suggesting they are hypomorphic. That is in line with the observation that complete inactivation of processes such as translation initiation or tRNA transcription is not compatible with life, suggesting that most disease-causing mutations are hypomorphs [81].

3.2.7 The pathomechanism for the neurodevelopmental syndrome is still unclear

A plausible pathomechanism underlying the cerebellar-dental-skeletal syndrome is a reduced steady-state level of tRNAs in the cell due to decreased Brf1 recruitment to Pol III promoters and thus impaired transcription by Pol III, which would be predicted to impair translation. This may explain the growth defect in yeast and short stature in the affected individuals. Whether the major drivers of the phenotype are tRNAs or other Pol III-dependent transcripts remains to be determined. Additionally, it is currently unknown why partial disruption of transcription by Pol III leads to organ and tissue specific phenotypes. There are approximately 450 nuclear encoded tRNA genes in the human genome for only 61 anticodons specified by the genetic code. It has been previously shown that up to 26% of tRNA genes are active in one cell type versus another and tRNA expression varies by as much as tenfold among human tissues [85, 86]. This suggests the existence of active cell and tissue-specific regulation of Pol III-dependent transcription [87]. Although the genome-wide architecture of Pol III-mediated transcription in neurons or specific brain regions is currently unknown, a pilot study revealed a high overall tRNA expression level in the brain [86]. This may at least partially explain the vulnerability of the brain or specific brain regions towards impaired transcription by Pol III or processing of tRNAs. This demonstrates the important role of Brf1 in transcription by Pol III and brain development.

Whether other affected tissues, such as the teeth, also show specific tRNA expression patterns remains to be investigated. Interestingly, dental anomalies such as delayed dentition or abnormally placed or shaped teeth are part of the Pol III-related leukodystrophies 4H syndrome and LO [71].

To sum up, our results define a rare syndrome with cerebellar hypoplasia, growth retardation and intellectual disability that is most likely caused by partial deficiency of the conserved Pol III transcription factor Brf1. This adds an example to the short list of genetic diseases caused by dysregulation of the Pol III machinery, most of which predominantly affect the central nervous system.

4 ADDITIONAL CONTRIBUTIONS

In the study of Treutlein et al. [39] single-molecule Förster resonance energy tranfer experiments and nano-positioning analysis were used to determine the 3D architecture of a minimal open complex. This consisted of a promoter DNA, including a TATA box and an 11 nt mismatch around the TSS, TBP, Pol II, TFIIB and TFIIF. They could show that in this minimal open complex the TBP-DNA complex resides above the Pol II cleft between the clamp and protrusion domains. Furthermore they suggested large overall structural changes during initiation-elongation transition due to the intrinsic flexibility of TFIIB.

For this study a fluorescently labeled TFIIB had to be used. To label TFIIB site-specific the Sfp phosphopantetheinyl transferase-catalyzed ybbR-tag technique was used [88]. Treutlein et al. introduced labels close to the cyclin domains, termed (C-term)- and (122)ybbR. To ensure their activity an *in vitro* transcription assay with a TFIIB temperature-sensitive nuclear extract was performed. This extract is inactive under normal reaction conditions. If 5 pmol recombinant TFIIB are supplied its full activity is recovered. For this study, the two differently labeled ybbR-TFIIB variants were added and their ability to recover the transcriptional activity was tested. Both variants support transcription on wild type level (Figure 22). Thus, the complexes used in this study contain a functional TFIIB.

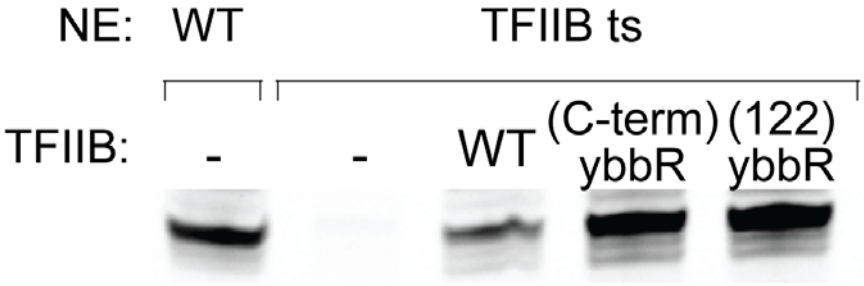

Figure 22 *In vitro* transcription of ybbR TFIIB variants. Treutlein et al., 2012, Mol Cell [39].
In vitro transcription assay testing *HIS4* promoter activity of TFIIB-ybbR mutants. Nuclear extract (NE) from wild type (WT) yeast strain shows full *in vitro* transcription activity (lane 1), whereas NE from a TFIIB temperature-sensitive yeast strain is essentially inactive (lane 2). Transcriptional activity can be restored when recombinant WT TFIIB and the mutants TFIIB-(C-term) ybbR and TFIIB-(122) ybbR are supplied (lane 3-5).

5 CONCLUSION AND OUTLOOK

In the first part of this study, a novel core promoter element in yeast, the GA element (GAE), was characterized. The GAE is found almost exclusively in TATA-less promoters. It is characterized by a guanine followed by a poly(dA)-stretch of at least four adenines. Similar to TATA box-containing promoters, the GAE-containing *TMT1* promoter is bound by TBP and supports formation of a TBP-TFIIB-DNA complex. Further *in vitro* and *in vivo* analysis showed a function of the GAE-containing region in TATA-less RNA polymerase II transcription. Moreover the GAE is mainly found in TFIID-dominated promoters. Together with the anti-correlation with canonical TATA boxes, this suggests a functional similarity of the TATA box and the GAE-containing region.

Several studies studies have linked poly(dA:dT) tracts to the function of defining the center of the nucleosome-free region, or in other words having a function in nucleosome positioning [47, 48, 59-61]. Since the GAE might also be seen as a poly(dA:dT) tract, it is important to note that the *in vitro* transcription experiments were done on a nucleosome-free template. Generating a poly(dA:dT) tract or mutation of the GAE-containing region resulted in decreased transcriptional activity. This demonstrates a direct role of the GAE-containing region in transcription, and suggests a function independent of nucleosome positioning.

This is the first time that a TATA-less promoter is used for *in vitro* transcriptional analysis and that an element with a function similar to the TATA box is described. In the future, the *TMT1* model promoter can be used for further *in vitro* studies of TATA-less transcription in comparison to TATA box-dependent transcription. Additionally, several aspects of GAE-dependent transcription remain to be elucidated.

First, it is still unclear where and in which orientation TBP and TFIIB bind to the GAE-containing region. This can be addressed by performing site-specific cleavage experiments or DNAse I footprinting [89]. For site specific cleavage, the protein is labeled at an (introduced) cysteine residue. If this residue is close to DNA in a protein-DNA complex, Cu^{2+}-ion addition leads to site-specific cleavage of the DNA. The DNA, labeled with radioactivity on one strand, can be separated on polyacrylamide gels revealing the cleavage site via its length. For DNAse I footprinting, a protein-DNA complex is formed and DNase I is added, which digests the uncovered DNA. Subse-

quently, the radioactivity-labeled fragments are separated on polyacrylamide gels where the region protected by the proteins can be determined.

After publication of this study, new ChIP-exo data were published where TATA-like elements were suggested [90]. Rhee and Pugh observed that 99% of the TATA-less TFIID-dominated promoters actually contain TATA-like elements differing only 1-2 nt from the TATA box consensus sequence. TFIIB is positioned around this element in a similar manner as around the TATA box [90]. Re-analysis of these published data by in-house bioinfomaticians revealed that the found GAE is positioned slightly upstream of those TATA-like elements. The implication of that positioning is unclear but the GAE might assist in PIC formation.

Second, the function of the guanine in the GAE is not clear. Since it does not impair TBP-TFIIB-DNA complex formation but does show a decreased functionality *in vitro* and *in vivo*, it must have a function independent from initial PIC assembly. It might be important for binding another protein which assists in orienting the PIC during the assembly or could support a distinct DNA conformation enhancing PIC assembly. In addition, it is not clear whether there is a GAE-specific binding protein or even proteins specific for GAE-containing promoters.

These points can be investigated by using the unbiased label-free mass spectrometry approach established in the PhD thesis of Martin Seizl [91]. In this approach, the GAE-containing and a GAE mutant or TATA box-containing promoter would be immobilized on beads and incubated with nuclear extract. The bound proteins would then be washed and after elution differently labeled, followed by mass spectrometry analysis. Comparison of two data sets would show the enrichment of a distinct set of proteins specific for the GAE-containing promoter. Interestingly, in a publication some years ago about the *ILV1* promoter region Moreira et al. described a protein called Datin. This binds to the poly(dA:dT) element of the *ILV1* promoter and regulates its activity [92]. Inspection of this poly(dA:dT) element revealed that it is actually a GAE. This raises the possibility that Datin might bind to GAEs. Further binding studies such as EMSAs and the above mentioned mass spectrometry approach would be needed to investigate this hypothesis.

As the consensus TATA box is only 8 nt in length, it would be interesting to determine the minimal GAE-containing sequence that is required to replace the TATA box and still shows transcriptional activity. To investigate this additional promoter con-

structs have to be designed and tested in *in vivo* reporter assays and *in vitro* transcription assays as described in this study.
Finally, another remaining question is the possibility of additional core promoter elements correlating with the GAE. Further bioinformatic analysis would be required to identify those elements.

In the second part of this study, four mutant variants of Brf1, a component of the general Pol III transcription factor TFIIIB, were characterized. TFIIIB is recruited by TFIIIC and recruits Pol III to promoters. It is composed of three proteins, namely TBP, Bdp1 and Brf1. Guntram Borck and colleagues from the University of Ulm identified biallelic point mutations in the human Brf1 in two pairs of siblings with cerebellar, skeletal and dental anomalies. Each pair of siblings showed the same pair of mutations, inherited in an autosomal-recessive manner.
The N-terminal half of Brf1 is homologous to the Pol II transcription factor TFIIB and all of the described mutations are predicted to lie within a domain interacting with DNA. This study could show that these mutations lead to severe growth defects, reduce Brf1 recruitment at tRNA promoters and impair *in vitro* transcription in the yeast *Saccharomyces cerevisiae*. These results indicate that hypomorphic *BRF1* mutations cause a neurodevelopmental syndrome, and that Brf1-mediated transcription by Pol III might be required for normal cerebellar and cognitive development. It is still unclear why these mutations lead to impaired transcription, hence, one of the main future goals would be to study the underlying mechanism.
One possible cause is a decreased affinity to DNA since in the hTFIIB homology structure two of the mutated residues are predicted to interact with DNA. Techniques to study DNA-binding, as EMSA or thermophoresis, would be an appropriate tool to investigate that hypothesis. Unfortunately, EMSAs are quite difficult to establish in this case and seem to depend on another Pol III transcription factor, TFIIIC [34, 93]. An approach developed in the group of Prof. Dr. Ulrike Gaul (Gene Center LMU) to test binding in solution, did not work due to aggregation. Hence no interaction between Brf1, TBP and DNA, respectively, was observed. Further optimization is needed to investigate these interactions.
One of the mutations (Thr259Met) shows only a weak effect in *in vitro* transcription (decrease to 70%) but a severe growth defect and decreased recruitment to tRNA promoters. In contrast, another mutation (Ser226Leu) affects transcription strongly

(decrease to 35%) but leads to no growth defect and change in recruitment. The former might have a regulatory role *in vivo* and the latter might be suppressed in the *in vivo* context. It might be possible to study these variants and their effects on transcription in a cell cycle dependent manner or upon a change in growth conditions. As reported by Fairley et al. Thr270 is phosphorylated during mitosis and represses Pol III-dependent transcription in human cells [94]. If this Thr is lost due to mutation, the repression is lost as well and transcription by Pol III is misregulated. Misregulation of Pol III-dependent transcription can also lead to cancer [67]. However, it is possible that the role of Thr259 in regulation is yeast-specific.

Interestingly, when these two mutant variants are introduced at the same time – as present in the affected children –yeast cells show impaired *in vitro* transcription, a growth defect and decreased recruitment of both Brf1 variants to tRNA promoters *in vivo*. Most likely, the simultaneous presence of the two mutations enhances the effect of the Ser226Leu mutation *in vivo*. In addition, Fairley et al reported that chromosome segregation is vulnerable to changes in Pol III-dependent transcription. Yeast cells harboring the mutations characterized in this study have the tendency to form aggregates without proper segregation of the cells. This might be another hint for implications of Pol III-dependent transcription in the cell cycle [94].

Additionally, it would be interesting to investigate the mutant variants not only with ChIP followed by qPCR but also genome-wide by ChIP-seq or RNA sequencing to verify the trends seen on single tRNA genes and investigate their (putative) effect on gene expression. Moreover, the effect of these mutations on Bdp1 and TBP or Pol III recruitment to tRNA promoters could be tested via ChIP to see whether Pol III initiation complex formation is disturbed. Complex formation could be investigated with purified factors *in vitro* as well. Finally, the mutations could be tested in a human system *in vivo* and *in vitro* to confirm the observations made in yeast. To study the impact of *BRF1* mutations on development a *Drosophila melanogaster* model system could be used.

6 MATERIALS AND METHODS

6.1 Materials

6.1.1 Strains

Table 4 ***Saccharomyces cerevisiae*** **strains used in this study.**

ID	Strain	Genotype	Source
	Wild type (BY4741)	MATa; his3Δ1; leu2Δ0, met15Δ0; ura3Δ0	Euroscarf (Y00000)
	BRF shuffle	MATα, ade2Δ::hisG, his3Δ200, leu2Δ0, lys2Δ0, met15Δ0, trp1Δ63, ura3Δ0, brf1Δ::HIS3/pSH524	Steve Hahn laboratory [77] (SHY285)

Table 5 ***Escherichia coli*** **strains used in this study.**

Strain	Genotype	Source
XL1-Blue	Rec1A; endA1; gyrA96; thi-1; hsdR17; supE44; relA1; lac[F'proAB lacIqZΔM15 tn10(Tet^r)]	Stratagene
BL21-Codon Plus (DE3) RIL	B; F^-; opmT; hsdS(r_s^- m_s^-); dcm+; Tetr; gal Δ(DE3); endA; Hte, [argU, ileY, leuW, Cam^r]	Stratagene

6.1.2 Plasmids

A detailed list with all plasmids generated is available through the Cramer laboratory.

Table 6 Plasmids used for protein expression in *E.coli*.

ID	Insert	Vector	Restriction site	Source
1990	*Sc* BRF1	pOPINB	*Kpn*I, *Hind*III	This study
1991	*Scbrf1 R218W*	pOPINB	*Kpn*I, *Hind*III	This study
1992	*Scbrf1 A221S*	pOPINB	*Kpn*I, *Hind*III	This study
1993	*Scbrf1 A221L*	pOPINB	*Kpn*I, *Hind*III	This study
1994	*Scbrf1 T254M*	pOPINB	*Kpn*I, *Hind*III	This study
1995	*Scbrf1 P288H*	pOPINB	*Kpn*I, *Hind*III	This study
1421	*Sc* Gcn4-6His	pET21a		Steve Hahn laboratory (pJF28)
1986	*Sc* TBPcore	pET		Lab stock
1985	*Sc* TFIIB FL	pOPINE	*Nco*I, *Pme*I	Lab stock

Table 7 Plasmids used for yeast complementation assay (BRF knock out strain) and ChIP assays.

ID	Insert	Vector	Restriction site	Source
1997	*Sc* BRF (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
1998	*Sc brf R218W*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
1999	*Sc brf A221S*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2000	*Sc brf A221L*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study

ID	Insert	Vector	Restriction site	Source
2001	*Sc brf T254M*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2002	*Sc brf P288H*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2003	*Sc* BRF-TAP (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2004	*Sc brf R218W*-TAP (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2005	*Sc brf A221S*-TAP (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2006	*Sc brf A221L*-TAP (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2007	*Sc brf T254M*-TAP (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2008	*Sc brf P288H*-TAP (-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS315	*Not*I, *Sac*I	This study
2009	*Sc brf R218W*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS314	*Not*I, *Sac*I	This study
2010	*Sc brf A221L*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS314	*Not*I, *Sac*I	This study
2011	*Sc brf T254M*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS314	*Not*I, *Sac*I	This study
2012	*Sc brf P288H*(-500bp upstream from ATG and +500bp downstream from STOP codon)	pRS314	*Not*I, *Sac*I	This study

Table 8 Plasmids used for *in vitro* transcription assays.

ID	Insert	Vector	Restriction site	Source
792	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) → HIS4 (TATA)	pBluescript KS$^+$	*Hind*III/*Bam*HI	Lab stock
1492	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) TATA mut to GGCCGGCC → HIS4 (GC-8)	pBluescript KS$^+$	*Hind*III/*Bam*HI	Lab stock
2013	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with 8bp TA-TAbox replaced by 12bp GAE of *TMT1* promoter → HIS4 (GAE-12)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2014	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with [-82] – [+24] replaced by [-188] – [+24] of TMT1 promoter sequence → HIS4 ($TMT1_{core}$)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2015	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with 8bp TA-TAbox -/+10 bp down/upstream replaced by 12bp GAE -/+ 10bp up/downstream of *TMT1* promoter → HIS4 (GAE-32)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2016	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with [-157] – [+24] replaced by [-139] – [+24] of *TMT1* promoter sequence → HIS4 ($TMT1_{down}$)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2017	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with [-133] – [-45] replaced by [-113] – [-31] of *TMT1* promoter sequence → HIS4 ($TMT1_{up}$)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study

ID	Insert	Vector	Restriction site	Source
2020	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) → TMT1 (GAE)	pBluescript KS$^+$	*Hind*III/*Bam*HI	Lab stock
2021	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) GAE mut 12bp → TMT1 (GC-12)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2022	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with 12bp GAE replaced by 8bp TATA box of the *HIS4* promoter → TMT1 (TATA)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2023	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with G to A in 12bp GAE → TMT1 (A-12)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2031	Native *SER3* promoter ([-230] – [+97] with respect to the A in the Inr region) → SER (TATA)	pBluescript KS$^+$	*Hind*III/*Bam*HI	Lab stock
2032	Native *SER3* promoter ([-230] – [+97] with respect to the A in the Inr region) TATAmut→ SER3 (GC-8)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2033	Native *SER3* promoter ([-230] – [+97] with respect to the A in the Inr region) with 8bp TATA-box replaced by 12bp GAE of *TMT1* promoter → SER3 (GAE)	pBluescript KS$^+$	*Hind*III/*Bam*HI	This study
2034	Native *SUP4* promoter ([-159] – [+244] with respect to TSS)	pBluescript KS	*Xho*I/*Bam*HI	This study

Table 9 Plasmids used for *in vivo* β-Galactosidase reporter assay.

ID	Insert	Vector	Restriction site	Source
2035	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) → HIS4 (TATA)	pRS315	*Hin*dIII/*Bam*HI	Lab stock
2036	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) TATA mut to GGCCGGCC → HIS4 (GC-8)	pRS315	*Hin*dIII/*Bam*HI	Lab stock
2037	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with 8bp TATA box replaced by 12bp GAE of *TMT1* promoter → HIS4 (GAE-12)	pRS315	*Hin*dIII/*Bam*HI	This study
2038	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with [-82] – [+24] replaced by [-188] – [+24] of *TMT1* promoter sequence → HIS4 ($TMT1_{core}$)	pRS315	*Hin*dIII/*Bam*HI	This study
2039	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with 8bp TATAbox -/+10 bp down/upstream replaced by 12bp GAE -/+ 10bp up/downstream of *TMT1* promoter → HIS4 (GAE-32)	pRS315	*Hin*dIII/*Bam*HI	This study
2040	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with [-157] – [+24] replaced by [-139] – [+24] of *TMT1* promoter sequence → HIS4 ($TMT1_{down}$)	pRS315	*Hin*dIII/*Bam*HI	This study
2041	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with [-133] – [-45] replaced by [-113] – [-31] of *TMT1* promoter sequence → HIS4 ($TMT1_{up}$)	pRS315	*Hin*dIII/*Bam*HI	This study

ID	Insert	Vector	Restriction site	Source
2042	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with GAE inserted at [-73]→ HIS4 (GAE-TATA)	pRS315	*Hin*dIII/*Bam*HI	This study
2043	native *HIS4* promoter ([-428] – [+24] with respect to the A in the start codon) with GAE inserted at [-46]→ HIS4 (TATA-GAE)	pRS315	*Hin*dIII/*Bam*HI	This study
2044	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) → TMT1 (GAE)	pRS315	*Hin*dIII/*Bam*HI	Lab stock
2045	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) GAE mut 12bp → TMT1 (GC-12)	pRS315	*Hin*dIII/*Bam*HI	This study
2046	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with 12bp GAE replaced by 8bp TATA box of the *HIS4* promoter → TMT1 (TATA)	pRS315	*Hin*dIII/*Bam*HI	This study
2047	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with G to A in 12bp GAE → TMT1 (A-12)	pRS315	*Hin*dIII/*Bam*HI	This study
2048	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with ∆GAE (5bp) → TMT1 (∆GAE)	pRS315	*Hin*dIII/*Bam*HI	This study
2049	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with 3nt downstream of GAE replaced by GCC → TMT1 (GC-3)	pRS315	*Hin*dIII/*Bam*HI	This study
2050	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with 4nt upstream of GAE replaced by GGCC → TMT1 (GC-4)	pRS315	*Hin*dIII/*Bam*HI	This study

ID	Insert	Vector	Restriction site	Source
2051	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with GAE replaced by GGCCG → TMT1 (GC-5)	pRS315	*Hin*dIII/*Bam*HI	This study
2052	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with 4nt up-stream/3nt downstream of GAE replaced by GGCC/ CGG → TMT1 (GC-7)	pRS315	*Hin*dIII/*Bam*HI	This study
2053	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with GAE and 3nt downstream replaced by GGCCGGCC → TMT1 (GC-8)	pRS315	*Hin*dIII/*Bam*HI	Lab stock
2054	native *TMT1* promoter ([-273] – [+24] with respect to the A in the start codon) with GAE and 4nt upstream replaced by GGCCGGCCG → TMT1 (GC-9)	pRS315	*Hin*dIII/*Bam*HI	This study
2055	native *SER3* promoter ([-230] – [+97] with respect to the A in the Inr region) → SER (TATA)	pRS315	*Hin*dIII/*Bam*HI	Lab stock
2056	native *SER3* promoter ([-230] – [+97] with respect to the A in the Inr region) TATAmut→ SER3 (GC-8)	pRS315	*Hin*dIII/*Bam*HI	This study
2057	native *SER3* promoter ([-230] – [+97] with respect to the A in the Inr region) with 8bp TATAbox replaced by 12bp GAE of TMT1 promoter → SER3 (GAE)	pRS315	*Hin*dIII/*Bam*HI	This study

6.1.3 Oligonucleotides

A detailed list with all oligonucleotides used during this study is available through the Cramer laboratory.

Table 10 List of Oligonucleotides used in this study.

Oligonucleotide	modification	Sequence 5' to 3'
EMSA *HIS4*(TATA) fw	5'-Cy5	ACAGTAGTATACTGTGTATATAATA-GATAT GGAACGTTAT
EMSA *HIS4*(TATA) rev		ATAACGTTCC ATATCTATTA TATACA-CAGT ATACTACTGT
EMSA *HIS4* (GC-8) fw	5'-Cy5	ACAGTAGTAT ACTGTGGGCC GGCCAGATAT GGAACGTTAT
EMSA *HIS4* (GC-8) rev		ATAACGTTCC ATATCTGGCC GGCCCACAGT ATACTACTGT
EMSA *HIS4*(GAE) fw	5'-Cy5	TAGTATACTGTGAAAGGAAAAGGAA-GATAT GGAACGTTAT
EMSA *HIS4*(GAE) rev		ATAACGTTCC ATATCTTCCTTTTC CTTTCA CAGTATACTA
EMSA *TMT1*(GAE) fw	5'-Cy5	ATTTTCATTTTTAAAGGAAAAG-GAATGTAA CTAATTTAGT
EMSA *TMT1*(GAE) rev		ACTAAATTAG TTACATTCCT TTTCCTTTAA AAATGAAAAT
EMSA *TMT1*(GC-12) fw	5'-Cy5	ATTTTCATTT TTGGCCGGCC GGCCATGTAA CTAATTTAGT
EMSA *TMT1*(GC-12) rev		ACTAAATTAG TTACATGGCC GGCCGGCCAA AAATGAAAAT
EMSA *TMT1*(TATA) fw	5'-Cy5	ATTTTCATTTTTTATATAATATGTAAC-TAA TTTAGT
EMSA *TMT1*(TATA) rev		ACTAAATTAG TTACATATTA TA-TAAAAAAT GAAAAT
EMSA *TMT1* (A-12) fw	5'-Cy5	ATTTTCATTT TTAAAAAAAA AAAAATGTAA CTAATTTAGT
EMSA *TMT1* (A-12) rev		ACTAAATTAG TTACATTTTT TTTTTTTTAA AAATGAAAAT

Oligonucleotide	modification	Sequence 5' to 3'
Pol II *in vitro* transcription	5'-Cy5	TTCACCAGTGAGACGGGCAAC
Pol III *in vitro* transcription	5'-Cy5	TCTCCCGGGGGCGAGTCGAACGCCC
SOE1 TSS fw		TTTGTAATGGAATTTTTCCAAC
SOE1 TSS rev		CGTGATGTGATAGCCGTTAC
SNR6 TSS fw		CGTCCACTATTTTCGGCTA
SNR6 TSS rev		GGTTACTTCGCGAACACAT
SUP4 TSS fw		TCTCTTCCATCCCTTAGCTT
SUP4 TSS rev		TAATTATCCGGTCCTTCCAA
SUP56 TSS fw		AAACATTGTGACCCAAATATCA
SUP56 TSS rev		AATCAGGCGCCTTAGACC
SUF10 TSS fw		TGATGTTGAGGTACCATATTTTG
SUF10 TSS rev		TCATACCTCTAGACCACACG
YER heteroch-romatin fw		TGCGTACAAAAAGTGTCAAGAGATT
YER heteroch-romatin rev		ATGCGCAAGAAGGTGCCTAT

6.1.4 Growth media and additives

Table 11 Composition of Growth media used in this study.

Growth medium	Compostion	Organism
LB	1% (w/v) trpytone; 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl; (+2% (w/v) agar for selective media plates)	*Escherichiacoli*
YPD	2% (w/v) peptone; 2% (w/v) gluxose; 1% (w/v) yeast extract; (+2% (w/v) agar for selective media plates)	*Saccharomyces cerevisiae*
Synthetic complete (SC)	0.67% (w/v) yeast nitrogen base; 0.06% (w/v) complete synthetic mix of amino acids, drop out as required; 2% (w/v) glucose; (+2% (w/v) agar for selective media plates)	*Saccharomyces cerevisiae*
5-FOA plates	SC; 0.01% (w/v) uracil; 0.2% (w/v) 5-FOA; 2% (w/v) agar	*Saccharomyces cerevisiae*

Table 12 Antibiotic concentration in LB media.

Antibiotic	Concentration
Ampicillin	100µg/ml
Chloramphenicol	50µg/ml
Kanamycin	30µg/ml

6.1.5 Buffers and solutions

Table 13 General buffers and solutions.

Buffer	Composition
TAE	40 mM tris acetate; 1 mM EDTA; pH 8.0$^{25°C}$
TBE	8.9 mM Tris/HCl; 8.9 mM boric acid, 2 mM EDTA, pH 8.0$^{25°C}$
TFB-1	30 mM KOAc; 50 mM $MnCl_2$; 100 mM RbCl; 10 mM $CaCl_2$; 15% (v/v) glycerol; pH 5.8$^{25°C}$
TFB-2	10 mM MOPS pH 7.0$^{25°C}$; 10 mM RbCl; 75mM CaCl2; 15% (v/v) glycerol
TELit	155 mM LiOAc; 10 mM Tris/HCl pH 8.0; 1 mM EDTA pH 8.0
LitSorb	18.2% (w/v) D-sorbitol in TELit pH 8.0
LitPEG	40% (w/v) PEG 3350 in TELit pH 8.0
MOPS running buffer	20 mM 3-(N-morpholino) propanesulfonic acid; 5 mM sodium acetate·3H_2O; 1 mM EDTA
5x SDS sample buffer	250 mM Tris/HCl pH 7.0$^{25°C}$; 50% (v/v) glycerol; 0.5% (w/v) bromophenol blue; 7.5% (w/v) SDS; 500 mM DTT
100x protease inhibitor cocktail	0.028 mg/ml leupeptin; 0.137 mg/ml pepstatin A: 0.017 mg/ml PMSF; 0.33 mg/ml benzamidine; in 100% ethanol p.a.

Table 14 Buffers used for Gcn4 purification.

Buffer	Composition
Lysis buffer	20 mM Tris/HCl pH 8.0$^{25°C}$, 500 mM NaCl, 5 mM DTT
Buffer A	20 mM HEPES pH 7.5$^{25°C}$, 10% glycerol, 1 mM EDTA, 2 mM DTT
Buffer B	20 mM HEPES pH 7.5$^{25°C}$, 150 mM potassium acetate, 10% (v/v) glycerol, 1 mM EDTA, 2 mM DTT

Table 15 Buffers used for TBP purification.

Buffer	Composition
Lysis buffer/buffer A	25 mM Tris/HCl pH $8.0^{25°C}$, 500 mM NaCl, 50 mM ammonium acetate, 10% glycerol
Buffer B	25 mM Tris/HCl pH $8.0^{25°C}$, 500 mM NaCl, 50 mM ammonium acetate, 10% glycerol; 250 mM imidazole
Buffer C	25 mM Tris/HCl pH $8.0^{25°C}$, 1 M NaCl, 50 mM ammonium acetate, 10% glycerol
Pol II buffer	5 mM HEPES pH $7.25^{20°C}$, 40 mM $(NH_4)_2SO_4$, 10µM $ZnCl_2$, 10mM DTT

Table 16 Buffers used for TFIIB purification.

Buffer	Composition
Lysis buffer	50 mM Tris/HCl pH $7.5^{25°C}$, 300 mM NaCl, 5 mM imidazole, 10 µM $ZnCl_2$, 2 mM DTT, 0.2% Tween-20
Wash buffer	50 mM Tris/HCl pH $7.5^{25°C}$, 300 mM NaCl, 10 mM imidazole, 10 µM $ZnCl_2$, 2 mM DTT,
Elution buffer	50 mM Tris/HCl pH $7.5^{25°C}$, 300 mM NaCl, 200 mM imidazole, 10 µM $ZnCl_2$, 2 mM DTT,
Buffer A	50 mM Tris/HCl pH $7.5^{6°C}$, 100 mM NaCl, 2 mM DTT
Buffer B	50 mM Tris/HCl pH $7.5^{6°C}$, 1 M NaCl, 2 mM DTT
Pol II buffer	5 mM HEPES pH $7.25^{20°C}$, 40mM $(NH_4)_2SO_4$, 10µM $ZnCl_2$, 10mM DTT

Table 17 Buffers used for Brf purification.

Buffer	Composition
Lysis buffer	25 mM Tris/HCl pH $7.5^{25°C}$; 200 mM KCl; 12.5 mM $MgCl_2$; 10% (v/v) glycerol; 1x PI
H.35 buffer	20 mM HEPES pH $8.0^{25°C}$; 350 mM KCl; 2 mM $MgCl_2$; 1% NaOH; 20% (v/v) glycerol; 0.1% (v/v) IGEPAL CA-630; 1x PI
G6 buffer	100 mM Tris/HCl pH $7.5^{4°C}$; 6 M GdmCl; 2 mM β-mercaptoethanol
Ni-NTA elution buffer	100 mM Tris/HCl pH $7.5^{4°C}$; 6 M GdmCl; 10 mM β-mercaptoethanol; 500 mM imidazole; 1xPI
Dialysis buffer	20 mM tris acetate (80% cation = pH $7.5^{4°C}$); 200 mM KCl; 2 mM $MgCl_2$; 20% (v/v) glycerol; 0.1% (v/v); IGEPAL CA-630; 1xPI

Table 18 Buffers used for electrophoretic mobility shift assay.

Buffer	Composition
Binding buffer	4% glycerol, 4 mM Tris-HCl pH $8.0^{23°C}$, 60 mM KCl, 5 mM $MgCl_2$, 100 µg/ml BSA, 0.1% Tween 20
Protein dilution buffer	20 mM Tris pH $7.9^{23°C}$, 150 mM KCl, 1 mM DTT, 10% glycerol, 50 µg/ml BSA

Table 19 Buffers used for yeast nuclear extract preparation.

Buffer	Composition
Resuspension buffer	50 mM Tris; 20 mM EDTA; 30 mM DTT; pH 7.5
YPDS	1% (w/v) yeast extract; 2% (w/v) bactopeptone; 2% (v/v) glucose; 1 M sorbitol
Buffer A	18% (w/v) polysucrose 400; 10 mM Tris pH $7.5^{25°C}$; 20 mM KOAc; 5 mM MgOAc; 1 mM EDTA pH $8.0^{25°C}$; 0.5 mM spermidine; 0.15 mM spermine; 3 mM DTT; 1xPI
Buffer B	100 mM Tris pH $7.9^{25°C}$; 50 mM KOAc; 10 mM $MgSO_4$; 20% glycerol; 2 mM EDTA
Buffer C	20 mM HEPES pH$7.6^{25°C}$; 10 mM $MgSO_4$; 1 mM EGTA pH 8.0; 20% glycerol
Buffer C +$NH_4(SO_4)_2$	20 mM HEPES pH $7.6^{25°C}$; 10 mM $MgSO_4$; 1 mM EGTA pH 8.0; 20% glycerol; 75 mM $NH_4(SO_4)_2$

Table 20 Buffer used for RNA polymerase II and III *in vitro* transcription assay.

Buffer	Composition
5x transcription buffer	500 mM KOAc, pH 7.6; 100 mM HEPES pH 7.6; 5 mM EDTA; 25 mM MgOAc
Phosphocreatine (64mg/ml)	128 mg phosphocreatine; 2 ml TE pH 7.5; Add 4 µl 1 M acetic acid, check pH with paper, should be ~7.5
HA + 1M KOAc	20 mM HEPES pH 7.9; 10% glycerol;1 mM EDTA;100 mM KOAc;1 mM DTT
5x primer annealing buffer	25 mM Tris pH 8.3; 375 mM KCl; 5 mM EDTA pH 8.0
5x synthesis buffer	250 mM Tris pH 8.3; 375 mM KCl; 22.5 mM $MgCl_2$; 75 mM DTT
Formamide sample buffer	80% formamide; 25 mM EDTA; 1.5% bromophenolblue

Table 21 Buffer used for chromatin immunoprecipitation.

Buffer	Composition
TBS	50 mM Tris/HCl pH $7.5^{4°C}$;150 mM NaCl
FA-lysis buffer 150	50 mM HEPES pH $7.5^{4°C}$; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 01% SDS; 150 mM NaCl; 1x PI
FA-lysis buffer 500	50 mM HEPES pH $7.5^{4°C}$; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 01% SDS; 500 mM NaCl
Wash buffer	10 mM Tris/HCl pH $8.0^{4°C}$; 0.25 M LiCl; 1 mM EDTA; 0.5% NP-40; 0.5% sodium deoxycholate
Elution buffer	50 mM Tris/HCl pH $7.5^{4°C}$; 10 mM EDTA; 1% SDS
PBS	137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; pH $7.4^{25°C*}$
TE	10 mM Tris/HCl pH $7.5^{4°C}$; 1 mM EDTA
LiCl wash buffer	100 mM Tris/HCl pH $7.5^{4°C}$, 500 mM LiCl, 1% NP40, 1% sodiumdeoxycholate

6.2 Methods

6.2.1 Molecular cloning

Preparation of chemically competent Escherichia coli *cells*

It is absolutely necessary to work sterile at all steps. *E.coli* XL-1 Blue or BL21(DE3), for plasmid preparation and protein overexpression, respectively, were grown in 200 ml LB medium at 37°C. For growth of BL21 (DE3) Chloramphenicol was added. After reaching an OD_{600} of 0.5 the cells were put on ice for 10 min. All wash and centrifugation steps were carried out at 4°C. The cells were centrifuged (4°C/ 3000 g/10 min) and washed with 50 ml TFB-1. After centrifugation (4°C/ 3000 g/10 min) the cell pellet was resuspended in 4 ml TFB-2. 50 µl aliquots were distributed in tubes on dry ice and afterwards stored at -80°C.

Preparation of chemically competent Saccharomyces cerevisiae *cells*

It is required to work sterile at all steps. A 50 ml culture was inoculated with an overnight culture at start OD_{600} of 0,2 (150 rpm, 30°C). The cells were harvested (5 min, 4,000 rpm, RT) and the supernatant discarded. The pellet was washed in 25 ml sterile H2O and 5 ml LitSorb. Finally the pellet was resuspended in 360 µl LitSorb and mixed with 40 µl salmon sperm DNA. The salmon sperm DNA was heated at 100°C for 10 min and cooled down on ice before use. 50 µl aliquots were prepared and used directly or stored at -80°C.

Polymerase chain reaction (PCR)

A PCR primer introducing restriction sites usually encodes a 5’ overhang GGGCCCGGG followed bythe restriction site and a sequence complementary to the target sequence ensures specific amplification. In addition to the polymerase and 0.2 mM dNTPs, $MgCl_2$ and DMSO could be added to increase product yield. Changing the buffers is another option for optimizing the PCR condition. *Taq* polymerase and *Phu* polymerase were used depending on the required application. Typically 5-10 ng of plasmid or 200 ng of yeast genomic DNA served as a template. Annealing temperature and elongation time were adjusted to the specific reactions. The biometra T3000 thermocycler was used.

To visualize the PCR products they were run on a 1% Agarose gel (w/v) containing 1:10 000 SYBR Safe DNA gel stain (Invitrogen)in 1xTAE buffer.
The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) or QIAquick Gel Extraction Kit (Qiagen).

Mutagenesis

To introduce mutations the overlap extension PCR was used [95]. Two complementary oligonucleotides harboring the mutation embedded in 20-25 nucleotides up- and downstream of the respective sequence were designed. First, the up- and downstream region of the mutated site, respectively, including each time the desired mutation was amplified in two independent PCRs. The PCR was carried out in the house-made Phu master mix containing buffer, dNTPs and nucleotides. PCR products were purified via gel extraction. Subsequently the PCR products were used as a template with the flanking primer. The product contains the full length product and the desired mutation.

Colony PCR in yeast

For colony PCRs of yeast a pinhead size amount of yeast cells was suspended in 100 µl 0.02 M NaOH. 50-100 µl soda lime glass beads (0.5 mm) were added. The sample was incubated for 10-15 min at 95°C vigorously shaking followed by a 5 min, full speed centrifugation step at room temperature. The supernatant can be used as a template for a PCR using the house-made Phu master mix.

Restriction digest, dephosphorylation and ligation

To perform the restriction digest, PCR products and vectors were usually incubated at 37°C at least one hour and maximum over night. Reaction buffers were chosen according to manufacturer's manual. PCR products were purified using the QIAquick PCR purification kit (Qiagen).To the vector sample 1 unit alkaline phosphatase (FastAP by Fermentas) was added according to the manufacturer's manual. Subsequently the restricted vector was purified using the QIAquick PCR purification kit (Qiagen).

To ligate the restricted vector and the PCR product they were mixed in a molar ratio of 1:5 with 10 units T4 Ligase as described in the manufacturer's manual. Samples were incubated at 16°C for at least 2 hours.

Transformation into Escherichia coli *and plasmid preparation*

To transform ligation reactions and plasmids into chemically competent *E.coli* cells 10 µl of ligation and 0.5 µl of purified plasmid, respectively, were added to a 50 µl cell aliquot. The cells were incubated on ice for 15 min followed by heat shock at 42°C for 45 seconds. The cells were placed on ice again. After addition of 250 µl LB medium the cells were incubated at 37°C for 45 min, shaking with 600 rpm. The cell suspension was spread on plates containing the respective antibiotics and incubated at 37°C. Plasmids were prepared from a 5 ml over night culture using the QIAprep Spin MiniPrep kit (Qiagen).
To verify new constructs test restriction digest and DNA sequencing was applied.

Transformation into Saccharomyces cerevisiae

2 µl of plasmid DNA and 360 µl of LitPEGwere added to 10 µl cellsand incubated for 30 min at RT. 8 µl DMSO were added before incubation for 15 min at 42°C. The cells were centrifuged (3 min, 2000 rpm, RT) and resuspended in 100 µl H2O. Cells were spread out on selection plates and incubated at 30°C or 24°C if the expected strains will be temperature sensitive.

Yeast cryo stocks

For yeast cryo stocks colonies from plates were resuspended in 30% sterile glycerol and frozen at -80°C. Do not flash-freeze in liquid nitrogen since this will lead to cell death.

6.2.2 General protein methods

Protein overexpression

Gcn4

Proteins were expressed in *E.coli* BL21-Codon plus (DE3) RIL cells. The expression plasmid (see Table 6) was transformed and cells were grown on Ampicillin and Chloramphenicol media plates. The 3 l main culture was inoculated at OD_{600} 0.1 with a preculture in LB medium containing Ampicillin and Chloramphenicol. The cells were grown at 37°C and 140 rpm shaking. Before protein expression cells were cooled down in cold room on ice. Protein expression was induced with 0.5 mM IPTG at an OD_{600} 0.7. Cells were grown over night at 18°C and harvested (20 min, 4000 rpm, 4°C). Pellet was washed with 40 ml LB medium and pelleted (10 min, 4000 rpm, 4°C). Pellet was flash-frozen in liquid nitrogen and stored at -80°C.

TBP

TBP core with an N-terminal His-tag (amino acids 61-240, a gift from Z. S. Juo) was transformed into Rosetta (DE3) pLysS cells and cells were grown on Ampicillin and Chloramphenicol plates. The 4 l main culture was inoculated at OD_{600} 0.1 with a preculture. The cells were grown at 37°C, 140 rpm. Protein expression was induced with 1 mM IPTG at OD_{600} 0.7 at 20°C. The cells were harvested after 18 h (20 min, 4 000 rpm). The pellet can be flash-frozen in liquid nitrogen and stored at -80°C.

TFIIB

TFIIB with an N-terminal His-tag on the pOPINE vector was transformed into Rosetta (DE3) pLysS cells and cells were grown on Ampicillin and Chloramphenicol plates. The 1 l main culture was inoculated at OD_{600} 0.1 with a preculture. The cells were grown at 37°C, 140 rpm. Protein expression was induced with 1 mM IPTG at OD_{600} 0.6 at 20°C. The cells were harvested after 18 h (20 min, 4 000 rpm). The pellet can be flash-frozen in liquid nitrogen and stored at -80°C.

Brf1

Proteins were expressed in *E.coli* BL21-Codon plus (DE3) RIL cells. The respective expression plasmids (see Table 6) were transformed and cells were grown on Kanamycin and Chloramphenicol media plates. The 2 l main culture was inoculated at

OD_{600} 0.1 with a preculture in LB medium containing Kanamycin and Chloramphenicol. Cells were grown at 37°C and 140 rpm shaking. Protein expression was induced with 1 mM IPTG at an OD_{600} 0.7. Cells were grown for further 3 h and harvested (20 min, 4000 rpm, 4°C). The pellet was washed with 40ml LB medium and centrifuged again (10 min, 4000 rpm, 4°C). The pellet was flash-frozen in liquid nitrogen and stored at -80°C.

Cell lysis

For cell disruption the cell pellets were thawed on ice and resuspended in 50 ml lysis buffer. Afterwards they were placed on ice and lysed by sonication (40 output value, 25% duty cycle, 15 min). Finally the insoluble fraction was separated from the soluble fraction by centrifugation (30 min, 15 000 rpm, 4°C).

Protein concentration determination

Nanodrop

Gcn4 protein concentration was calculated from absorbance at 280 nm using the specific absorption coefficient. This was calculated using ProtParam [96].Absorbance was measured with a NanoDrop spectrophotometer.

Bradford

For the Brf1 protein and its variants the concentration was determined with the help of the Bradford assay [97]. All samples were treated according to the manufacturer's manual (Bio-Rad).

For each batch of dye solution a separate standard curve was measured with bovine serum albumin (Roth).

SDS-Polyacrylamide gel electorphoresis (SDS-PAGE)

Proteins were analyzed on 4-12% gradient pre-casted NuPAGE Novex Bis-Trisminigels (Invitrogen) in a Bio-Rad gel system. Gels were run in MOPS buffer for 45 min at 200V. SDS buffer [98]was added and samples were boiled for 2 min at 95°C before loading on the gel. Gels were stained in InstantBlue (expedion).

Mass spectrometry

To identify purified proteins and their potential impurities protein samples were separated by SDS-PAGE. The bands of interest were excised and sent to the protein analysis core facility (Adolf-Butenandt-Institut, LMU) for analysis.

6.2.3 Recombinant protein purification

Gcn4 purification

For composition of buffers please see Table 14.

After cell lysis, the lysate was cleared by centrifugation (30 min, 15 000 rpm, 4°C). The supernatant was loaded twice onto a 2 ml Ni-NTA column (Qiagen) equilibrated with lysis buffer. The column was washed three times with 10 column volumes (CV) of lysis buffer containing 20 mM imidazole. Bound protein was eluted with 10 CV of lysis buffer containing 500 mM imidazole. Protein was further purified by cation exchange chromatography using a HiTrap SP column (GE Healthcare) equilibrated with buffer A. The protein was eluted with a linear gradient of 10 CVs from 0 to 1 M NaCl in buffer A. After concentration, the sample was applied to a Superdex 200 size exclusion column (Amersham) equilibrated with buffer B. The sample was concentrated to approximately 0.5 mg/ml, flash-frozen in small aliquots in liquid nitrogen, and stored at -80°C. All buffers contained protease inhibitor cocktail.

TBP purification

For composition of buffer please see of buffer please see Table 15.

After cell lysis the sample was cleared by centrifugation (30 min, 15 000 rpm, 4°C). The supernatant was loaded onto 2 ml Ni-NTA bead resin (Qiagen) and washed with 7.5 CV of buffer A. The proteins were eluted with 3 CV buffer B and the conductivity was adjusted to the conductivity of buffer A. The sample was loaded onto a 5 ml Heparin column and wash with buffer A until it reaches the baseline. The proteins were eluted in 1 ml fractions over a 5 CV gradient from buffer A to buffer C. The peak fraction contains TBP which is pooled and applied to a Superdex 75 10/300 GL size exclusion column. The column was run in Pol II buffer. The TBP-containing fractions were combined and flash-frozen in liquid nitrogen. TBP can be stored at -80°C.

TFIIB purification

For the composition of buffers please refer to Table 16.

The cell pellet was resuspended in 30 ml lysis buffer and lysed by sonication. The supernatant was centrifuged twice for 30 min, 15 000 rpm, 4°C and bound in batch to 2 ml Ni-NTA bead volume (Qiagen) for 20 min at 4°C. The beads were washed with 10 CV wash buffer on a gravity flow column. The protein was eluted with 3 CV elution buffer. Before loading the protein sample on a MonoS 10/100 GL column its conductivity was adjusted to buffer A and it was filtered. The proteins were eluted in 1 ml fractions over a 15 CV gradient from 0 to 100% buffer B. The peak fraction containing TFIIB was pooled and concentrated to a volume of 700 µl for loading on the size exclusion column Superdex 75 10/300 GL. The protein sample was separated over 1.5 CV in Pol II buffer. The peak fraction containing TFIIB was collected, concentration determined and samples were frozen in liquid nitrogen.

Brf purification

For composition of buffers please see of buffers please see Table 17.

The cells were thawed on ice and resuspended in 50 ml lysis buffer. Subsequently 0.8 mg lysozyme (Roth) per ml lysate was added and incubated rotating in the cold room. After 10 min 0.2% IGEPAL CA-630 (MP Biomedicals) was added for further 5 min. For cell disruption lysate was transferred into a glas beaker and sonicated for 15 min, 25% duty cycle and 40 output value. Afterwards the lysate was centrifuged (30 min, 17 500 rpm, 4°C) and the supernatant removed. The pellet contained Brf1 in inclusion bodies. Upper layer of white and grey pellet was removed and the remaining parts were washed twice in H.35 buffer. Inclusion bodies were extracted with 5 ml G6 buffer and rotation for 1 h at 4°C. To remove insoluble parts the sample was centrifuged (15 min, 15 000 rpm, 4°C). The supernatant was bound in batch to 2.5 ml Ni-NTA bead volume in G6 buffer over night at 4°C. Beads were washed with 12.5 ml G6 buffer and protein eluted in batch with three times 2.5 ml Ni-NTA elution buffer. To the combined eluate $ZnSO_4$ and DTT were added to final concentrations of 10 µM and 5 mM respectively. Finally the denatured Brf1 was refolded via rapid dilution and dialysis. For rapid dilution 100 µl of the eluate was placed in the lid of a 1.5 ml Eppendorf tube containing 1 ml dialysis buffer. The lid was closed and the tube flipped around quickly. For every tube this could be repeated twice. After rapid dilution the

protein was dialyzed in dialysis buffer followed by centrifugation (15 min, 15 000 rpm, 4°C) to remove aggregated proteins. Since concentrating the protein was not possible aliquots were directly frozen in liquid nitrogen and stored at -80°C. Usually the purification resulted in 30 ml of 0.4 – 0.8 mg/ml protein solution.

6.2.4 Biochemical assays

Nuclear extract preparation

Nuclear extracts were prepared from 3 l of BY4741 wild type yeast culture or SHY285, a Brf1 knock-out strain containing a Brf1 W107R mutation on a plasmid as described [99].

Brf1 W107R yeast strain could be grown at 30°C. Composition of buffers can be found in can be found in Table 19.

Yeast cells were grown to an OD_{600} 3-5 and harvested (10 min, 4 000 rpm, 18°C). The supernatant was discarded and the pellet resuspended in 35 ml resuspension buffer. The cell suspension was incubated for 15 min in a 30°C water bath and pelleted again (10 min, 4 000 rpm, 18°C). Pellets were resuspended in 20 ml YPDS and 3 ml of 2 M sorbitol was added. Cell wall disruption was achieved by adding 3 ml resuspension buffer containing 18 mg Zymolyase, 1xPI and 1xPMSF and incubating for 30 min in a 30°C water bath. Progress of the cell wall disruption was followed by measuring OD_{600}. The value should decrease by 70 – 80%. 100 ml YPDS were added and spheroblasts pelleted for 10 min, 4 000 rpm, 18°C. Cells were resuspended in 250 ml YPDS and again incubated for 30 min in a 30°C water bath. From now on all following steps were carried out at 4°C. After centrifugation (10 min, 4 000 rpm, 18°C) cells were washed twice in 200 ml YPDS and once in 250 ml 1 M Sorbitol. After the final wash cells were resuspended in 100 ml buffer A. The cell membrane was disrupted by passing the suspension three times through a pre-chilled Dounce glass homogenizer with a narrow pestle. The crude nuclei were isolated by centrifugation (8 min, 5 000 rpm, 4°C) in GSA bottles. This step was repeated twice and each time the pellet was discarded. Supernatant was transferred to SS34 tubes and the nuclei were pelleted (30 min, 13 000 rpm, 4°C). The nuclei were resuspended in 15 ml buffer B and washed once with 15 ml buffer B. The nuclei can be flash-frozen in liquid nitrogen at that point and stored at -80°C.

The frozen nuclei were thawed in the cold room on a spinning wheel. To disrupt the nuclear membrane 3 M ammonium sulfate solution was added to a final concentration of 0.5 M. Samples were incubated for 30 min in the cold room on a spinning wheel. After centrifugation (90 min, 28 000 rpm, 4°C – SW28 tubes) all proteins in the supernatant were precipitated by addition of 0.35 g solid ammonium sulfate per ml supernatant. Again the samples were incubated for 30 min in the cold room. To pellet the precipitated proteins the solution was centrifuged in SW28 tubes again (20 min, 28 000 rpm, 4°C). The supernatant was removed and samples re-spun (5 min, 10 000 rpm, 4°C). The pellet contained all nuclear proteins and was resuspended in a maximum of 1.5 ml buffer C. The nuclear extract was dialyzed over 4.5 hours in 500 ml buffer C + $(NH_4)_2SO_4$ with buffer exchange every 1.5 hours. Afterwards the nuclear extract was aliquoted and flash-frozen in liquid nitrogen. Protein concentration was determined after first freeze and appropriate dilution with 0.1% SDS with Bradford.

RNA polymerase II in vitro *transcription*

In vitro transcription and analysis by primer extension were performed as described [99]. Template plasmids were generated by inserting the respective promoter sequence as described above in pBluescript KS+ with *Hind*III and *Bam*HI (Table 8). Primer extension for all constructs was done using the same 5'-Cy5-labeled oligonucleotide (Table 10). The composition of the buffers can be found inTable 20. For the *in vitro* transcription reaction 1x transcription buffer, 2.5 mM DTT, 150 ng template, 192 µg phosphocreatine, 0.2 µg creatine phosphokinase, 10 U RiboLock RNase inhibitor (Fermentas) and 200 ng recombinant full-length Gcn4 were mixed. Gcn4 was allowed to bind to the template for 10 min at 18°C. Afterwards 200 µg yeast nuclear extract wild type were added and transcription initiated by addition of 0.1 mM NTPs. The total reaction volume was 25 µl. Transcription reaction was stopped by RNA isolation using the RNeasy MinElute kit (Qiagen) according to the manufacturer's manual. For primer annealing 1x annealing buffer and 0.125 pmol primer were added to the isolated RNA (total volume: 20 µl). The samples were boiled for 2 min at 95°C and incubated for 45 min at 48°C. Before primer extension 2µg Actinomycin D were added, followed by 1x synthesis buffer, 0.1 mM dNTPs and 0.25 U MuLV reverse transcriptase (Roche) resulting in a volume of 60 µl. Primer were extended for 30 min

at 48°C. The resulting cDNA was isolated via ethanol precipitation. The dried pellet was resuspended in 4 µl 0.04 mg/ml RNase A and 4 µl formamide sample buffer. Before loading samples were boiled for 2 min at 95°C and directly put on ice.
Samples were run on a 7 M urea, 8% polyacrylamide (35:1) gel in 1x TBE for 45 min, 180 V. The gel was pre-run for 5 to 10 min and the pockets were rinsed before loading.
Gels were analyzed with a typhoon scanner FLA9400 and ImageQuant Software (GE Healthcare).

RNA polymerase III in vitro *transcription*

The *in vitro* transcription for RNA polymerase III was basically done as described for RNA polymerase II. The template contained the *SUP4* gene from [-159] – [+244] with respect to the transcription start site of the tRNA. *SUP4* encodes for one tyrosine tRNA. The transcription reaction contained 1x transcription buffer, 2.5 mM DTT, 150 ng template, 192 µg phosphocreatine, 0.2 µg creatine phosphokinase, 10 U RiboLock RNase inhibitor (Fermentas), 50µg temperature-sensitive nuclear extract - harboring a temperature-sensitive W107R mutation in Brf -, 40 µM α-amanitin and 100 ng of recombinant protein where indicated. Proteins were added to the transcription reaction mix for 5 min before addition of NTPs. The transcription reaction was carried out at 30°C. The primer was annealed at 60°C. All other following steps were performed as described above.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described [100] with minor modifications. Templates were generated by annealing the complimentary PAGE-purified oligonucleotides (coding strand was 5'-Cy5-labeled, Table 10). The buffer composition can be found in Table 18. The binding reaction contained 3 nM of 40 bp 5'-Cy-5-labeled dsDNA, 300 nM TBP_{core} and 3 µM TFIIB, 1 mM DTT, 50 µg/ml Heparin in 1x binding buffer in a total reaction volume of 20 µl. Recombinant TBP_{core} and TFIIB were expressed in *E.coli* and purified as described [101]. Proteins were diluted in dilution buffer. Samples were incubated for 30 min at 18°C and loaded on a 6% native polyacrylamide gel (acrylamide:bisacrylamide 60:1, 190 mM glycine, 25 mM Tris pH $8.3^{23°C}$with acetic

acid, 0.5 mM DTT, 0.1% APS, 1% TEMED). The gel was pre-run for 5 min and then run at 160 V for 15 min at room temperature in 190 mM glycine, 25 mM Tris pH $8.3^{23°C}$. The gels were analyzed with a typhoon scanner FLA9400 and ImageQuant Software (GE Healthcare).

In vivo *reporter assay in yeast*

The native promoter sequences of *HIS4* (428 bp upstream to 24 bp downstream of the start codon) and *TMT1* (273 bp upstream to 24 bp downstream of the start codon) and their mutant variants were cloned between *Hind*III/*Bam*HI into a pRS315 plasmid a pRS315 plasmid (see Table 9) with the lacZ gene inserted between *Not*I/*Sal*I. The reporter plasmids were transformed into BY4741 wild type yeast cells and grown in biological duplicates in SD (–leu) medium to an OD_{600} of 0.5-1.0. β-galactosidase levels were determined using the Yeast β-galactosidase Assay Kit according to the manufacturer's manual (Thermo Scientific, #75768).

Chromatin immunoprecipitation

For compositions of buffer please refer to Table 21. Experiments were basically done as described [102] with minor modifications.

For the ChIP assay TAP-tagged Brf1 and its mutant variants were introduced in the vector pRS315 which contains Brf expressed under the control of its own promoter. For combinations of the mutations S226L and T259M were cloned into pRS314 vector and co-transformed with the TAP-tagged Brf variants T259M-TAP and S226L-TAP in the pRS315 vector, respectively.

For duplicates two colonies from one plate were grown to an OD_{600} 0.8 in 50 ml. 1 ml 37% formaldehyde (Sigmal-Aldrich) was added and the cell suspension shook for 20 min at 20°C. To stop cross-linking 5 ml 3 M glycine (pre-warmed to 30°C) were added and the cell suspension shook for further 5 min at 20°C. The cells were harvested (5 min, 4 000 rpm; 4°C) and pellets washed with 20 ml ice-cold TBS, 2 ml ice-cold FA-lysis buffer 150 and 1 ml ice-cold FA-lysis buffer 150. The supernatant was discarded and the pellet flash-frozen in liquid nitrogen. The pellets can be stored at -80°C. For further treatment the cells were thawed on ice and resuspended in ice-cold FA-lysis buffer 150. To 1 ml cooled down Zirconia beads (0.5 mm diameter, BioSpec

products) in 2 ml DNase free tubes 1 ml cell suspension was added. The cells were lysed by bead beating (Retsch) for 30 min, 30/sec at 4°C in the cold room. The solution containing the chromatin was separated from the beads by punching a hole in the bottom and top of the tube and centrifuging (1 min, 1 000 rpm, 4°C) into a 15 ml Bioruptor tube. 200 µl ice-cold FA-lysis buffer 150 were added. The chromatin was fragmented by sonication for 35 cycles, 30 seconds on/off, high intensity in the Biorupter (daigenode). Afterwards the samples were centrifuged for 10 min, 13 000 rpm, 4°C and the supernatant was transferred into a new DNase free tube and centrifuged for a second time (30 min, 13 000 rpm, 4°C). The chromatin concentration was measured with Nanodrop. 100 µl of chromatin solution can be used for fragment size control on a 1% agarose gel. 30 µl of the chromatin solution were transferred to a 0.5 ml low binding tube and labeled as input. 670 µl chromatin solution were added to 100 µl magnetic beads with coupled anti-rabbit IgG and labeled as IP (immunoprecipitation).

Magnetic beads were prepared as follows. If the IgG was frozen it needs to be centrifuged (20 min, 15 000 rpm, 4°C) and the concentration newly determined. 18 µl of Dynabeads Protein G (invitrogen) per IP were washed four times with 500 µl PBS containing 5 mg/ml BSA. Beads for more than one IP can be prepared together. Finally beads were resuspended in a total volume of 500 µl PBS containing 5 mg/ml BSA and 82.5 µg antibody per 100 µl beads. The antibody was allowed to bind for 30 min at 4°C on a rotating wheel. The coupled antibody was washed three times with 500 µl PBS containing 5 mg/ml BSA and resuspended in 109 µl PBS containing 5 mg/ml BSA per 18 µl beads.

The IP (670 µl chromatin solution plus 100 µl magnetic beads with coupled antibody) was carried out for 1 hour at 4°C on a rotating wheel. The beads were washed five times with 1 ml LICl wash buffer and once with 1 ml TE. Every time they were allowed to mix with the buffer for 3 min on a rotating wheel. Finally the beads were resuspended in 120 µl ChIP elution buffer, vortexed and incubated for 5 min at 95°C. The supernatant was transferred to a 0.5 ml low binding tube labeled IP.

Cross-links of IP samples were reversed by addition of 80 µl TE and 20 µl Proteinase K (20 mg/ml, Sigma). To ‘input’ samples 100 µl ChIP elution buffer, 60 µl TE and 20 µl Proeinase K were added. Both samples were incubated for 2 hours at 37°C and over night at 65°C.

The DNA was purified using the Qiaquick PCR Purification Kit (Qiagen) with the exception that DNA was loaded with 3 000 rpm for 1 min onto the columns. The DNA was eluted with 50 µl H_2O.

For analysis of chromatin fragments quantitative real time PCR (qPCR) was applied. A typical qPCR reaction mix contained 10 µl SensiFast Sybr NO-ROX Kit (Bioline), 0.8 µl forward primer (10 pmol/µl), 0.8 µl reverse primer (10 pmol/µl) and 7.4 µl H_2O. The reaction was performed on a CFX96 Real-Time System (Bio-Rad) using a 3 min denaturing step at 95°C followed by 49 cycles of 30 seconds 95°C, 30 seconds 61°C and 15 seconds at 72°C. The threshold cycle values (Ct) were determined using the Ct determination mode 'regression' of Bio-Rad CFX Manager software package (version 3). The fold enrichment over a heterochromatic control region was determined and calculated as described[103].

Yeast complementation assay for BRF *mutations*

To introduce Brf1 mutations in yeast the shuffle vector system developed by Sikorski and Hieter was used [104]. Brf1 mutations were introduced in the vector pRS315, which contains yeast Brf1 expressed under the control of its own promoter (see Table 7). Mutations were introduced using site-directed mutagenesis and confirmed by DNA sequencing. The identified mutations from humans (h) were transferred to yeast (y). The corresponding numbers are as follows: hR223 = yR218, hS226 = yA221, hT259 = yT254, hP292 = yP288. For combinations of the mutations Brf1 variants T259M and S226L were cloned into pRS314 vector and co-transformed with the S226L and T259M mutation in the pRS315 vector, respectively. Plasmids were transformed into the yeast strain SHY285 and the wild type *BRF1* gene was replaced with the mutant gene by plasmid shuffle at 30°C. Cells were spread on selective media and restreaked twice on selective media plates containing 5-FOA. Cells were tested for growth phenotypes on synthetic complete glucose plates (-leucine and –leucine/tryptophane for combinations) at 30°C. For spot dilutions an equal amount of yeast cells was tenfold diluted in a serial dilution and spotted on YPD plates. Subsequently plates were incubated at 30°C.

7 REFERENCES

1. Crick, F., *Central Dogma of Molecular Biology.* Nature, 1970. **227**(5258): p. 561-563.

2. Cramer, P., *Common structural features of nucleic acid polymerases.* Bioessays, 2002. **24**(8): p. 724-9.

3. Ringel, R., et al., *Structure of human mitochondrial RNA polymerase.* Nature, 2011. **478**(7368): p. 269-273.

4. Cramer, P., *Multisubunit RNA polymerases.* Current Opinion in Structural Biology, 2002. **12**(1): p. 89-97.

5. Ruet, A., Sentenac, A., and Fromageot, P., *A Specific Assay for Yeast RNA Polymerases in Crude Cell Extracts.* European Journal of Biochemistry, 1978. **90**: p. 325-330.

6. Haag, J.R. and Pikaard, C.S., *Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing.* Nature Reviews Molecular Cell Biology, 2011. **12**(8): p. 483-492.

7. Geiduschek, E.P. and Ouhammouch, M., *Archaeal transcription and its regulators.* Molecular Microbiology, 2005. **56**(6): p. 1397-1407.

8. Saecker, R.M., Record, M.T., and Dehaseth, P.L., *Mechanism of Bacterial Transcription Initiation: RNA Polymerase - Promoter Binding, Isomerization to Initiation-Competent Open Complexes, and Initiation of RNA Synthesis.* Journal of Molecular Biology, 2011. **412**(5): p. 754-771.

9. Roeder, R.G., *Nuclear RNA polymerases: Role of general initiation factors and cofactors in eukaryotic transcription*, in *Methods in Enzymology; RNA polymerase and associated factors, Part A*, S. Adhya, Editor 1996. p. 165-171.

10. Cormack, B.P. and Struhl, K., *The TATA-binding protein is required for transcription by all 3 nuclear-RNA polymerases in yeast-cells.* Cell, 1992. **69**(4): p. 685-696.

11. Vannini, A. and Cramer, P., *Conservation between the RNA Polymerase I, II, and III Transcription Initiation Machineries.* Molecular cell, 2012. **45**(4): p. 439-446.

12. Moss, T., et al., *A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis.* Cellular and Molecular Life Sciences, 2007. **64**(1): p. 29-49.

13. Thomas, M.C. and Chiang, C.M., *The general transcription machinery and general cofactors.* Critical Reviews in Biochemistry and Molecular Biology, 2006. **41**(3): p. 105-178.

14. Basehoar, A.D., Zanton, S.J., and Pugh, B.F., *Identification and distinct regulation of yeast TATA box-containing genes.* Cell, 2004. **116**(5): p. 699-709.

15. Juven-Gershon, T., et al., *The RNA polymerase II core promoter - the gateway to transcription.* Current opinion in cell biology, 2008. **20**(3): p. 253-259.

16. Shandilya, J. and Roberts, S.G.E., *The transcription cycle in eukaryotes: From productive initiation to RNA polymerase II recycling.* Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms, 2012. **1819**(5): p. 391-400.

17. Nechaev, S. and Adelman, K., *Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation.* Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 2011. **1809**(1): p. 34-45.

18. Koleske, A.J. and Young, R.A., *An RNA polymerase-II holoenzyme responsive to activators.* Nature, 1994. **368**(6470): p. 466-469.

19. Ranish, J.A., Yudkovsky, N., and Hahn, S., *Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB.* Genes & Development, 1999. **13**(1): p. 49-63.

20. Nikolov, D.B., et al., *Crystal structure of a TFIIB-TBP-TATA-element ternary complex.* Nature, 1995. **377**(6545): p. 119-28.

21. Kohler, A. and Hurt, E., *Exporting RNA from the nucleus to the cytoplasm.* Nature reviews. Molecular cell biology, 2007. **8**(10): p. 761-773.

22. Bywater, M.J., et al., *Dysregulation of the basal RNA polymerase transcription apparatus in cancer.* Nature Reviews Cancer, 2013. **13**(5): p. 299-314.

23. Egloff, S., Dienstbier, M., and Murphy, S., *Updating the RNA polymerase CTD code: adding gene-specific layers.* Trends in Genetics, 2012. **28**(7): p. 333-341.

24. Mayer, A., et al., *Uniform transitions of the general RNA polymerase II transcription complex.* Nature structural & molecular biology, 2010. **17**(10): p. 1272-1278.

25. Schramm, L. and Hernandez, N., *Recruitment of RNA polymerase III to its target promoters.* Genes & development, 2002. **16**(20): p. 2593-620.

26. Orioli, A., et al., *RNA polymerase III transcription control elements: Themes and variations.* Gene, 2012. **493**(2): p. 185-194.

27. Geiduschek, E.P. and Kassavetis, G.A., *The RNA polymerase III transcription apparatus.* Journal of Molecular Biology, 2001. **310**(1): p. 1-26.

28. Schramm, L., et al., *Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters.* Genes & development, 2000. **14**(20): p. 2650-2663.

29. Arimbasseri, A.G. and Maraia, R.J., *Distinguishing core and holoenzyme mechanisms of transcription termination by RNA polymerase III.* Molecular and Cellular Biology, 2013.

30. Lee, Tong i. and Young, Richard a., *Transcriptional Regulation and Its Misregulation in Disease.* Cell, 2013. **152**(6): p. 1237-1251.

31. Fuda, N.J., Ardehali, M.B., and Lis, J.T., *Defining mechanisms that regulate RNA polymerase II transcription in vivo.* Nature, 2009. **461**(7261): p. 186-192.

32. Dikstein, R., *The unexpected traits associated with core promoter elements.* Transcription, 2011. **2**(5): p. 201-6.

33. Wang, Y., Fairley, J.A., and Roberts, S.G., *Phosphorylation of TFIIB links transcription initiation and termination.* Current biology : CB, 2010. **20**(6): p. 548-53.

34. Kassavetis, G.A., et al., *Functional and Structural Organization of Brf, the TFIIB-Related Component of the RNA Polymerase III Transcription Initiation Complex.* Molecular and Cellular Biology, 1998. **18**(9): p. 5587-5599.

35. Yang, C.H., et al., *Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters.* Gene, 2007. **389**(1): p. 52-65.

36. Huisinga, K.L. and Pugh, B.F., *A genome-wide housekeeping role for TFIID and a highly regulated stress-related role for SAGA in Saccharomyces cerevisiae.* Molecular cell, 2004. **13**(4): p. 573-585.

37. Burley, S.K., *The TATA box binding protein.* Current Opinion in Structural Biology, 1996. **6**(1): p. 69-75.

38. Pugh, B.F. and Tjian, R., *Transcription from a TATA-less promoter requires a multisubunit TFIID complex.* Genes & Development, 1991. **5**(11): p. 1935-1945.

39. Treutlein, B., et al., *Dynamic architecture of a minimal RNA polymerase II open promoter complex.* Molecular cell, 2012. **46**(2): p. 136-46.

40. Lifton, R.P., et al., *The organization of the histone genes in Drosophila melanogaster: functional and evolutionary implications.* Cold Spring Harbor symposia on quantitative biology, 1978. **42 Pt 2**: p. 1047-51.

41. Pribnow, D., *Nucleotide-sequence of an RNA polymerase binding-site at an early T7 promoter.* Proceedings of the National Academy of Sciences of the United States of America, 1975. **72**(3): p. 784-788.

42. Reiter, W.D., Hudepohl, U., and Zillig, W., *Mutational analysis of an archaebacterial promoter - essential role of a TATA box for transcription efficiency and start-site selection in vitro* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(24): p. 9509-9513.

43. Chen, W. and Struhl, K., *Saturation mutagenesis of a yeast his3 TATA element - genetic evidence for a specific TATA-binding protein.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(8): p. 2691-2695.

44. Kuehner, J.N. and Brow, D.A., *Quantitative analysis of in vivo initiator selection by yeast RNA polymerase II supports a scanning model.* Journal of Biological Chemistry, 2006. **281**(20): p. 14119-14128.

45. Singer, V.L., Wobbe, C.R., and Struhl, K., *A wide variety of DNA-sequences can functionally replace a yeast TATA element for transcriptional activation. .* Genes & Development, 1990. **4**(4): p. 636-645.

46. Sugihara, F., Kasahara, K., and Kokubo, T., *Highly redundant function of multiple AT-rich sequences as core promoter elements in the TATA-less RPS5 promoter of Saccharomyces cerevisiae.* Nucleic acids research, 2011. **39**(1): p. 59-75.

47. Struhl, K., *Naturally-occuring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast.* Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(24): p. 8419-8423.

48. Iyer, V. and Struhl, K., *Poly(dA-dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA-structure.* The EMBO journal, 1995. **14**(11): p. 2570-2579.

49. Hahn, S., et al., *Yeast TATA-binding protein TFIID binds to TATA elements with both consensus and nonconsensus DNA-sequences.* Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(15): p. 5718-5722.

50. Smale, S.T., *Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes.* Biochimica et biophysica acta, 1996. **1351**: p. 73-88.

51. Sikorski, T.W. and Buratowski, S., *The basal initiation machinery: beyond the general transcription factors.* Current Opinion in Cell Biology, 2009. **21**(3): p. 344-351.

52. Chalkley, G.E. and Verrijzer, C.P., *DNA binding site selection by RNA polymerase II TAFs: a TAF(II)250-TAF(II)150 complex recognizes the Initiator.* The EMBO journal, 1999. **18**(17): p. 4835-4845.

53. Lagrange, T., et al., *New core promoter element in RNA polymerase II-dependent transcription: sequence-specific DNA binding by transcription factor IIB.* Genes & Development, 1998. **12**(1): p. 34-44.

54. Tokusumi, Y., et al., *The new core promoter element XCPE1 (X Core Promoter Element 1) directs activator-, mediator-, and TATA-binding protein-dependent but TFIID-independent RNA polymerase II transcription from TATA-less promoters.* Molecular and Cellular Biology, 2007. **27**(5): p. 1844-58.

55. Lim, C.Y., et al., *The MTE, a new core promoter element for transcription by RNA polymerase II.* Genes & development, 2004. **18**(13): p. 1606-17.

56. Auble, D.T., et al., *Mot1, a global repressor of RNA-polyermase-II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism.* Genes & Development, 1994. **8**(16): p. 1920-1934.

57. Muldrow, T.A., et al., *MOT1 can activate basal transcription in vitro by regulating the distribution of TATA binding protein between promoter and nonpromoter sites.* Molecular and Cellular Biology, 1999. **19**(4): p. 2835-2845.

58. Lariviere, L., et al., *Structure-system correlation identifies a gene regulatory Mediator submodule.* Genes & Development, 2008. **22**(7): p. 872-877.

59. Thiryblaise, L.M. and Loppes, R., *Deletion analysis of the ARG4 promoter of Saccharomyces cerevisiae - a poly(dA-dT) stretch involved in gene-transcription.* Molecular & General Genetics, 1990. **223**(3): p. 474-480.

60. Moreira, J.M.A., Horz, W., and Holmberg, S., *Neither Reb1p nor Poly(dA-dT) elements are responsible for the highly specific chromatin organization at the ILV1 promoter.* Journal of Biological Chemistry, 2002. **277**(5): p. 3202-3209.

61. Graham, I.R. and Chambers, A., *A Reb1p-binding site is required for efficient activation of the yeast RAP1 gene, but multiple binding-sites for Rap1p are not essential.* Molecular Microbiology, 1994. **12**(6): p. 931-940.

62. Wu, R. and Li, H., *Positioned and G/C-capped poly(dA:dT) tracts associate with the centers of nucleosome-free regions in yeast promoters.* Genome Research, 2010. **20**(4): p. 473-484.

63. Kuras, L. and Struhl, K., *Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme.* Nature, 1999. **399**(6736): p. 609-613.

64. Muller, F., Demeny, M.A., and Tora, L., *New problems in RNA polymerase II transcription initiation: Matching the diversity of core promoters with a variety of promoter recognition factors.* Journal of Biological Chemistry, 2007. **282**: p. 14685-14689.

65. Faiger, H., et al., *TBP flanking sequences: asymmetry of binding, long-range effects and consensus sequences.* Nucleic acids research, 2006. **34**(1): p. 104-119.

66. Tsukihashi, Y., et al., *Impaired core promoter recognition caused by novel yeast TAF145 mutations can be restored by creating a canonical TATA element within the promoter region of the TUB2 gene.* Molecular and Cellular Biology, 2000. **20**(7): p. 2385-2399.

67. Marshall, L. and White, R.J., *Non-coding RNA production by RNA polymerase III is implicated in cancer.* Nature Reviews Cancer, 2008. **8**(12): p. 911-914.

68. Cabarcas, S. and Schramm, L., *RNA polymerase III transcription in cancer: the BRF2 connection.* Molecular Cancer, 2011. **10**: p. 47.

69. Namavar, Y., et al., *Classification, diagnosis and potential mechanisms in pontocerebellar hypoplasia.* Orphanet Journal of Rare Diseases, 2011. **6**: p. 50.

70. Budde, B.S., et al., *tRNA splicing endonuclease mutations cause pontocerebellar hypoplasia.* Nature genetics, 2008. **40**(9): p. 1113-1118.

71. Bernard, G., et al., *Mutations of POLR3A encoding a catalytic subunit of RNA polymerase Pol III cause a recessive hypomyelinating leukodystrophy.* American Journal of Human Genetics, 2011. **89**(3): p. 415-23.

72. Tetreault, M., et al., *Recessive mutations in POLR3B, encoding the second largest subunit of Pol III, cause a rare hypomyelinating leukodystrophy.* American Journal of Human Genetics, 2011. **89**(5): p. 652-5.

73. Daoud, H., et al., *Mutations in POLR3A and POLR3B are a major cause of hypomyelinating leukodystrophies with or without dental abnormalities and/or hypogonadotropic hypogonadism.* Journal of Medical Genetics, 2013. **50**(3): p. 194-7.

74. Saitsu, H., et al., *Mutations in POLR3A and POLR3B encoding RNA Polymerase III subunits cause an autosomal-recessive hypomyelinating leukoencephalopathy.* American Journal of Human Genetics, 2011. **89**(5): p. 644-51.

75. White, R.J., *Transcription by RNA polymerase III: more complex than we thought.* Nature reviews. Genetics, 2011. **12**(7): p. 459-63.

76. Vannini, A. and Cramer, P., *Conservation between the RNA polymerase I, II, and III transcription initiation machineries.* Molecular cell, 2012. **45**(4): p. 439-46.

77. Hahn, S. and Roberts, S., *The zinc ribbon domains of the general transcription factors TFIIB and Brf: conserved functional surfaces but different roles in transcription initiation.* Genes & development, 2000. **14**: p. 719-730.

78. Colbert, T. and Hahn, S., *A yeast TFIIB-related factor involved in RNA polymerase III transcription.* Genes & development, 1992. **6**(10): p. 1940-1949.

79. Zoghbi, H.Y. and Bear, M.F., *Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities.* Cold Spring Harbor perspectives in biology, 2012. **4**(3).

80. Pavlowsky, A., Chelly, J., and Billuart, P., *Emerging major synaptic signaling pathways involved in intellectual disability.* Molecular Psychiatry, 2012. **17**(7): p. 682-93.

81. Najmabadi, H., et al., *Deep sequencing reveals 50 novel genes for recessive cognitive disorders.* Nature, 2011. **478**(7367): p. 57-63.

82. Tsai, F.T. and Sigler, P.B., *Structural basis of preinitiation complex assembly on human pol II promoters.* The EMBO journal, 2000. **19**(1): p. 25-36.

83. Van Roon-Mom, W.M., et al., *TATA-binding protein in neurodegenerative disease.* Neuroscience, 2005. **133**(4): p. 863-72.

84. Zuhlke, C. and Burk, K., *Spinocerebellar ataxia type 17 is caused by mutations in the TATA-box binding protein.* Cerebellum, 2007. **6**(4): p. 300-7.

85. Barski, A., et al., *Pol II and its associated epigenetic marks are present at Pol III-transcribed noncoding RNA genes.* Nature structural & molecular biology, 2010. **17**(5): p. 629-34.

86. Dittmar, K.A., Goodenbour, J.M., and Pan, T., *Tissue-specific differences in human transfer RNA expression.* PLoS genetics, 2006. **2**(12): p. e221.

87. Oler, A.J., et al., *Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors.* Nature structural & molecular biology, 2010. **17**(5): p. 620-8.

88. Yin, J., et al., *Site-specific protein labeling by Sfp phosphopantetheinyl transferase.* Nature protocols, 2006. **1**(1): p. 280-5.

89. Cox, J.M., et al., *Bidirectional binding of the TATA box binding protein to the TATA box.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(25): p. 13475-13480.

90. Rhee, H.S. and Pugh, B.F., *Genome-wide structure and organization of eukaryotic pre-initiation complexes.* Nature, 2012. **483**(7389): p. 295-301.

91. Seizl, M., *The Mediator head module and regulation of RNA polymerase II transcription initiation*, in *Department of Chemistry and Biochemistry*2011, Ludwig-Maximilians-Universität München: Munich. p. 111.

92. Moreira, J.M.A., et al., *Datin, a yeast poly(dA : dT)-binding protein, behaves as an activator of the wild-type ILV1 promoter and interacts synergistically with Reb1p.* Molecular and General Genetics, 1998. **258**(1-2): p. 95-103.

93. Liao, Y., Moir, R.D., and Willis, I.M., *Interactions of Brf1 peptides with the tetratricopeptide repeat-containing subunit of TFIIIC inhibit and promote preinitiation complex assembly.* Molecular and Cellular Biology, 2006. **26**(16): p. 5946-56.

94. Fairley, Jennifer a., et al., *Direct Regulation of tRNA and 5S rRNA Gene Transcription by Polo-like Kinase 1.* Molecular cell, 2012. **45**(4): p. 541-552.

95. Higuchi, R., Krummel, B., and Saiki, R.K., *A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions.* Nucleic acids research, 1988. **16**(15): p. 7351-67.

96. Gasteiger, E., et al., *ExPASy: The proteomics server for in-depth protein knowledge and analysis.* Nucleic acids research, 2003. **31**(13): p. 3784-8.

97. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical biochemistry, 1976. **72**: p. 248-54.

98. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.

99. Seizl, M., et al., *Mediator head subcomplex Med11/22 contains a common helix bundle building block with a specific function in transcription initiation complex stabilization.* Nucleic acids research, 2011. **39**(14): p. 6291-6304.

100. Auble, D.T. and Hahn, S., *An ATP-dependent inhibitor of TBP binding to DNA.* Genes & Development, 1993. **7**(5): p. 844-856.

101. Kostrewa, D., et al., *RNA polymerase II-TFIIB structure and mechanism of transcription initiation.* Nature, 2009. **462**(7271): p. 323-330.

102. Mayer, A., et al., *CTD tyrosine phosphorylation impairs termination factor recruitment to RNA polymerase II.* Science, 2012. **336**(6089): p. 1723-5.

103. Aparicio, O., et al., *Chromatin immunoprecipitation for determining the association of proteins with specific genomic sequences in vivo.* Current Protocols in Molecular Biology, 2005. **Chapter 21**: p. Unit 21 3.

104. Sikorski, R.S. and Hieter, P., *A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae.* Genetics, 1989. **122**(1): p. 19-27.

ABBREVIATIONS

Bdp1 ..... *B double prime 1*
bp ..... *base pairs*
BRE ..... *TFIIB-recognition element*
Brf1 ..... *B-related factor 1*
CF ..... *core factor*
ChIP ..... *chromatin immuno precipitation*
CTD ..... *C-terminal domain*
CV ..... *column volumes*
DCE ..... *downstream core element*
DNA ..... *deoxyribonucleic acid*
DPE ..... *downstream promoter element*
EMSA ..... *electrophoretic mobility shift assay*
GAE ..... *GA element*
GTF ..... *general transcription factor*
IC ..... *initiation complex*
ICR ..... *internal control region*
INR ..... *initiator*
min ..... *minute*
MTE ..... *motif ten element*
NFR ..... *nucleosome-free region*
nt ..... *nucleotides*
NTP ..... *nucleotide*
PCH ..... *pontocerebellar hypoplasias*
PCR ..... *polymerase chain reaction*
PIC ..... *pre-initiation complex*
Pol ..... *RNA Polymerase*
PSE ..... *proximal sequence element*
qPCR ..... *quantitative real time PCR*
RNA ..... *ribonucleic acid*
rpm ..... *rounds per min*
rRNA ..... *ribosomal RNA*
RT ..... *room temperature*

Sc ..... *Saccharomyces cerevisiae*
SDS-PAGE ..... *SDS-Polyacrylamide gel electrophoresis*
SNAPc ..... *small nuclear RNA-activating protein complex*
snRNA ..... *small nuclear RNA*
TAF ..... *TBP associated factor*
TAP ..... *tandem affinity purification*
TBP ..... *TATA box-binding protein*
tRNA ..... *transfer RNA*
TSS ..... *transcription start site*
UE ..... *upstream promoter element*
WT ..... *wild type*
XCPE1 ..... *X core promoter element 1*

LIST OF FIGURES

LIST OF TABLES

APPENDIX

List of *Sc* promoters containing the identifed GA element described and characterized in chapter 2.

YAL002W	YBL076C	YBR171W	YCL028W
YAL007C	YBL090W	YBR172C	YCL030C
YAL012W	YBL093C	YBR175W	YCL031C
YAL016W	YBL095W	YBR181C	YCL035C
YAL018C	YBL105C	YBR183W	YCL037C
YAL020C	YBR003W	YBR195C	YCL038C
YAL023C	YBR009C	YBR197C	YCL040W
YAL025C	YBR014C	YBR204C	YCL043C
YAL027W	YBR015C	YBR207W	YCL044C
YAL033W	YBR018C	YBR214W	YCL051W
YAL035W	YBR019C	YBR216C	YCL056C
YAL053W	YBR022W	YBR220C	YCL057W
YAL055W	YBR024W	YBR228W	YCL059C
YAL056W	YBR034C	YBR234C	YCR026C
YAL058W	YBR036C	YBR243C	YCR032W
YAR002C-A	YBR037C	YBR248C	YCR033W
YAR002W	YBR040W	YBR251W	YCR034W
YAR007C	YBR042C	YBR252W	YCR042C
YAR023C	YBR044C	YBR254C	YCR045C
YAR027W	YBR048W	YBR257W	YCR053W
YAR028W	YBR052C	YBR258C	YCR054C
YAR033W	YBR056W-A	YBR259W	YCR059C
YBL004W	YBR057C	YBR260C	YCR079W
YBL006C	YBR059C	YBR265W	YCR083W
YBL007C	YBR077C	YBR267W	YCR086W
YBL010C	YBR091C	YBR268W	YCR088W
YBL011W	YBR097W	YBR269C	YCR094W
YBL023C	YBR101C	YBR270C	YCR097W
YBL026W	YBR103W	YBR272C	YDL002C
YBL028C	YBR111W-A	YBR273C	YDL010W
YBL034C	YBR123C	YBR279W	YDL015C
YBL039W-B	YBR127C	YBR282W	YDL025C
YBL040C	YBR138C	YBR286W	YDL029W
YBL045C	YBR142W	YBR287W	YDL040C
YBL051C	YBR143C	YBR296C	YDL043C
YBL056W	YBR152W	YCL001W	YDL048C
YBL058W	YBR153W	YCL002C	YDL049C
YBL061C	YBR154C	YCL005W-A	YDL053C
YBL068W	YBR166C	YCL010C	YDL057W
YBL069W	YBR168W	YCL011C	YDL061C
YBL071W-A	YBR169C	YCL016C	YDL063C

YDL069C
YDL072C
YDL074C
YDL076C
YDL077C
YDL078C
YDL081C
YDL082W
YDL084W
YDL086W
YDL087C
YDL088C
YDL092W
YDL098C
YDL099W
YDL100C
YDL103C
YDL109C
YDL111C
YDL116W
YDL122W
YDL123W
YDL130W-A
YDL134C
YDL142C
YDL144C
YDL147W
YDL153C
YDL155W
YDL157C
YDL160C
YDL165W
YDL168W
YDL170W
YDL173W
YDL176W
YDL178W
YDL181W
YDL185W
YDL188C
YDL193W
YDL202W
YDL204W
YDL205C
YDL207W
YDL208W
YDL212W
YDL213C
YDL219W
YDL220C
YDL232W
YDL234C
YDL235C
YDR002W
YDR004W
YDR005C
YDR012W
YDR013W
YDR016C
YDR020C
YDR021W
YDR023W
YDR042C
YDR045C
YDR052C
YDR054C
YDR067C
YDR071C
YDR075W
YDR076W
YDR083W
YDR084C
YDR091C
YDR100W
YDR101C
YDR110W
YDR115W
YDR116C
YDR131C
YDR135C
YDR158W
YDR159W
YDR163W
YDR165W
YDR166C
YDR167W
YDR180W
YDR184C
YDR195W
YDR197W
YDR204W
YDR205W
YDR206W
YDR212W
YDR233C
YDR237W
YDR238C
YDR239C
YDR245W
YDR247W
YDR255C
YDR257C
YDR260C
YDR266C
YDR267C
YDR272W
YDR276C
YDR277C
YDR285W
YDR288W
YDR289C
YDR294C
YDR296W
YDR298C
YDR300C
YDR304C
YDR305C
YDR308C
YDR311W
YDR313C
YDR315C
YDR317W
YDR319C
YDR323C
YDR328C
YDR330W
YDR331W
YDR334W
YDR341C
YDR342C
YDR347W
YDR348C
YDR350C
YDR353W
YDR354W
YDR361C
YDR365C
YDR367W
YDR368W
YDR371W
YDR372C
YDR379C-A
YDR383C
YDR391C
YDR393W
YDR403W
YDR404C
YDR405W
YDR411C
YDR415C
YDR418W
YDR425W
YDR429C
YDR432W
YDR435C
YDR441C
YDR454C
YDR461W
YDR463W
YDR469W
YDR476C
YDR477W
YDR478W
YDR481C
YDR483W
YDR485C
YDR486C
YDR488C
YDR494W
YDR495C
YDR496C
YDR497C
YDR510W
YDR511W
YDR522C
YDR524C
YDR530C
YEL002C
YEL003W
YEL004W
YEL006W
YEL013W
YEL015W
YEL016C

YEL018W
YEL020W-A
YEL024W
YEL025C
YEL026W
YEL027W
YEL036C
YEL040W
YEL044W
YEL054C
YEL056W
YEL058W
YEL059C-A
YEL063C
YEL066W
YER001W
YER002W
YER006W
YER007C-A
YER009W
YER012W
YER016W
YER019W
YER020W
YER021W
YER022W
YER023W
YER024W
YER025W
YER027C
YER029C
YER030W
YER042W
YER046W
YER047C
YER048C
YER048W-A
YER050C
YER055C
YER059W
YER061C
YER063W
YER068W
YER070W
YER071C
YER076C
YER082C
YER083C
YER086W
YER087C-B
YER091C
YER092W
YER094C
YER100W
YER113C
YER115C
YER123W
YER126C
YER133W
YER134C
YER146W
YER148W
YER150W
YER156C
YER162C
YER170W
YER174C
YER175C
YER177W
YER180C
YER182W
YFL001W
YFL002C
YFL007W
YFL010C
YFL013C
YFL025C
YFL028C
YFL029C
YFL033C
YFL034C-B
YFL037W
YFL038C
YFL040W
YFL041W
YFL045C
YFL046W
YFL055W
YFL056C
YFR001W
YFR002W
YFR004W
YFR009W
YFR011C
YFR012W
YFR040W
YFR041C
YFR042W
YFR043C
YFR044C
YFR045W
YFR046C
YFR051C
YGL004C
YGL010W
YGL012W
YGL015C
YGL028C
YGL033W
YGL043W
YGL047W
YGL053W
YGL054C
YGL056C
YGL058W
YGL070C
YGL077C
YGL078C
YGL083W
YGL092W
YGL093W
YGL096W
YGL100W
YGL105W
YGL117W
YGL122C
YGL127C
YGL130W
YGL142C
YGL159W
YGL161C
YGL164C
YGL172W
YGL174W
YGL180W
YGL192W
YGL198W
YGL200C
YGL206C
YGL213C
YGL215W
YGL219C
YGL220W
YGL222C
YGL223C
YGL225W
YGL226W
YGL237C
YGL245W
YGL247W
YGL255W
YGR008C
YGR020C
YGR023W
YGR026W
YGR029W
YGR030C
YGR036C
YGR038W
YGR042W
YGR048W
YGR049W
YGR052W
YGR057C
YGR063C
YGR066C
YGR072W
YGR078C
YGR080W
YGR083C
YGR087C
YGR088W
YGR089W
YGR101W
YGR103W
YGR111W
YGR117C
YGR123C
YGR128C
YGR130C
YGR143W
YGR145W
YGR152C
YGR155W

YGR156W
YGR157W
YGR158C
YGR166W
YGR172C
YGR174W-A
YGR175C
YGR178C
YGR179C
YGR180C
YGR185C
YGR193C
YGR199W
YGR208W
YGR209C
YGR211W
YGR220C
YGR227W
YGR229C
YGR230W
YGR238C
YGR239C
YGR241C
YGR245C
YGR248W
YGR253C
YGR262C
YGR267C
YGR272C
YGR275W
YGR277C
YGR281W
YGR282C
YGR283C
YGR284C
YGR288W
YGR295C
YHL001W
YHL003C
YHL009C
YHL010C
YHL021C
YHL023C
YHL024W
YHL025W
YHL026C
YHL031C
YHL034C
YHL039W
YHL040C
YHR003C
YHR004C
YHR007C-A
YHR013C
YHR018C
YHR020W
YHR024C
YHR025W
YHR026W
YHR034C
YHR035W
YHR037W
YHR038W
YHR050W
YHR051W
YHR052W
YHR058C
YHR060W
YHR061C
YHR062C
YHR065C
YHR068W
YHR069C
YHR071W
YHR072W-A
YHR076W
YHR077C
YHR081W
YHR084W
YHR085W
YHR088W
YHR089C
YHR092C
YHR109W
YHR115C
YHR124W
YHR127W
YHR131C
YHR132C
YHR132W-A
YHR135C
YHR137W
YHR138C
YHR142W
YHR143W-A
YHR148W
YHR152W
YHR153C
YHR162W
YHR175W
YHR176W
YHR179W
YHR184W
YHR185C
YHR187W
YHR191C
YHR192W
YHR193C
YHR195W
YHR196W
YHR199C-A
YHR208W
YIL004C
YIL006W
YIL007C
YIL008W
YIL010W
YIL016W
YIL021W
YIL030C
YIL031W
YIL034C
YIL035C
YIL038C
YIL039W
YIL040W
YIL041W
YIL043C
YIL051C
YIL066C
YIL076W
YIL078W
YIL085C
YIL093C
YIL108W
YIL112W
YIL116W
YIL117C
YIL122W
YIL127C
YIL128W
YIL138C
YIL143C
YIL144W
YIL152W
YIL162W
YIR001C
YIR006C
YIR009W
YIR016W
YIR029W
YIR035C
YIR037W
YJL006C
YJL034W
YJL054W
YJL065C
YJL066C
YJL068C
YJL069C
YJL072C
YJL079C
YJL081C
YJL097W
YJL107C
YJL110C
YJL115W
YJL116C
YJL127C-B
YJL145W
YJL148W
YJL160C
YJL161W
YJL162C
YJL166W
YJL171C
YJL174W
YJL176C
YJL178C
YJL179W
YJL180C
YJL186W
YJL191W
YJL192C

YJL194W
YJL196C
YJL209W
YJL218W
YJR001W
YJR007W
YJR010C-A
YJR017C
YJR025C
YJR031C
YJR044C
YJR045C
YJR055W
YJR056C
YJR057W
YJR063W
YJR070C
YJR075W
YJR076C
YJR085C
YJR094W-A
YJR097W
YJR104C
YJR106W
YJR116W
YJR118C
YJR122W
YJR133W
YJR143C
YKL002W
YKL003C
YKL004W
YKL006C-A
YKL006W
YKL009W
YKL015W
YKL018C-A
YKL024C
YKL028W
YKL033W-A
YKL038W
YKL039W
YKL042W
YKL045W
YKL049C
YKL050C
YKL057C
YKL062W
YKL068W
YKL074C
YKL077W
YKL078W
YKL080W
YKL082C
YKL095W
YKL096W
YKL099C
YKL100C
YKL105C
YKL108W
YKL117W
YKL121W
YKL122C
YKL125W
YKL126W
YKL127W
YKL128C
YKL129C
YKL133C
YKL140W
YKL142W
YKL143W
YKL145W
YKL154W
YKL160W
YKL163W
YKL167C
YKL178C
YKL181W
YKL189W
YKL194C
YKL195W
YKL205W
YKL213C
YKL218C
YKR008W
YKR009C
YKR015C
YKR016W
YKR019C
YKR025W
YKR026C
YKR035W-A
YKR043C
YKR048C
YKR051W
YKR057W
YKR060W
YKR065C
YKR066C
YKR070W
YKR071C
YKR074W
YKR085C
YKR088C
YKR100C
YLL002W
YLL008W
YLL010C
YLL011W
YLL018C
YLL018C-A
YLL024C
YLL036C
YLL050C
YLL051C
YLL055W
YLR003C
YLR009W
YLR013W
YLR017W
YLR018C
YLR019W
YLR028C
YLR030W
YLR038C
YLR040C
YLR043C
YLR050C
YLR051C
YLR052W
YLR054C
YLR060W
YLR064W
YLR065C
YLR066W
YLR068W
YLR069C
YLR070C
YLR073C
YLR074C
YLR083C
YLR093C
YLR109W
YLR113W
YLR118C
YLR126C
YLR133W
YLR154C
YLR165C
YLR167W
YLR175W
YLR186W
YLR189C
YLR193C
YLR196W
YLR197W
YLR200W
YLR201C
YLR206W
YLR209C
YLR213C
YLR215C
YLR216C
YLR221C
YLR223C
YLR226W
YLR229C
YLR231C
YLR241W
YLR243W
YLR250W
YLR254C
YLR257W
YLR262C
YLR262C-A
YLR270W
YLR275W
YLR283W
YLR285W
YLR287C-A
YLR290C
YLR291C
YLR293C

YLR295C
YLR298C
YLR300W
YLR303W
YLR307C-A
YLR307W
YLR326W
YLR336C
YLR340W
YLR341W
YLR343W
YLR347C
YLR355C
YLR360W
YLR362W
YLR363W-A
YLR368W
YLR371W
YLR372W
YLR390W
YLR395C
YLR404W
YLR405W
YLR409C
YLR412W
YLR417W
YLR438C-A
YLR439W
YLR440C
YLR442C
YLR443W
YLR449W
YLR453C
YML004C
YML009C
YML016C
YML018C
YML019W
YML025C
YML026C
YML029W
YML030W
YML036W
YML043C
YML048W
YML057W
YML062C
YML064C
YML065W
YML067C
YML070W
YML074C
YML077W
YML079W
YML081C-A
YML085C
YML086C
YML093W
YML105C
YML107C
YML108W
YML110C
YML118W
YML119W
YML121W
YML125C
YML127W
YML128C
YML130C
YMR001C
YMR002W
YMR004W
YMR005W
YMR013C
YMR015C
YMR035W
YMR036C
YMR037C
YMR038C
YMR048W
YMR054W
YMR059W
YMR067C
YMR071C
YMR077C
YMR080C
YMR081C
YMR093W
YMR099C
YMR102C
YMR116C
YMR119W
YMR121C
YMR122W-A
YMR123W
YMR131C
YMR134W
YMR146C
YMR147W
YMR153W
YMR155W
YMR158W
YMR164C
YMR177W
YMR180C
YMR181C
YMR182W-A
YMR183C
YMR187C
YMR190C
YMR192W
YMR199W
YMR203W
YMR205C
YMR213W
YMR215W
YMR220W
YMR222C
YMR233W
YMR234W
YMR235C
YMR238W
YMR241W
YMR243C
YMR256C
YMR258C
YMR260C
YMR265C
YMR274C
YMR277W
YMR281W
YMR282C
YMR295C
YMR298W
YMR307W
YMR309C
YMR311C
YMR312W
YMR314W
YMR316W
YMR318C
YNL002C
YNL004W
YNL006W
YNL025C
YNL026W
YNL032W
YNL038W
YNL044W
YNL048W
YNL052W
YNL053W
YNL067W
YNL069C
YNL075W
YNL076W
YNL078W
YNL079C
YNL082W
YNL083W
YNL085W
YNL086W
YNL088W
YNL092W
YNL097C
YNL098C
YNL099C
YNL112W
YNL113W
YNL121C
YNL130C
YNL138W-A
YNL141W
YNL145W
YNL146C-A
YNL146W
YNL151C
YNL158W
YNL162W-A
YNL165W
YNL168C
YNL175C
YNL182C
YNL186W

YNL188W
YNL193W
YNL197C
YNL202W
YNL207W
YNL211C
YNL219C
YNL222W
YNL227C
YNL232W
YNL233W
YNL237W
YNL241C
YNL246W
YNL248C
YNL249C
YNL251C
YNL252C
YNL261W
YNL263C
YNL264C
YNL265C
YNL268W
YNL271C
YNL281W
YNL283C
YNL294C
YNL300W
YNL301C
YNL305C
YNL307C
YNL318C
YNL322C
YNL323W
YNL326C
YNL330C
YNR006W
YNR010W
YNR015W
YNR017W
YNR018W
YNR019W
YNR021W
YNR022C
YNR026C
YNR030W
YNR032C-A
YNR032W
YNR034W
YNR036C
YNR038W
YNR041C
YNR051C
YNR054C
YOL002C
YOL005C
YOL006C
YOL007C
YOL012C
YOL015W
YOL020W
YOL022C
YOL028C
YOL031C
YOL036W
YOL038W
YOL044W
YOL048C
YOL051W
YOL055C
YOL061W
YOL067C
YOL071W
YOL073C
YOL076W
YOL077C
YOL077W-A
YOL080C
YOL083W
YOL090W
YOL093W
YOL094C
YOL097C
YOL101C
YOL115W
YOL117W
YOL119C
YOL121C
YOL124C
YOL125W
YOL136C
YOL139C
YOL143C
YOL145C
YOL146W
YOL149W
YOL151W
YOL162W
YOR001W
YOR005C
YOR006C
YOR014W
YOR020C
YOR027W
YOR038C
YOR039W
YOR045W
YOR047C
YOR059C
YOR060C
YOR067C
YOR077W
YOR078W
YOR083W
YOR087W
YOR089C
YOR090C
YOR097C
YOR103C
YOR104W
YOR111W
YOR118W
YOR120W
YOR123C
YOR125C
YOR131C
YOR133W
YOR136W
YOR137C
YOR143C
YOR148C
YOR149C
YOR159C
YOR164C
YOR167C
YOR168W
YOR174W
YOR175C
YOR179C
YOR194C
YOR196C
YOR204W
YOR206W
YOR209C
YOR210W
YOR215C
YOR230W
YOR231W
YOR233W
YOR234C
YOR239W
YOR243C
YOR253W
YOR254C
YOR257W
YOR258W
YOR270C
YOR276W
YOR281C
YOR283W
YOR286W
YOR288C
YOR296W
YOR297C
YOR301W
YOR305W
YOR307C
YOR310C
YOR323C
YOR324C
YOR339C
YOR346W
YOR347C
YOR360C
YOR370C
YOR372C
YOR374W
YOR375C
YOR376W-A
YPL004C
YPL005W
YPL008W
YPL010W
YPL013C

YPL024W
YPL030W
YPL033C
YPL046C
YPL053C
YPL057C
YPL063W
YPL064C
YPL074W
YPL084W
YPL093W
YPL097W
YPL105C
YPL107W
YPL118W
YPL121C
YPL124W
YPL127C
YPL129W
YPL131W
YPL132W
YPL133C
YPL146C
YPL157W
YPL160W
YPL164C
YPL167C
YPL168W
YPL169C
YPL170W
YPL173W
YPL175W
YPL178W
YPL184C
YPL191C
YPL198W
YPL199C
YPL204W
YPL208W
YPL211W
YPL214C
YPL215W
YPL217C
YPL218W
YPL224C
YPL227C
YPL229W
YPL232W
YPL234C
YPL235W
YPL237W
YPL239W
YPL242C
YPL243W
YPL244C
YPL249C
YPL253C
YPL255W
YPL263C
YPL264C
YPL266W
YPL273W
YPR004C
YPR010C
YPR016C
YPR020W
YPR023C
YPR026W
YPR027C
YPR028W
YPR030W
YPR034W
YPR036W
YPR037C
YPR041W
YPR054W
YPR063C
YPR075C
YPR082C
YPR084W
YPR086W
YPR088C
YPR098C
YPR110C
YPR112C
YPR115W
YPR124W
YPR129W
YPR137W
YPR139C
YPR143W
YPR147C
YPR158W
YPR161C
YPR163C
YPR166C
YPR169W
YPR173C
YPR174C
YPR175W
YPR180W
YPR183W