# Charles University in Prague
# Faculty of Science

## Department of Cell Biology



# Properties of DNA-binding mutations of CSL proteins

**Diploma Thesis**

Mikoláš Teska
2012

Supervised by doc. RNDr. Petr Folk, CSc.

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Prague, August 2012                                                      Mikoláš Teska

# Acknowledgements

# Table of contents

# Abstract and keywords

Notch pathway plays a critical role during the development and life of metazoan organisms. CSL proteins are the component of the Notch pathway that mediates the regulation of target genes. The discovery of CSL-like proteins in yeast raised the question of their function in unicellular organisms which did not utilize the canonical Notch pathway. CSL-homologues in yeast are conserved in parts that are important for DNA binding and for fission yeast proteins it was shown that they bind to CSL recognition elements *in vitro*. In fission yeast, CSL paralogues Cbf11 and Cbf12 play antagonistic roles in cell adhesion and the coordination of cell and nuclear division. Yeast CSL proteins have long and intrinsically unstructured N-terminal domains compared to metazoan CSL proteins. In this study, we investigated the functional significance of these extended N-termini of CSL proteins by their complete removal. For newly constructed truncated variants of proteins Cbf11 and Cbf12 in *Schizosaccharomyces pombe* we observed the lack of ability to bind CSL recognition RBP probe. The removal of N-terminal parts of CSL proteins in fission yeast led to the change in their cellular localization. Once strongly preferred nuclear localization changed by the removal of N-terminal domains to cytoplasmic localization with a weaker or equal presence in the nucleus. We also observed an effect of the overexpression of mutated variants of Cbf11 that cannot bind RBP probe on abundance of endogenic Cbf11 bound to RBP probe in the ΔCbf12 strain. Our data showed that N-terminal parts of yeast CSL proteins effect DNA-binding abilities of these proteins and cellular localization. Revealing the function of the yeast CSL proteins could contribute to the knowledge of Notch-independent functions of CSL proteins in metazoa.

## Keywords

## Abstrakt a klíčová slova

Signální dráha Notch hraje klíčovou roli ve vývoji a životě mnohobuněčných organismů. Proteiny CSL jsou klíčovou součástí dráhy Notch, kde zprostředkovávají regulaci cílových genů. Objev CSL-homologních proteinů v kvasinkách nastolil otázku ohledně jejich funkce v jednobuněčných organismech před vznikem kanonické Notch dráhy. CSL-homologní proteiny v kvasinkách jsou konzervovány v částech, které jsou důležité pro vazby na DNA, a prokázalo se, že tyto proteiny se vážou na CBF1-vazebné elementy *in vitro*. V kvasince *Schizosaccharomyces pombe* hrají paralogy CSL (Cbf11 a Cbf12) protichůdné role v buněčné adhezi a v koordinaci buněčného a jaderného dělení. Kvasinkové proteiny CSL mají oproti proteinům CSL u metazoa dlouhé a vnitřně nestrukturované N-koncové domény. V této studii jsme zkoumali funkční význam těchto N-koncových částí CSL proteinů pomocí jejich úplného odstranění. U nově vytvořených zkrácených variant proteinů Cbf11 a Cbf12 v *S. pombe* jsme pozorovali absenci jejich vazby na próbu RBP, kterou rozpoznávají proteiny CSL. Odstranění N-terminálních částí proteinů CBF u *S. pombe* vedlo ke změně v buněčné lokalizaci. Původně silná jaderná lokalizace u proteinů Cbf divokého typu se odstraněním N-koncových částí změnila na smíšenou lokalizaci v cytoplazmě a jádře. Overexprese nevazebných mutací genu Cbf11 měla za následek snížení vazby endogenního proteinu Cbf11 ve kmeni ΔCbf12. Naše data ukázala, že N-terminální části kvasinek proteinů CSL mají vliv na DNA-vazebné vlastnosti a buněčnou lokalizaci těchto proteinů. Odhalení funkce proteinů CSL u kvasinek by mohlo přispět k rozšíření současného poznání Notch-nezávislých funkcí CSL bílkovin u metazoí.

## Klíčová slova

signální dráha Notch, CSL, *Schizosaccharomyces pombe*, N-terminální doména, DNA-vazebná mutace

# Abbreviations

| | |
|---|---|
| bHLH | basic Helix Loop Helix |
| BTD | Beta trefoil domain |
| CDK | Cyclin-dependent kinase |
| CIR | CBF1 interacting corepressor |
| CSL | (CBF1, Su(H), Lag-1) |
| DBC | DNA-binding core |
| DBM | DNA-binding mutation |
| DTT | Dithiothreitol |
| EBNA2 | EBV nuclear antigen 2 |
| EBV | Epstein-Barr virus |
| EGF | Epidermal growth factor |
| EMAS | Electrophoretic Mobility Shift Assay |
| EMM | Edinburgh Minimal Media |
| GFP | Green fluorescent protein |
| HDAC | Histone deacetylase |
| HPV | Herpes virus |
| NECD | Notch extracellular domain |
| NICD | Notch intracellular domain |
| NLS | Nuclear localization signal |
| PCR | Polymerase chain reaction |
| PTF1 | Pancreas transcription factor 1 |
| RBPJ | Recombining Binding Protein suppressor of hairless |
| RHR | Rel homology region |
| SDS | Sodium dodecyl sulphate |
| SHARP | (SMRT/HDAC1 Associated Repressor Protein) |
| SKIP | Ski-interacting protein |
| SMRT | Silencing mediator for retinoid or thyroid-hormone receptors |
| TBS | Tris-buffered saline |
| TTBS | Tween Tris-buffered saline |

# 1. The objectives of the Diploma thesis

- To analyze the influence of N-terminal parts of fission yeast CSL proteins Cbf11 and Cbf12 on DNA-binding propreties of these proteins.

- To test possible interactions between protein variants and endogenic Cbf11 and analyze the influence of such interactions on DNA-binding properties of tested proteins

- To analyze the adhesivity and toxicity phenotype of overexpressed proteins with mutations in fission yeast

- To analyze the cellular location of all protein variants using the overexpression of GFP tagged constructs.

# 2.    Literature review

## 2. 1 Notch Pathway

### 2. 1. 1        Notch pathway introduction

The Notch pathway is one of the fundamental signaling mechanisms that influence embryonic development and maintaining tissue homeostasis in most multicellular organisms. The Notch pathway functions during processes, such as somitogenesis, the development of gut, bone and cardiovascular system (Bolos et al., 2007), the development of neural (Gaiano and Fishell, 2002) and pancreatic tissue (Murtaugh et al., 2003), cell fate decision in mammary glands development (Dontu et al., 2004) and T-cell lineage commitment (Laky and Fowlkes, 2008). Notch pathway allows signaling between neighboring cells through contact of transmembrane proteins on the cell surface. There are two main steps of the Notch pathway: the interaction of a ligand with the Notch receptor and signal transduction at the level of transcription factor from the CSL family.

### 2. 1. 2        Notch receptor and ligand

The Notch receptor is synthesized in the Golgi apparatus as an inactive precursor. Then it undergoes proteolytic cleavage (S1) (Figure 2. 1) catalyzed by a protein Furin in the Golgi apparatus. The resulting fragments of the receptor stay connected and are exported to the plasma membrane (Logeat et al., 1998). Extracellular part of the receptor called NECD (Notch extracellular domain) consists of 10 to 36 EGF (Epidermal growth factor) repeats and repetitions of the LN (Leu-Asn) sequence which are next to the plasma membrane (Gazave et al., 2009). To receive the signal from neighboring cell and to activate Notch pathway Notch receptor must bind to its ligand. Notch ligand is a transmembrane protein with large extracelluar portion. Two common types of Notch ligands are Delta or Jagged/Serrate and they can mediate a different effect of Notch signaling. For example, during the differentiation of hematopoetic stem cells into T-cell vs. B-cell / natural killer (NK) precursors Delta inhibits the differentiation to T-cell vs. B-cell / natural killer (NK) while Jagged doesn't inhibit such lineage commitment and allows commitment to both lineages (Jaleco et al., 2001). The extracellular part of ligands consists of N-terminal DSL domain, epidermal growth factor (EGF) like repeats and in case of Jagged there is cysteine

11

rich CR domain close to the transmembrane part. DSL domain together with the last (EGF)-like repeats are required for binding the receptor (D'Souza et al., 2008; Rebay et al.,1991). Intracellular part of the receptor called NICD (Notch intracellular domain) is cleaved off after the receptor-ligand interaction and travels to the nucleus.
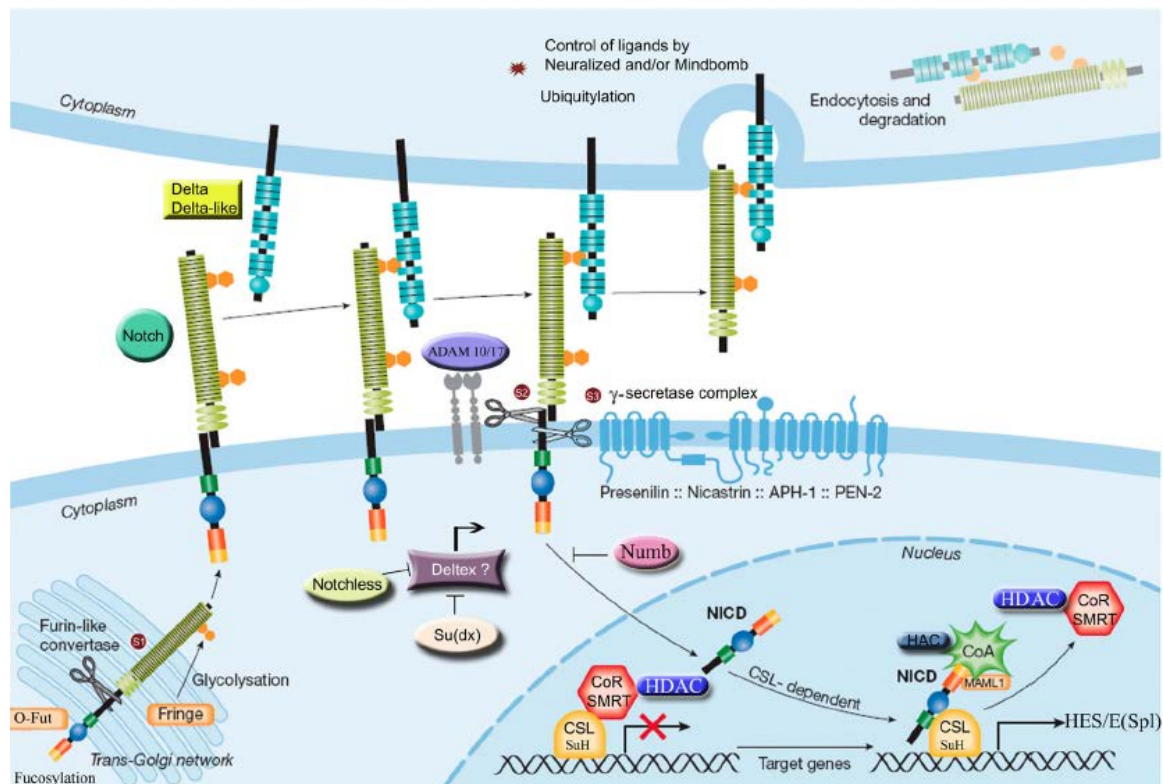


**Figure 2. 1** - Overview of processes taking place during signal transduction in Notch pathway. S1, S2 and S3 are the sites of proteolysis. The main steps are: binding of a ligand to the receptor, proteolytic cleavage, translocation to the nucleus and incorporation of NICD to the activating complex (adopted from Bray 2006) .

### 2. 1. 3    Notch receptor and ligand interaction

When the Notch receptor binds its ligand, a series of two proteolytic cleavages (Figure 2. 1) take place within the receptor molecule. First cleavage (S2) is located just outside of the cell membrane; it is catalyzed by an enzyme from the ADAM (A Disintegrin And Metalloproteinase) metalloprotease family (Brou et al., 2000; Mumm et al., 2000; Jarriault et al., 2005). This modification becomes possible because of a conformational change induced by the ligand binding. As a result, the extracellular part of the receptor is cut off and stays bound to the ligand molecule. This is followed by the second cleavage (S3), which takes place within the intramembrane portion of the receptor. It is catalyzed by γ-secretase complex that contains presenilin (Mumm and Kopan, 2000). As a result of this

second cleavage, NICD is separated from the membrane and can translocate to the nucleus (Fig. 2. 2). NICD consists of the RAM domain (RBPJ Associated Molecule), which interacts with CSL transcription factors, followed by NLS and a series of 3-6 ankyrine repeats, of which the last ankyrine repeat stays partly unstructured until the NICD binds to the complex of CSL in the nucleus. At the C-terminal end of the NICD there is another NLS, a transactivation domain and PEST sequence which plays role in ubiquitinylation (Artavanis-Tsakonas et al., 1999).
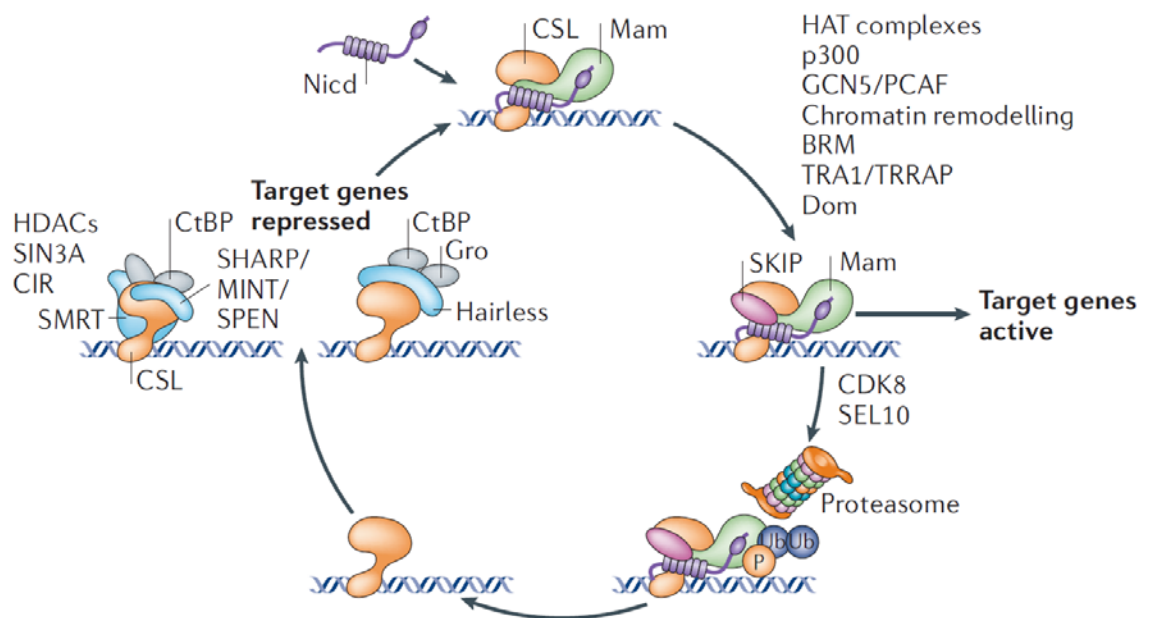


**Figure 2. 2** – The dynamics of the CSL-complex composition between different stages of transcription regulation. CSL without interaction with NICD or other activating partners is in the form of transcription repressing complex and target genes are repressed. When NICD binds to the complex, the repressing complex breaks down and transcription activating complex, which activates target genes, is formed. Later, the NICD is phosphorylated, which leads to the proteasomal degradation of transcription activating complex and subsequently to the reestablishment of the transcription repressing complex (adopted from from Bray 2006).

### 2. 1. 4    Notch pathway inside the nucleus

In the nucleus, NICD regulates transcription of target genes trough its interaction with transcription factors from the CSL family. CSL proteins are named after three homologues (CBF1, Su(H), Lag-1) and compose family of transcription factors essential for the development of most multicellular organisms. Interestingly RBP-J from the CSL family was originally identified as a repressor (Dou et al., 1994). Later it was discovered that CSL

13

both represses and activates transcription. Without the presence of NICD in the nucleus, a transcription repressing complex is formed on CSL proteins (Olave et al., 1998). This repression complex was shown to include silencing mediator for retinoid or thyroid-hormone receptors SMRT (Kao et al., 1998), CBF1 interacting corepressor CIR (Hsieh et al., 1999), Hairless and SHARP (Barolo et al., 2002; Oswald et al., 2002). All of these proteins help to recruit histone deacetylases (HDACs), which remove acetyle groups from N-terminal lysines of histones changing their charge and binding properties. This strengthens the histone – DNA interaction, subsequently leading to transcription repression (Thiagalingam et al., 2003). Another protein found to interact with CSL complex is SKIP, which seems to be present in both the repressing and activating complex (Zhou et al., 2000). This protein is involved in signaling of many transcription factors, e. g., vitamin D receptor, CSL, Smad2/3, and MyoD (Folk et al., 2004). When NICD binds CSL it displaces the repression complex and on this interaction a new transcription activating complex is built. The core of the activating complex is composed of four base components: CSL, NICD, Mastermind and SKIP. Mastermind is a transcription coactivator which also has a role in NICD degradation (Petcherski and Kimble, 2000; Fryer et al., 2004). This four-membered complex then recruits general transcription activators like histone acetyl transferase p300, which then acetylates N-terminal lysines of histones and changes the charge of N-terminal parts of histones. p300 can also interact with other transcription activators and acetylate them eventually helping to activate transcription (Kurooka and Honjo, 2000; Wallberg et al., 2002). Transcription is then turned off by the decomposition of transcription activating complex, induced by the degradation of NICD. Degradation of NICD is promoted by Mastermind and SKIP recruitment of CycC:CDK8 kinase that phosphorylates NICD at the C-terminal PEST sequence. PEST sequence is associated with protein degradation; phosphorylation of the PEST leads to the recruitment of Fbw7/Sel10 E3 ubiquitin-protein ligase complex that ubiquitylates NICD resulting in proteolysis of NICD (Frey et al., 2004) (figure 2. 2). NICD degradation in the nucleus leads rapidly to reconstitution of repressing complex at the CSL site.

### 2. 1. 5     Notch target genes and interaction with viruses

Although the Notch pathway is critical in various developmental processes, the number of genes directly regulated by Notch is limited. Typical examples of regulated genes are the subfamilies of Hes (Hairy/Enhancer of Split), HERP (HES-related repressor protein), and

Hey genes, bHLH transcription factors, which play a critical role in somitogenesis, neurogenesis, nephrogenesis and cardiovascular development (Iso et al., 2003; Fischer and Gessler, 2007). Other genes that were shown to be targets of Notch are, e. g., c-myc, cyclinD, p21, NFκB2(Palomero et al., 2006; Ronchini et al., 2001; Rangarajan et al., 2001; Oswald et al., 1998). Notch also targets Notch3 from the Notch receptor family in karetinocytes (Ohashi et al., 2010).

Notch pathway is exploited by several viruses in their strategies. For example, EBV (Epstein-Barr virus) protein EBNA2 binds CSL with the assistance of SKIP (Zhou et al., 2000) and mimics the presence of NICD, which leads to the transcription activation of specific genes that help to stimulate B-cells survival and growth proliferation during EBV infection of B-cells (Hsieh et al., 1995). Analogically, adenovirus protein 13SE1A binds CSL, which leads to the change of CSL complex from transcription repressing to activating (Ansieau et al., 2001). In herpes virus (HPV) positive cervical carcinoma cells, Notch-1 expression is significantly reduced or absent while Notch-2 expression remains elevated. Constitutive expression of Notch-1 reduces the expression of viral proteins E6 and E7 in CSL-independent way and reduces cell invasive growth. Down-regulation of Notch-1 signaling plays an important role in late stages of HPV-induced carcinogenesis (Talora et al., 2002). Other authors reported that Notch-signaling is up-regulated by E6 and E7 proteins and helps the transformation of cervical intraepithelial lesions to invasive cervical carcinoma (Weijzen et al., 2003). In the case of SV40 virus infection of human mesothelial cells, the expression of Notch-1 is up-regulated leading to increased proliferation (Bocchetta et al.,2003).

## 2. 2   *Notch-idependent functions of CSL*

### 2. 2. 1        Notch-independent functions of Su(H) and Lag-1

During the mesectoderm specification of *Drosophila* blastoderm embryo, CSL protein Su(H) has a dual function. First function is Notch-dependent up-regulation of protein sim (Single-minded) in the mesoectoderm. The second function is the Notch-independent role of Su(H) in the repression of ectopic expression of *sim* dorsally in the neuroectoderm (Morel and Schweisgut, 2000). Protein *sim* is important for mesoectoderm specification (Kosman et al. 1991; Nambu et al., 1991; Thomas et al. 1988). This example shows that in

specific tissues repressive function of CSL can stay without the influence of Notch. Su(H) has also notch-independent activating role in *Drosophila.* During the development of external mechanosensory organs, Notch pathway plays a critical role in securing the right amount and position of these organs through lateral inhibition and also helps the development of four distinct cell types that form the mechanoreceptor; a sheath cell (thecogen), a shaft cell (trichogen), a socket cell (tormogen), and one or more glial cells (Heitzler and Simpson, 1991; Van De Bor et al., 2000). In the socket cell, Su(H) is dramatically up-regulated, which is due to the recruitment of Su(H) to its own downstream enhancer that is different from promoter proximal enhancer used by the Notch pathway. This activation from the downstream enhancer is Notch-independent. Deletion of this enhancer doesn't inhibit the growth or generation of cuticular structure, but the function of mechanorecption is defective (Barolo et al., 2000; Koelzer a Klein, 2003).

CSL can repress genes that are not targeted by the Notch pathway and this repression can be overcome by other activating factors. For example, Lag-1, a member of CSL family in *C. elegans,* negatively regulates transcription of hlh-6, a basic helix-loop-helix transcription factor specific for pharyngeal glands, until the repression is overcome
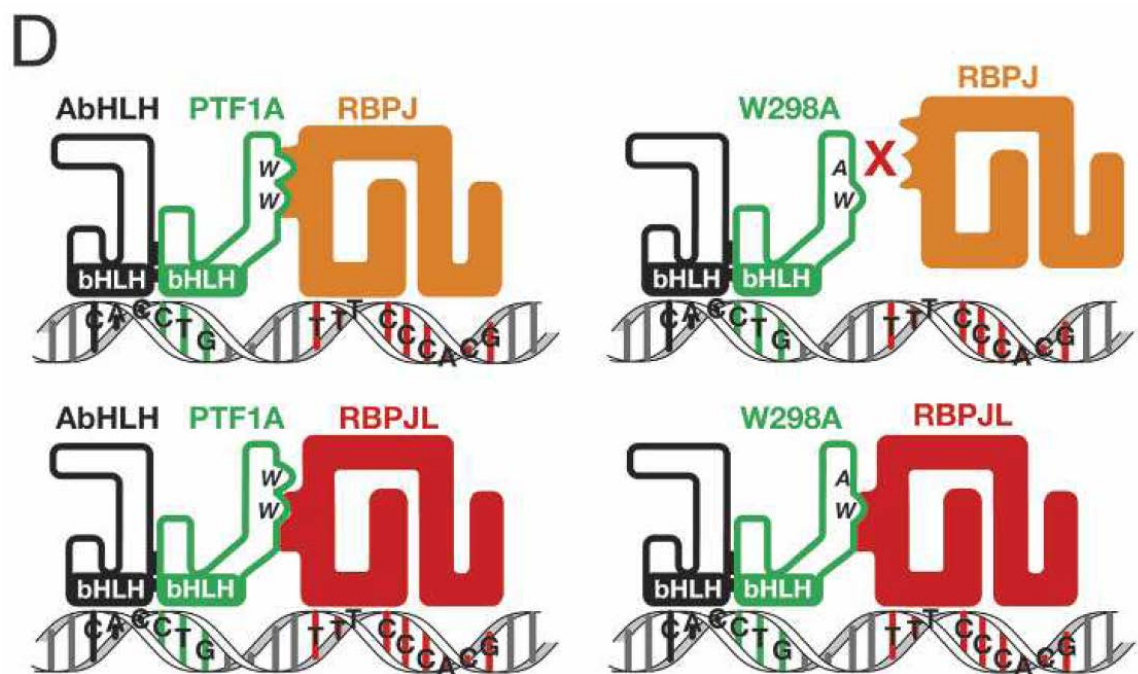


**Figure 2. 3** – Dimer of bHLH factors AbHLH-PTF1A(p48) bound to RBPJ and RBPJL, respectively. RBPJ utilizes two motives carrying the conservative amino-acid (W) on PTF1A whereas RBPJL uses only one of the motives (W), the other motive can be absent (W298A substitution). Because of that mutation W298A in PTF1A disturbs only the binding to RBPJ, rather than to RBPJL (adopted from Masui et al., 2007).

by activators including Fkd and Peb-1 (Ghai and Gaudet, 2008; Raharjo and Gaudet, 2007). In *Xenopus,* the CSL parolog XSu(H)2 seems to play a role in an early gastrulation in a Notch-independent manner (Ito et al., 2007).


## 2. 2. 2        Role of RBPJ(L) in Ptf1 complex

In mammals, CSL parologues RBPJ and RBPJL are differently recruited to a complex regulating the transcription of pancreas specific genes. Transcription of these genes is controlled largely by PTF1 (pancreas transcription factor 1) (Rose et al., 2001), which is a transcription complex composed of 3 proteins: basic helix loop helix (bHLH) transcription factor p48/Ptf1a, a common class-A bHLH transcription factor and the third part is the Rbpj or Rbpjl protein. Binding site for PTF1 complex is composed of two elements: an E-box with the consensus sequence 5'-CACCTG-3' to which the two bHLH transcription factors bind, and a TC-box with the consensus sequence 5'-TTTCCCACG-3' where Rbpj or Rbpjl bind. These binding elements are spaced one to two helical turns apart. Dimer p48-Class A bHLH can bind separately to E-box sequence and the same can be observed for Rbpj/Rbpjl and TC-box, but as a trimeric complex they can only bind to a combination of E-box and TC-box. The architecture of the complex connections (Figure 2. 3) is such that p48 and class A bHLH transcription factor are connected via helix-loop-helix interaction, whereas Rbpj/Rbpjl is connected to p48 trough the C-terimnal domain motive of p48 (Beres et al., 2006). This motive is similar to CSL binding motives of NICD or EBNA2. Using mutation in the C-region of p48 (W298A) that disrupts the binding with Rbpj and not with Rbpjl, it was shown that Rbpj is required for proper formation and differentiation of pancreatic tissue. Rbpjl knock-out mice has a different phenotype compared to p48 (W298A) mice that cannot create Ptf1 complex including Rbpj and show pancreatic agenesis (Sellick et al., 2004). Pancreatic agenesis is a rare cause of neonatal diabetes mellitus and the knowledge of the clinical features is sparse (Hoveyda et al., 1999). Rbpjl knock-out mice show smaller but developed pancreas with lower acinar phenotype. Because PTF1 binding sites are found in the promoter of RBPJL it may be enhanced by the PTF1 complex. Observations indicate that first there is an activity of PTF1 (RBPJ) complex which also activates transcription of RBPJL. Subsequently Rbpjl substitute Rbpj in PTF1 complex and regulates target gene expression during later stages of pancreas development. PTF1 complex is conserved from mammals to insects although

there doesn't seem to be an orthologue of RBPJL in *Drosophila melanogaster*. The conservation is so high that chimeric complex made out of insect and mammalian parts binds to mouse Ela1 promoter and has transactivation potential (Masui et al., 2007). Beside the development of the pancreatic tissue PTF1 complex also plays a role in the formation of cerebellum, retina and spinal cord (Sellick et al., 2004; Glasgow et al., 2005; Fujitani et al., 2006). In the dorsal spinal cord and cerebellum, PTF1 including Rbpj regulates the development of specific GABAergic neuronal populations (Hori et al., 2008).

There is a growing evidence of Notch-independent functions of CSL including default repression CSL complex without the intervention of Notch signal. This repression can be overcome by other activating regulators without a change in the CSL complex as it happens in case of Notch signaling. CSL can also - in some cases - play a role in transcription activation without the Notch signaling. Best evidence comes from its role in the PTF1 complex. Some of these roles are largely conserved in metazoan species and can represent connection between these roles and the role of CSL proteins in fungi.

## *2. 3   CSL in metazoa*

### 2. 3. 1      CSL structure

Crystallographic study of CSL protein Lag-1 from *Caenorhabditis elegans* bound to DNA was conducted (Kovall and Hendrickson, 2004). This study showed that CSL proteins have a highly conserved structure of three core domains (RHR-N, BTD and RHR-C). N-terminal and C-terminal parts of CSL proteins are unstructured and show low level of conservation (Figure. 2. 4). RHR-N and RHR-C have imunoglobulin-like fold with significant similarity to the Rel domains of Rel family of transcription factors, such as NF-κB and NFAT (Chen et al., 1998; Ghosh et al., 1995). RHR-N interacts with the major groove of DNA analogically to the Rel proteins, but the RHR-C domain does not interact with DNA, in contrast to Rel transcription factors, where both domains interact with DNA (Figure 2. 5) (Wilson and Kovall, 2006). RHR-C in CSL transcription factors seem to undergo a substantial folding change upon the binding of ankyrine repeats of NICD (Kovall, 2008).
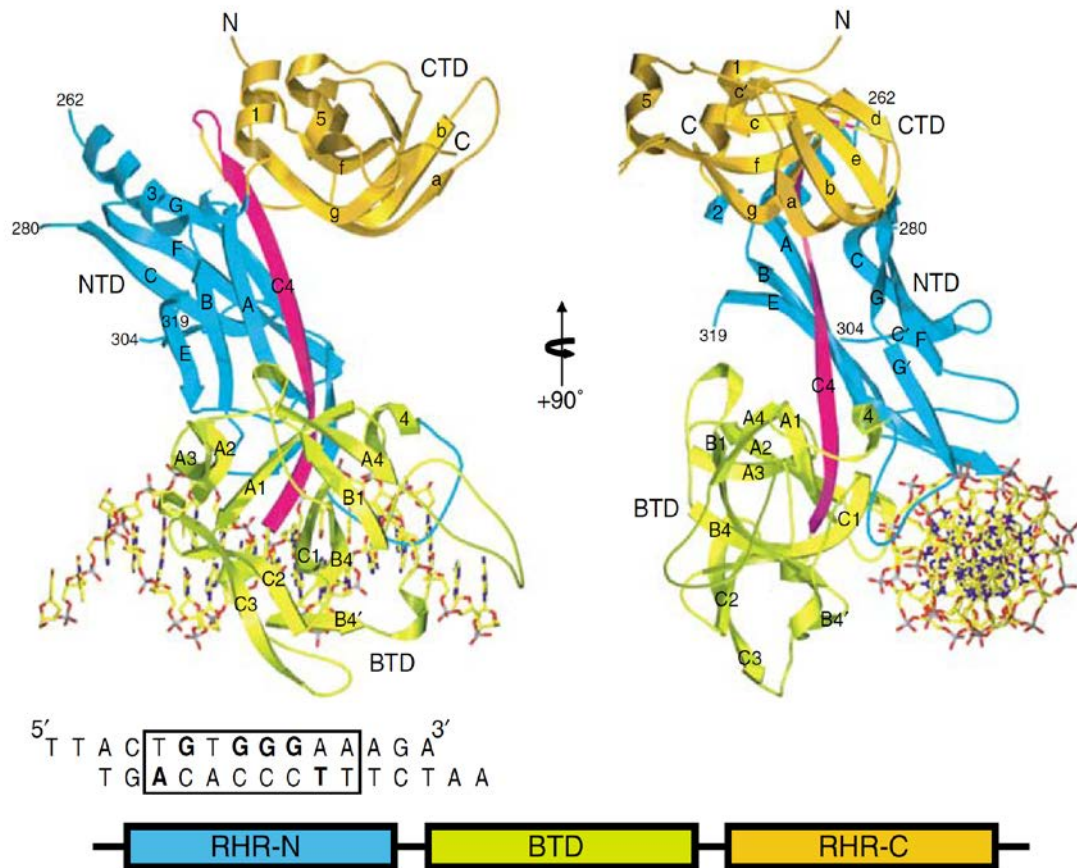
**Figure 2. 4** - 3D structure of the core part of the CSL protein Lag-1 from *Cenorhabditis elegans* in two perpendicular perspectives. The three domains are shown in different colors: RHR-N in blue, central BTD in green and RHR-C in orange. The β–strand C4 that participates in the structure of β-sheets of all three domains is in purple. This visualization of the structure lacks N-terminal and C-terminal extensions. At the left bottom left corner there is the canonical binding element that was used for the crystallization of CSL bound to DNA (adopted from Kovall and Hendrickson, 2004).

Beta trefoil domain (BTD), which lies between RHR-N and RHR-C, interacts with a small groove of DNA and also binds cofactors (Johnson et al., 2010; kovall and hendrickson, 2004). BTD has a structure of beta-barell with lid. This beta-barell has a three-fold symmetry, similarly to other proteins that are equipped with BTD domain; interleukins and cytokines (Murzin et al., 1992). The structure of beta-barrel is made of four fibers (B1-B4) in repeat (Figure 2. 6). The fibers B1 and B4 are involved in the barrel walls and B2 and B3 form the lid (Graves et al., 1990).

|  |  |  |
|---|---|---|
| NFAT/Fos-Jun | CSL | p53 |
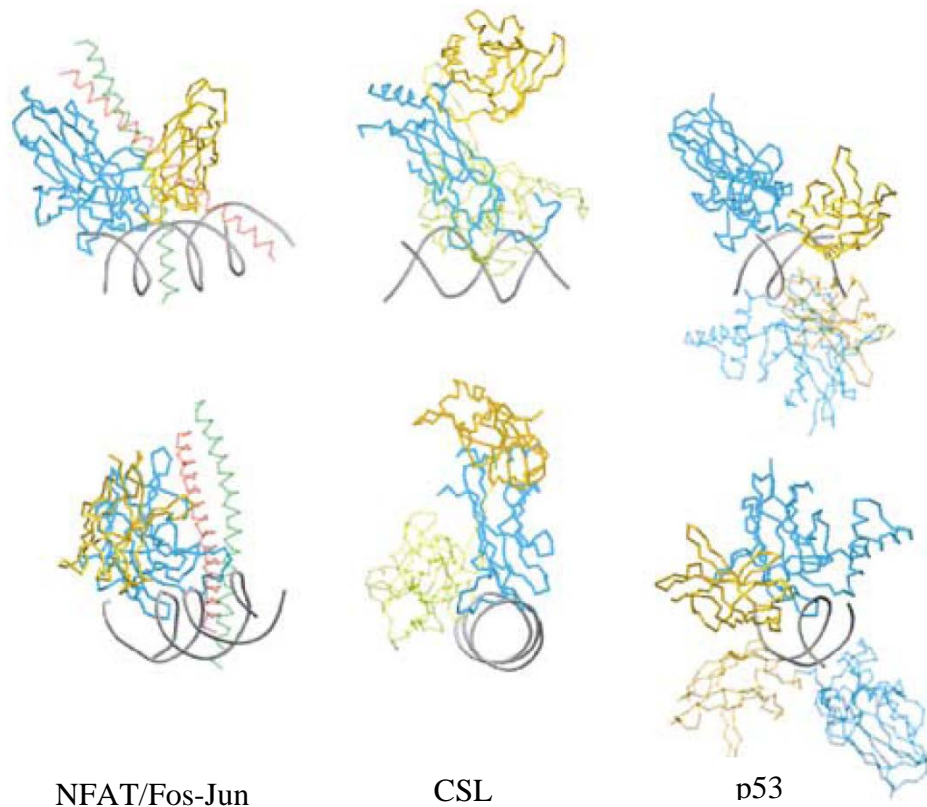
**Figure 2. 5** - Models of protein-DNA complexes showing the difference between domain organization in DNA-binding of three different types of transcription factors with Rel or Rel homology domains. On the left-hand side there is the NFAT/Fos-Jun/DNA structure. NFAT has two Rel domains where both are participating in interactions with DNA. In the middle there is CSL protein bound to DNA where out of two Rel homology domains only RHR-N is interacting with DNA and the second domain interacting with DNA is BTD. On the right-hand side there is the p52 homodimer bound to DNA. In that case, both Rel domains are interacting with DNA. In case of CSL proteins only one Rel domain is interacting with DNA compared to other two transcription factors where two Rel domains interact with DNA (adopted from Kovall and Hendrickson, 2004).

The BTD domain of CSL lacks the second pair of fibers B2 and B3, which is normally involved in the structure of the beta-barrel lid. Without these fibers a non-polar region is exposed. This non-polar pocket is preserved in the CSL family through evolution and is utilized by some interacting partners such as NICD, p48, EBNA2 and KyoT2 (Tamura et al., 1995; Beres et al., 2006; Ling and Hayward., 1995; Taniguchi et al., 1998). All of these proteins share consensus ΦWΦP motif (Φ stands for a hydrophobic amino-acid) which is probably responsible for the interaction with CSL. In case of the BTD domain from the CSL family, there are also some changes at the site of strands interconnections. The most significant change is a longer extended loop between the first B4 and second B1. This loop is parallel to the DNA and points away from the protein in a way that enables interaction

with other proteins (Kovall and Hendrickson, 2004). Mutations in this binding loop suppressed the function of CSL as a repressor by interrupting the interaction with CIR (CBF1 interacting corepressor) or EBNA2 (Hsieh et al., 1999; Fuchs et al., 2001; Sakai et al., 1998).
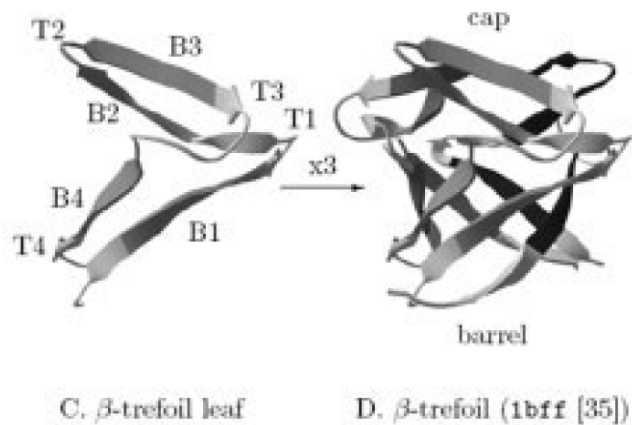


**Figure 2. 6** - Model of BTD. (C) Single β-trefoil leaf consisting of β-strands B1-B4 and turns T1-T4 (D)The whole β-trefoil structure consists of three of these leafs with cap (McDonnell et al., 2006).

### 2. 3. 2    DNA-binding properties of metazoan CSL proteins

CSL protein was originally identified as a factor that binds the immunoglobulin recombination signaling DNA site in B-cells (Kawaichi et al., 1992) and it was named RBP-J (recombination signal binding protein). Later another independent group showed that mammalian CSL protein CBF1 (C-promoter binding factor 1) binds to promoter C in Epstein-Barr virus sequence and that in fact this protein is identical to RBP-J (Henkel et al., 1994). Soon after the binding DNA sequence GTG $^G/_A$ GAA was identified as canonical motive along with other possible binding sites (TGGGAAA, TGGGAAAGAA, CATGGGAAA; Barolo et al., 2000; Dou et al., 1994; Lam and Bresnick, 1998; Morel and Schweisguth, 2000; Shirakata et al., 1996; Oswald et al. 1998; Tun et al., 1994; Lee et al., 2000). There are two parologues of CSL proteins found in mammalian genome called RBP-J and RBP-L and seem to bind the same recognition sequence (Beres et al., 2006). Notch-independent function of RBP-J and RBP-L in mammals with new recognition sequence was identified (look at chapter 2. 2 Notch independent functions of CSL). Domains RHR-N and BTD interact with negative charge of DNA helix through highly conserved surface with large positive charge. Specific interactions of RHR-N and BTD domains with major- and minor-groove of DNA are highly conserved among species.
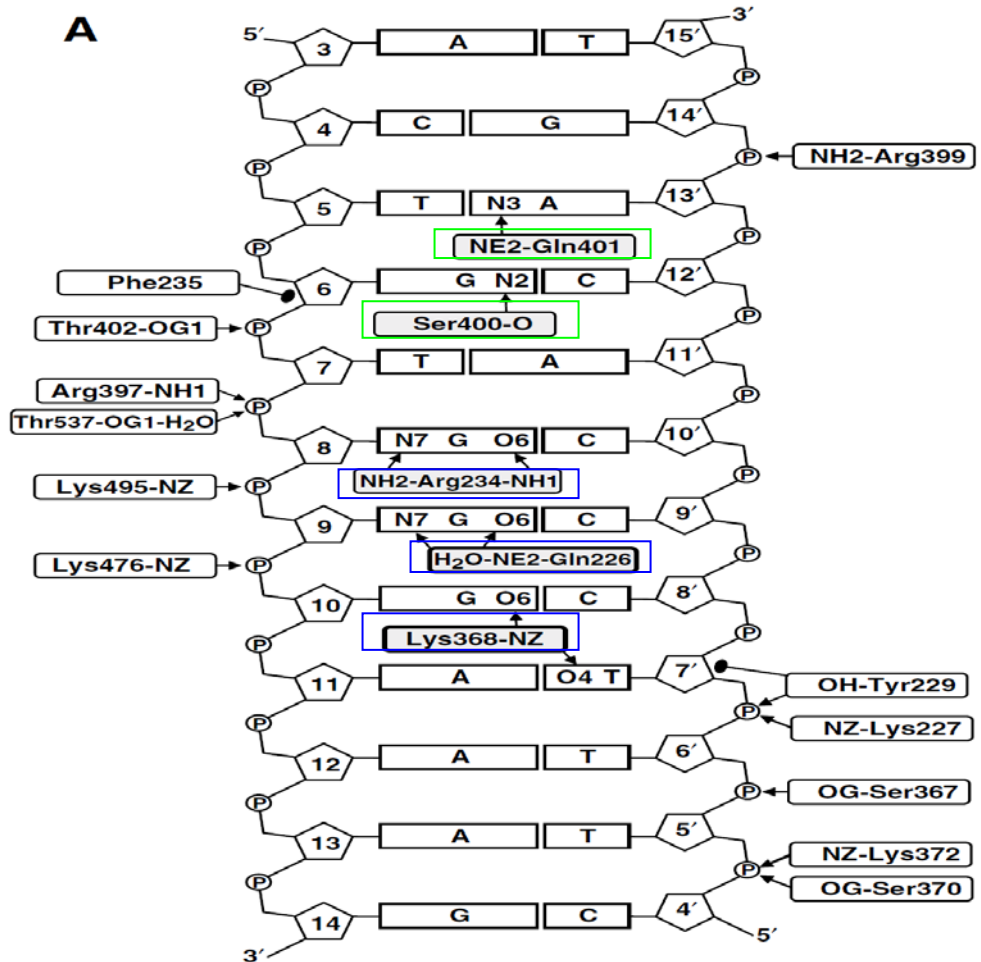
**Figure 2. 7** - Specific interactions of CSL domains with DNA are shown. Interactions of RHR-N or BTD with DNA are shown in blue or green, respectively. On the side there are the non-specific interactions of both domains with sugar-phosphate backbone of DNA (adopted from Kovall and Hendrickson, 2004).

Kovall and colleagues mapped a crystal structure of CSL protein Lag-1 from *C. elegans* bound to short doublestrand recognition element 5'-A5CTGTGGGAAAG14-3' and 5'-C4TTTCCCACAGT14-3', respectively, in the opposite direction. They identified specific interactions of amino-acid residues with specific groups of purines and pyrimidines within the DNA element (Figure 2. 7). In case of the RHR-N domain, they identified interactions of Arg234 with $G_8$, Lys368 with G10 and Gln226 with G9 in the major-groove. BTD provided specific interactions with minor-groove through Ser400 interacting with G6 and Gln401 interacting with A13. Beside these specific interactions, there are also some non-specific interactions of RHR-N and BTD with phosphate-ribose backbone (Kovall and Hendrickson, 2004).

## 2. 4   CSL in fungi

### 2. 4. 1  The aboundance of CSL proteins in fungi

Despite the fact that CSL-encoding genes are absent from genome of lower eukaryotes and plants, two distant homologues were identified in genome of *Schizosaccharomyces pombe* (Lai, 2002). Following this discovery a BLAST search of publicly available fungal genome was conducted revealing a group of fourteen fungal organisms coding homological genes of CSL proteins. These discovered genes were divided into two novel classes of CSL homologues F1 and F2 (Prevorovsky et al., 2007; Prevorovsky et al., 2011).

Phylogenetic distribution comparative study was conducted including newly found fungal CSL proteins and metazoan CSL proteins (Prevorovsky et al., 2011). The group of chosen CSL proteins from metazoa was denoted class M (Figure 2. 8). The knowledge of the fungal CSL proteins is not very deep and the only experimentally studied members are Cbf11 and Cbf12 found in fungi *S. pombe*.

*Schizosaccharomyces pombe* is a simple unicellular eukaryotic model organism with a completely sequenced genome of 12,57 Mbp (Wood et al.,2002). Because *S. pombe* belongs to Taphrinomycotina, the early branching group of ascomycetes (Hedges, 2002), it is a distant relative of other popular model organism *Saccharomyces cerevisiae*, but in fact it is an unique group of fungi extremely divergent in terms of the gene sequence. Many molecular characteristics of *S.pombe* resemble more closely higher eukaryotes than other groups of fungi.  This makes *S. pombe* a superior model for studying the cell cycle and its regulation, cell growth and polarity, the replication and repair of DNA, chromatin architecture and dynamics, and gene expression (Egel, 2004).

Two parologues of CSL proteins; Cbf11 (SPCC736.08) belonging to class F1 and Cbf12 (SPCC1223.13) belonging to class  F2 were identified in *S. pombe* (Prevorovsky et al., 2007). They are nonessential transcription factors localized to the nucleus excluding the nucleolus. Cbf11 is constitutively expressed in all growth phases of the cell culture in contrast to Cbf12 for which expression increases during the stationary phase and during meiozis (Prevorovsky et al., 2007). Cbf11 was shown to bind metazoan CSL binding sites compare to Cbf12, which did not bind any of the same sequences (Prevorovsky et al., 2007). Interestingly, N-terminally truncated variant of Cbf12 was shown to bind metazoan binding site (Prevorovsky et al., 2011).
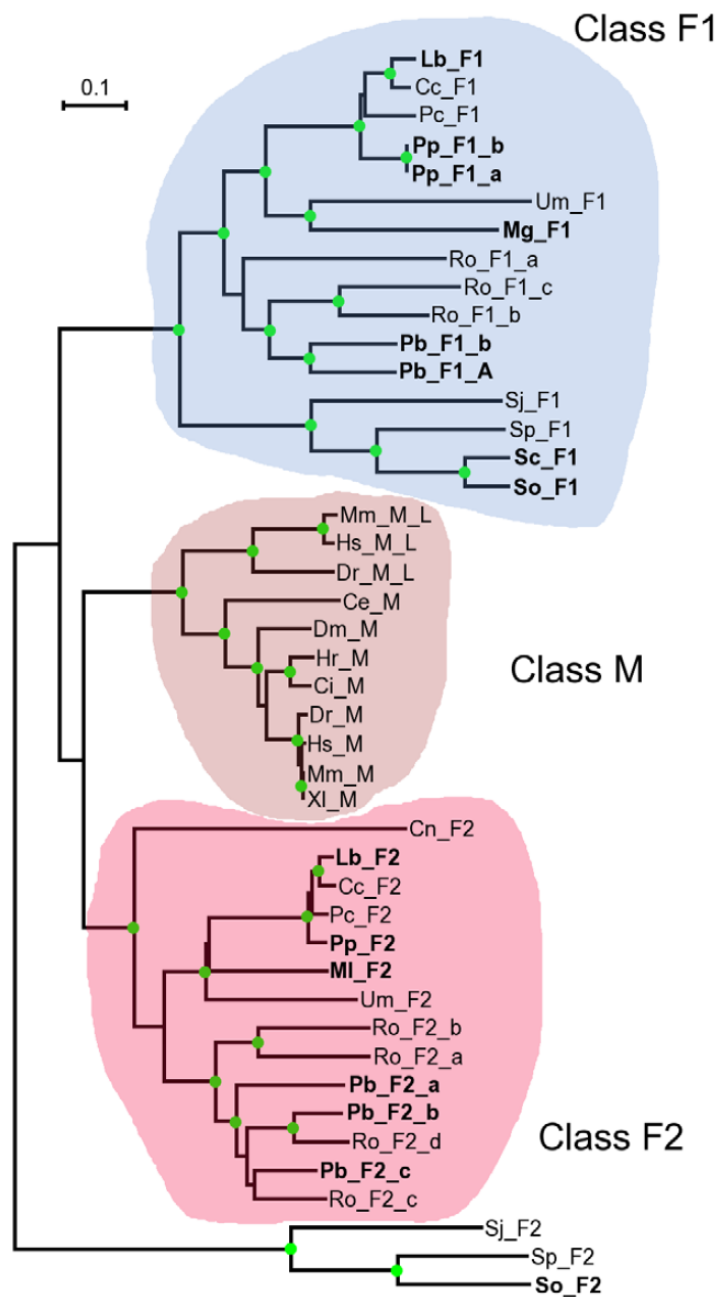
**Figure 2. 8** - An unrooted neighbor-joining phylogenetic tree of three CSL classes F1,F2,M. List of organisms: **Schizosaccharomyces pombe** (Sp), **S. octosporus** (So), **S. japonicus** (Sj) **S. cryophilus** (Sc) belong to ascomycetes. **Coprinus cinereus** (Cc), **Cryptococcus neoformans** (Cn), **Laccaria bicolor** (Lb), **Malassezia globosa** (Mg), **Melampsora laricispopulina** (Ml) **Phanerochaete chrysosporium** (Pc), **Ustilago maydis** (Um) and **Postia placenta** (Pp) belong to basidiomycetes. **Rhizopus oryzae** (Ro) and **Phycomyces blakesleeanus** (Pb) are zygomycetes. Representative metazoan CSL sequences are from **human** (Hs), **mouse** (Mm), **zebrafish** (Dr), **Xenopus laevis** (Xl), **Ciona intestinalis** (Ci), **Halocynthia roretzi** (Hr), **fruit fly** (Dm) and **Caenorhabditis elegans** (Ce).tree of class F1,F2 and M of CSL proteins (adopted from Prevorovsky et al., 2011).

24

In previous years studies with mutant strains were conducted to determine the functions of CSL genes in *S. pombe*. These mutant strains had deletion of one or both CSL genes. Due to comparing the mutant strains to the wild type strain interesting observations were made. Both Cbf11 and Cbf12 effect cell-cell and cell-surface adhesion, while Cbf11 acts as a negative regulator and Cbf12 as a positive regulator. Deletion of Cbf11 induced growth phenotype in 19°C (cold sensitivity) and also in 30°C, where the effect was strengthen by the deletion of Cbf12. In contrast the the overexpression of Cbf12 from plasmid seemed to be toxic for the double-knockout or wild type strains. The strains with deletion of Cbf11 or overexpression Cbf12 showed stable diploid-like populations which is abnormal for *S. pombe*. The same strains also displayed various cell separation defects highly resembling the fission yeast sep mutants (Prevorovský et al., 2009). Sep mutants show various defects in creation of septum and separation of dividing cells which can lead to formation of multinucleate cells (Sipiczki et al., 1993; Grallert et al., 1999). From the results obtained from experiments with Cbf11 and Cbf12 a model of CSL functioning in *S. pombe* was composed (Figure 2. 9)
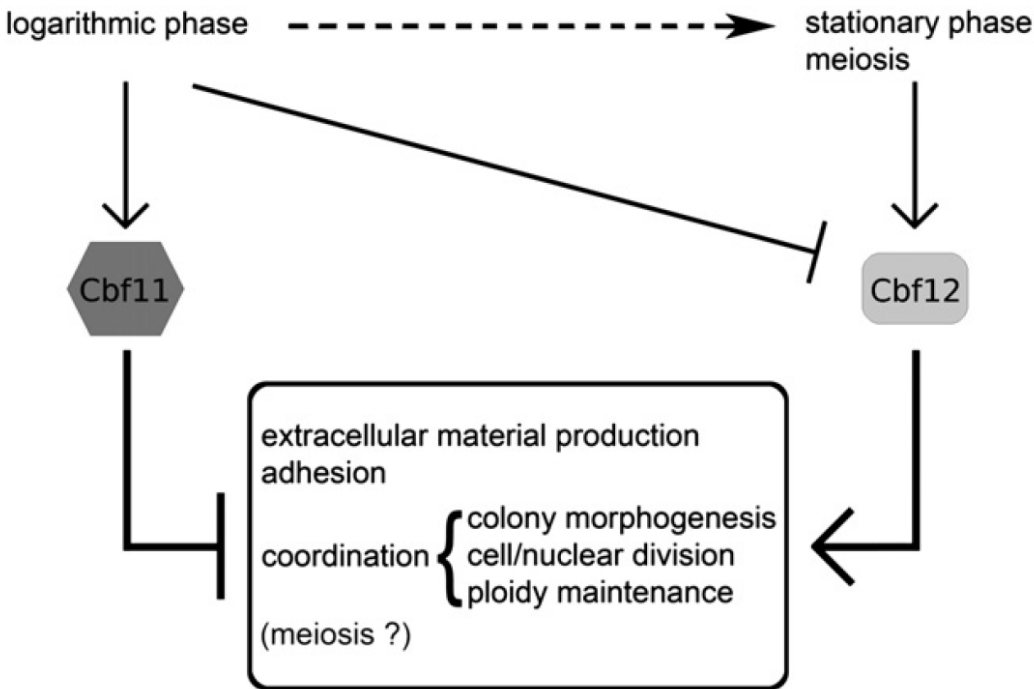


**Figure 2. 9** - Model of functions and interaction of CSL proteins in the fission yeast. CSL proteins Cbf11 and Cbf12 seem to play opposing roles such cell processes as adhesion, extracellular material production and coordination of colony morphogenesis, cell/nuclear division and ploidy maintenance (adopted Prevorovsky et al., 2009).

## 2. 4. 2    N-terminal domains of CSL proteins in fission yeast and other fungi

Newly discovered CSL proteins in fungi have some notable differences from metazoan CSL proteins. They are generally larger proteins and most notably the N-terminal domains in fungal CSL proteins are significantly longer then in case of metazoan proteins. These N-terminal extensions show low level of complexity suggesting the lack of 3D structure in this part of protein. CSL proteins in *S. pombe*, especially Cbf12, have significantly long N-terminal extension which is predicted to be highly disordered (Prevorovsky et al., 2011). Long disordered extensions are usually easily accessible and often serve as sites of posttranslational modifications as phosphorylation, ubiquitinylation, proteolytic processing or can also mediate protein-protein interactions (Xie et al., 2007). For example the N-terminal part of the cyclin-dependent kinase (CDK) inhibitor p21, approximately 60 amino-acids, is an intrinsically disordered part of the protein in the free solution. When it binds to Cdk2, it adopts an ordered stable conformation. This most likely contributes to the ability to bind and inhibit multiple Cdk complexes including cyclin A-Cdk2, cyclin E-Cdk2 and cyclin D-Cdk4 (Kriwacki et al., 1996).

For Cbf12 protein from *S. pombe* the phosphorylation sites were analyzed using mass spectrometry. Results showed 16 sites for N-terminal part, 3 sites for core part and 0 sites for C-terminal part. Western blotting experiments showed multiple sizes of tagged Cbf12 proteins in lysate suggesting a potential posttranslational cleavage (Prevorovsky et al., 2011). Truncated variant of protein $Cbf12_\Delta(1\text{-}394)$ that had the first 394 amino-acids cut off from the N terminus was constructed and showed the ability to bind CSL canonical binding motive RBP (see section 4) compared to full length Cbf12 which didn't bind the RBP motive (Prevorovsky et al., 2011). This result points to the importance of extended N-terminal domains of fungal CSL proteins.

# 3.    Materials and methods

## 3.1 Microorganisms and cultivations

### 3. 1. 1 *Escherichia coli*

**DH5α** strain          F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1

*hsd*R17(r$_K^-$, m$_K^+$) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻

### 3. 1. 2        *Schizosaccharomyces pombe*

| Strain | Genotype | Source |
|--------|----------|--------|
| PN559 | *h⁻ leu1-32 ura4-D18 ade6-M216* | (Decottignies et al., 2003) |
| MP09 | *h⁻ leu1-32 ura4-D18 ade6-M210 Δcbf11::kan$^r$ Δcbf12::pCloneNat1⁻* | (Prevorovsky et al., 2009) |
| MP03 | *h⁻ leu1-32 ura4-D18 ade6-M216 Δcbf12::pCloneNat1* | (Prevorovsky et al., 2009) |

**Table 3.1** – *S. pombe* strains used in the study.

### 3. 1. 3        Cultivation of microorganisms

All used microorganisms were cultured in standard media listed below. The G418 (100 µg/ml, Sigma) and/or ClonNAT (90 µg/ml, Werner Bioagents) antibiotics were added to YES for selection purposes when required.

All yeast strains were cultivated in 30°C.

*E. coli* strain was cultivated in 37°C.

When required, the tenfold serial dilution ($10^5$-$10^0$ cells) was prepared of washed cells from exponentially growing liquid cultures and spotted on solid media.

**Cultivation of *S. pombe* strains with inducible tagged protein expression the thiamine-repressible plasmids pREP41HA or pREP42GFP**

EMM minimal medium without appropriate amino acids and with thiamine was inoculated by desired *S. pombe* strain and left to grow to mid logarithmic phase. Then the cells were 3x centrifuged (Jouan MR 22i, rotor SWM 180.5; 5 000 rpm, 3 min., 4°C). Each time between the centrifugations the media in tubes was changed to media without thiamine and the sells were resuspended. After the third centrifugation the inoculum was diluted to 10-20 ml with OD 0.1 measured by (Specol 20, $\lambda$ = 595 nm). Then the strains were left shaking 200 rpm in 30°C for 20 hours diluting the culture to keep the OD under 0.8. After 20 hours the cultures were prepared for further use with high expression of tagged proteins from pREP41/42 plasmids.

**Medium *E. coli*:**

| | | |
|---|---|---|
| **LB** | 10g/l | Universalpepton M66 (Merck) |
| | 5g/l | yeast extract (Formedium Ltd) |
| | 5g/l | NaCl (Penta) |

| | | |
|---|---|---|
| **2x LB** | 20g/l | Universalpepton M66 (Merck) |
| (superLB) | 10g/l | yeast extract (Formedium Ltd) |
| | 5g/l | NaCl (Penta) |

**Agar medium (ŽA)** 40g/l Živný agar č. 2 (Imuna)

Ampiciline antibiotics (Biotika) were added to final concentration of 100 (µg/ml) if needed.

**Medium *S. pombe*:**

| | | |
|---|---|---|
| **YES** | 0.5% | yeast extract (Formedium Ltd) |
| | 3% | glucose (Sigma) |
| | 0.025% | SP supplements (Formedium Ltd) |

Glucose and medium were sterilized (autoclav) separately and mixed afterwards, 2% agar was added for solid medium.

**EMM (Edinburgh Minimal Media)**

EMM broth without dextrose          1.23 %
Glucose                             2 %

add appropriate  amino acids for selection:

| Leucin | 0.04 % |
|--------|--------|
| Adenin | 0.04 % |
| thiamine | 0.001 % |

- 2% agar was added for solid medium

Ampiciline antibiotics (Biotika) were added to final concentration of 100 (µg/ml).

**Strain storage:**

All prepared strains were stored for later use as glycerol stock. 60% glycerol (Sigma) was added to cell culture to final 30% glycerol concentration and stored at -80°C.

For short term storage during experiments strains were kept on Petri dishes at -20°C for maximum period of 3 weeks. After that new a stock was used.

### 3. 1. 4     Transformation

*E. coli* **by electroporation**

0.5–5 µl of solution with plasmid DNA (5 pg-0.5 µg DNA) was mixed with 30-50 µl electro-competent cells *E. coli*. Solution was kept on an Ice the whole time. Final solution with plasmid DNA was transferred to precooled 0.2 cm electroporation cuvette (Biorad). Gene Pulser Apparatus (Biorad) was set to capacitance 25 µF, electric potential 2.5 kV and resistance 200 Ω. After the cuvette was placed in the slot a pulse was generated optimally

4-5 ms. Immediately after the pulse the culture was resuspended to 0.5 mL of LB medium with 0.5 % of glucose and left shaking at 37°C for 50 minutes. Finally the cells were spread in a petri dish with ŽA medium with required selection antibiotics.

*S. pombe* **by lithium acetate technique**

Recipient cells grown for 2-3 days on solid medium were moved in sterile condition from plate and resuspended in reaction mix (74 μl 50% PEG-4000 (Sigma), 7 μl 1M LiAc (Sigma) a 7 μl 10x TE). 2 μl of denatured ssDNA (salmon sperm DNA, 10mg/ml, Sigma) was add along with 1-5 μl of plasmid DNA (>100 ng). The solution was gently mixed and incubated in 30ºC for 60-240 min. Then the heat shock 42ºC was applied fo 15 min. The solution was spread on appropriate plates and grown at 30ºC for 4-6 days.

**Reaction mix (**all solutions sterile)**:**

**50% (w/v) PEG-4000**

**1M LiAc**

**10x TE** (100 mM Tris-HCl, 10 mM EDTA; pH 7.5)

### 3. 1. 5 Adhesivity assay

Cells were spotted or patched on appropriate EMM plates and grown at 30°C for various times. The adhesivity assay (based on a protocol described in (Guldal and Broach, 2006)) consisted of washing the plates under en even stream of water for 1 min. The spot cell mass remaining attached to the plate was then documented as photography using an Olympus SP-350 digital camera.

## 3. 2 Protein techniques

### 3. 2. 1 Preparation of native and denatured cell lysates from *S. pombe*

**Native lysate -**

Day 1: Appropriate medium was inoculated by cells from Petri dish and left shaking at 30°C at 200 rpm until the optical density (OD) reached value of 1 measured by

spectrophotometer (Specol 20, $\lambda$ = 595 nm). If the cells with pREP41/42 plasmids were cultivated, they were kept growing for 20 hours to express proteins under the *nmt1* promoter (Craven et al., 1998).

Day 2: Lysis Buffer was supplemented with 1/500 of volume of 1M DTT and 1/100 of volume of protease inhibitor FY (Serva) and after that it was kept on an ice. Cell culture was cooled down on an ice for 10 min. After that cells were transferred to test tubes (Falcon) and harvested by centrifugation (Jouan MR 22i, rotor SWM 180.5; 5 000 rpm, 3 min., 4°C). Sediment was resuspended in STOP buffer and centrifuged (Jouan MR 22i, rotor SWM 180.5; 5 000 rpm, 3 min., 4°C) again and the sediment was kept at -80°C. Following procedures were performed on an ice. Bead beating tube was half filled with precooled beads (425-600 μm, Sigma). Sediment was resuspended with 40 μl of lysis buffer mix and added to bead beating tube. Sample was loaded to FastPrep®-24 (M.P. Biomedicals) and shaken 3x 20 seconds with the speed to 5. Sample rested for 5 min on ice between each run. After that the tube was punctured and 600 μl of lysis buffer mix were added. The tube was put to another tube and the solution was centrifuged (Jouan MR 22i, rotor SWM 180.5; 1 000 rpm, 1 min., 4°C) to the new tube. Whole volume was moved to a new microtube and centrifuged (20 000 g, 20 min., 4° C). The supernatant (native lysate) was collected to a new microtube and stored at -80°C.

**Solutions:**

**Lysis buffer 1**       25mM HEPES (pH 7.6), 0.1mM EDTA (pH 8), 150mM KCl,
                         0.1% Triton X100, 25% glycerol, 1M urea; pH 7.6
                         pH adjust using KOH,

**STOP buffer**          150 mM NaCl, 50 mM NaF, 25 mM HEPES, 1 mM $NaN_3$;
                         pH 8

**Denatured lysate -**
Inoculum of growen cells (10 ml) was harvested by centrifugation (Jouan 3000rpm, 5min, 4°C), resuspended in 1ml ice cold STOP buffer and transfered to Eppendorf tube. Then it was entrifuged (Jouan 6000rpm, 5min, 4°C) and the supernatant was disposed off while the sediment was put to freezer -80°C for 20min. Then the sediment was resuspended in 100ul

(93ul of 2M NaOH+7ul 2-mercaptoethanol), the rest of the procedure was performed on ice including pre-cooled reagents. Sample was vortexed for 2min (4ºC) and 100ul of 50% TCA was add and the sample was kept on ice for 5min. Sample was then centrifuged ( Jouan 12000rpm, 2min, 0ºC) and the supernatant was disposed of.  Then 500ul of 1M Tris was added and the sample was resuspended and centrifuged (Jouan 12000rpm, 2min, 0ºC). Supernatant was disposed of and sample was resuspended in 100ul of 2x Laemmli buffer preheated to 80ºC. Then the sample was incubated in +80ºC for 20min and after that cooled on ice. Finally the sample was centrifuged (12000rpm, 3min, Room Temperature) and the supernatant was transfered to a new Eppendorf tube. Samples were stored in -80ºC.

**Solutions:**

STOP buffer                 –    50mM NaF

                             -    1mM $NaN_3$ (azid)

                             -    25mM Hepes (sigma, USA) ($K^+$) pH 7.0

                             -    150mM NaCl

pH=8, sterilization (autoclave)

NaOH mix      – 1.85M NaOH

               - 7% 2-mercaptoethanol        ( add fresh each time)

50% TCA

1M Tris

2x Laemmli + DTT

### 3. 2. 2       Protein concentration measurement DC protein assay (Bio-Rad)

Before the procedure, standards were prepared to create graph to correlate data to.
Standards:

V = 50 μl

|       | Concentration BSA | BSA (2 mg/ml) (Bio-Rad) | Lysis buffer 1 | MP H$_2$O |
|-------|-------------------|-------------------------|----------------|-----------|
| S1    | 0.2 mg/ml         | 5 μl                    | 5 μl           | 40 μl     |
| S2    | 0.6 mg/ml         | 15 μl                   | 5 μl           | 30 μl     |
| S3    | 1.2 mg/ml         | 30 μl                   | 5 μl           | 15 μl     |
| S4    | 1.8 mg/ml         | 45 μl                   | 5 μl           | -         |
| Blank |                   | -                       | 5 μl           | 45 μl     |

Samples:  5 μl of native lysate + 45 μl of MP H$_2$O

Concentration of samples was measured according to manufacture instructions. First the standard curve was constructed by measuring the absorbance of Standards with known concentration by spectrophotometer (UV-1650 PC Shimadzu) at λ = 750 nm. Then the samples absorbance was measured and the concentration was calculated from standard curve.

### 3. 2. 3 SDS-polyacryleamid gel electrophoresis (SDS-PAGE)

Mini-ProteanIII (Bio-Rad) apparatus was used.

Aperture for gel preparation was put together according to manufacture instructions.

Acrylamide separation gel of relevant concentration was mixed.

Separation gel mixture:

| final concentration of acrylamide gel | AA/BIS (ml) | 4xTris-HCl/SDS pH 8.8 (ml) | MP H$_2$O (ml) |
|----------------------------------------|-------------|-----------------------------|-----------------|
| 7.5%                                   | 1.25        | 1.25                        | 2.5             |
| 10%                                    | 1.67        | 1.25                        | 2.08            |
| 12%                                    | 2.0         | 1.25                        | 1.75            |

Before applying the mix to glass 16.5 μl of 10% (w/v) APS (ammonium persulfate, Sigma) and 3.4 μl of TEMED (Bio-Rad) was added. Then the mix was quickly applied and

covered by isopropanol then left to polymerize. After the separation gel is polymerized the isopropanol layer was removed and stacking gel was mixed:

Stacking gel mixture:

| AA/BIS (ml) | 4x Tris-HCl/SDS pH 6.8 (ml) | MP H$_2$O (ml) |
|---|---|---|
| 0.325 | 0.625 | 1.55 |

12.5 μl of 10% (w/v) APS and 2.5 μl of TEMED was again added before the application of mix. The comb was quickly inserted and the gel was left to polymerize. When the gel was ready the comb was removed and glasses with the gel inside were moved to aperture and the rest of the aperture to run the gel was put together according o manufacture instructions and filled with 1xSDS buffer. Samples kept on a nice were mixed with 1/10 of 1M DTT and 1/5 of Leammli buffer. Final sample mix was boiled for 10 minutes and centrifuged (Hettich MIKRO 20; 10 000 rpm, 2 min., 20°C) and returned back to the ice. Samples were carefully applied to gel and the apparatus was run at 20 mA per gel. When the proteins were separated the gel was visualized by Coomassie Brilliant Blue R-250 or transferred to nitrocellulose membrane by Western blotting technique.

**Solutions:**

**AA/BIS**          30% acrylamide (Sigma),

0.8% (w/v) N,N´-methylenbisakrylamidu (Merck)

Filtrate with 0.45 μl filter, store in dark at 4°C

**4x Tris-HCl/SDS**    1.5 M Tris, 0.4% SDS; pH 8.8

pH modulate with HCl

Filtrate with 0.45 μl filter, store in dark at 4°C

**4x Tris-HCl/SDS**    0.5 M Tris, 0.4% SDS; pH 6.8

pH modulate with HCl

Filtrate with 0.45 μl filter, store in dark at 4°C

**5x SDS buffer**         125 mM Tris, 950 mM glycin, 0.5% SDS; pH 8.3
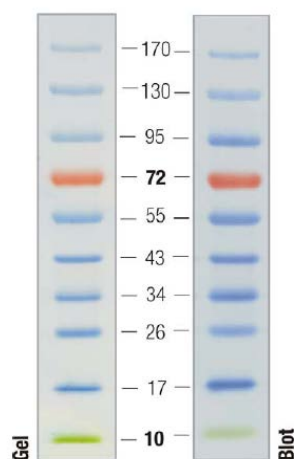
**Figure 3. 1** - Standard for protein size (PageRuler$^{TM}$ Prestained Protein Ladder, kDa, Fermentas).

### 3. 2. 4    Staining of proteins in polyacrilamide gel

Gels were stained in a dye solution for 30 minutes and then were left shaking in destaining solution over-night in room temperature.

**Solutions:**

**Dye solution**   20% methanol, 0.117% (v/v) Coomassie Brilliant Blue R-250 (Bio-Rad), 0.051% (v/v) Bismarck Brown R (Sigma-Aldrich), 7% acetic acid in $H_2O$

**Destaining solution**   7% Acetic acid, 5% methanol

### 3. 2. 5    Western blotting

Mini Trans-Blot Module (Bio-Rad) apparatus was used.

Gels were prepared from glass and cut off the stacking gel area. Gel was put on a filter paper resting on a pad lying on the blot cell and the nitrocellulose membrane was put over it. Then another filter paper was placed creating the blot sandwich and a top pad was placed. The blot cell was closed and put to the apparatus in correct direction. The apparatus was filled with the transfer buffer and a body of ice. The blot was run for 2 hours at 100 V.

**Solution:**

**Transfer buffer**        25 mM Tris, 192 mM glycine, 20% methanol; pH 8.05

### 3. 2. 6      Protein immunodetection

After Western blotting the nitrocellulose membrane was washed in TBS for 5 minutes. 30 mL of 3% non-fat dry milk in TTBS was prepared and the membrane is blocked in the mix shaking at 37°C for 1 hour. After the membrane was blocked it was incubated in 3% non-fat dry milk in TTBS with selected primary antibody with the right concentration (see table 3.2) over-night with continuous movement at 4°C. Next day the primary antibody was disposed and the membrane was washed 3x5 minutes in TTBS. Then it was incubated in 3% non-fat dry milk in TTBS with the secondary antibody with the right concentration (see table 3.3) for 1 hour. After that the membrane was removed from secondary antibody and washed 3x5 minutes in TTBS and 1x5 minutes in TBS. After that the membrane was developed according to type of secondary antibody. In case of Secondary antibody conjugated with peroxidase the luminogenic substrates (Amersham) were used to develop the membrane and LAS-4000 (Fujifilm) to document. In case of secondary antibody conjugated with alcalic phosphatase the membrane was washed in AP buffer and then put to developing mix consisting of (AP buffer, 1/2000 1M $MgCl_2$ and 1/100 of solution A and Solution B). The membrane was incubated in dark for 1 day. Developed membrane was scanned for documentation.

**Solutions:**

**TBS**          20 mM Tris, 500 mM NaCl; pH 7.5

                 Set the pH using HCl


**TTBS**         TBS s 0.05% Tween 20 (Serva)


**AP buffer**    100 mM Tris-HCl; pH 9.5


**substrate A**  30 mg NBT (nitroblue tetrazolium) in 70% DMF (dimenthylformamid), 100x concentrated


**substrate B**  15 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate) in $H_2O$, 100x concentrated

| Name, specifity | Dilution | company |
|---|---|---|
| His•Tag antibody (mouse monoclonal) | 1: 2 000 | Novagen (70796) |
| anti HA.11, purified (mouse monoclonal) | 1: 1 000 | Covance (MMS-101-P) |
| GFP Antibody (FL) (rabbit polyclonal IgG) | 1: 1 000 | Santa Cruz Biotechnology (sc-8334) |

**Tab. 3.2** - List of primar antibodies

| Name, specifity | Dilution | company |
|---|---|---|
| goat anti-mouse IgG-HRP | 1:10 000 | Santa Cruz Biotechnology (sc-2031) |
| goat anti-mouse IgG-AP conjugate, | 1: 2 000 | Bio-Rad (170-6520) |
| goat anti-mouse IgG-AP conjugate, | 1: 2 000 | Bio-Rad (170-6518) |

**Tab. 3.3**- List of secondar antibodies

Synthetic positive control: Posi-Tag Epitope Tag Control Protein (Covance)

## *3. 3   DNA techniques*

### 3. 3. 1   Isolation of Plasmid DNA from E. coli by Alkaline Lysis

Day 1:

20-50 ml of appropriate medium (LB medium) with selective antibiotics (ampicilin, 100 µg/ml, Biotika) in Erlenmeyer flask was inoculated by *E. coli* cells from Petri dish and left shaking at 37°C at 200 rpm overnight (minimum of 16 hours).

Day 2:

The culture was left to cool down on an ice and was transferred to centrifugation tube (50 ml Falcon). Then it was centrifuged (Jouan MR 22i, rotor SWM 180.5; 4 400 rpm, 10 min., 4°C). Sediment was resuspended in 3 ml of precooled solution I and mixed by vortex or pipette. The tube was removed from the ice and 6 ml of solution II was added. Tube was gently mixed and left to rest in a room temperature for 5 min. After that the tube was put back on an ice and 4.5 ml of precooled solution III was added. Tube was gently mixed and

left to rest on the ice for 15 min. White precipitate was formed. Mixture was tranfered to polypropylen centrigugation tube and span with (Jouan MR22i, rotor AM 38.15; 13 000 rpm, 20 min., 4°C). Supernatant was transferred to a new tube and exact volume of 0.6 of isopropanol was added. Immediately the mixture was shaken and cetrifuged (Jouan MR 22i, rotor SWM 180.5; 4 000 rpm., 6 min., 20°C). Supernatant was disposed of and the sediment was left to dry out and dissolved in 300 μl of MP $H_2O$. The mixture volume was measured and the mixture was transferred to a new Eppendorf tube. One volume of 10M LiCl was added and the mixture was put to -80°C for 20 minutes. The mixture was centrifuged with (Jouan MR 22i, rotor AM 38.15; 15 000 rpm, 5 min., 4°C) and the supernatant was transferred to a new Eppendorf tube. One volume of 96% ethanol was added and the tube was stored at -80°C for 20 minutes. After that the mixture was centrifuged with (Jouan MR 22i, rotor AM 38.15; 15 000 rpm, 5 min., 4°C), the supernatant was disposed of and the sediment was washed with 0.5 ml of 70% ethanol and centrifuged again with (Jouan MR 22i, rotor AM 38.15; 15 000 rpm, 5 min., 4°C). In the end the ethanol was removed and the sediment was left to dry 30 minutes at 37°C. Finally the dried sediment was dissolved in 100 μl of MP $H_2O$. The quality of procedure and DNA concentration was determined by DNA electrophoresis.

**Solutions:**

| | |
|---|---|
| **solution I (TEG)** | 25 mM Tris-HCl (pH 8.0), 10 mM EDTA-NaOH, 1% glucoses |
| **solution II** | 1% SDS, 0.2 M NaOH |
| **solution III** | 3M (KAc), 2M acetic acid; pH 4.8-5.4 |

### 3. 3. 2    Agarose gel DNA electrophoresis

Gel DNA electrophoresis was performed in horizontal apparatus in 1 x TAE buffer. Gels were 6-10 cm long and 3-5 mm thick. The distance between electrodes was 14 cm. SeaKem LE (FMC) agarose was used in 1-2% concentration depending on the size of DNA fragments. 5–50 μl of DNA sample (10 ng–2 μg DNA) was loaded mixed with 1/6 of volume of loading buffer (Fermentas). One of the loads was 6 μl of Marker. For separation the voltage of 0.3-10 V/cm was used. DNA was visualized with ethidium bromide solution (0.5 μg/ml) under an UV light. For documentation photos were taken with Panasonic DMC-F27 with red/UV filter on.

**Solutions:**

**1x TAE**                40 mM Tris, 20mM cold acetic acid, 2 mM $Na_2EDTA$;

                               pH 8.5

**6x sample buffer**    0.06% bromphenol blue, 60% glycerol, 60 mM EDTA
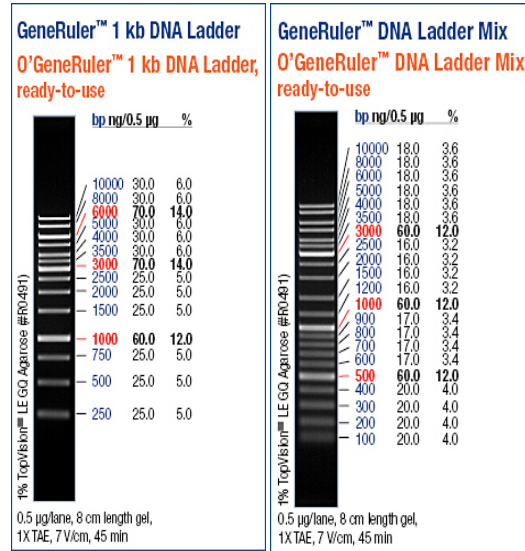


**Figure 3.2** – Standards of DNA fragment size and concentration.

### 3. 3. 3        DNA isolation from agarose gel

Fragment of the gel containing only desired DNA was cut out with a scalpel and put to an Eppendorf tube. The weight was then determined. Following procedure was taken from kit manual NucleoSpin®Extract II. For every 100 mg of gel 200 μl of buffer NT was added (if the agarose gel had higher concentration then 2%, another 400 μl of buffer NT was added). Mixture was incubated at 50°C while shaking until the agarose fully melted (5-10 min). Sample was loaded to Column placed into a collection Tube (2 ml) and cetrifuged for 1 min at 11 000 x g. The flow-through was discarded and 600 μl of Buffer NT3 was added. Then the sample was centrifuged 1 min at 11 000 x g and the flow-through was discarded again. The sample was once again centrifuged 2 min at 11 000 x g. Finally the column was placed into a clean 1.5 Eppendorf tube and 15-50 μl of Elution buffer were added. After resting the tube for 1 min the sample was cetrifuged 1 min at 11 000 x g. Isolated DNA was in the tube.

### 3. 3. 4      The use of DNA restriction modification enzymes

The reaction mix (20 ul is listed below) was prepared in a 0.5 ml micro tube:

| | |
|---|---|
| DNA sample in $H_2O$/TE buffer | x µl (0.1-4 µg DNA) |
| 10x restriction buffer | 2.0 µl |
| Deionized MP $H_2O$ | add the reaction to total volume of 20 µl |
| Restriction endonucleasis | 1-10 Units of enzym per 1 µg DNA |

Type of restriction buffer, time and temperature of reaction was determined according to enzyme manufacture instructions.

The reaction was left for 1 to 4 hours in optimal temperature (usualy 37°C). The reaction was stopped by freezing in -20°C or by heat shock at 65°C for 15 min. Another method was glycogen DNA precipitation.

The amount and quality of cut DNA was determined by DNA electrophoresis.

### 3. 3. 5      DNA ligation

Reaction mix (10 ul) was prepared in a 0.5 ml micro tube:

| | |
|---|---|
| DNA in MP $H_2O$/TE buffer | x µl (50-100 µg DNA) with chosen molar ratio between Vector and insert |
| 10x ligation buffer (Fermentas) | 1.0 µl |
| MP $H_2O$ | add to total volume of 10 µl |
| T4 DNA ligse | 0.5 Units of enzyme (1 U/µl, Fermentas) |

The tube with ligation was placed in a water bath of 19°C and placed into a fridge. There it was left overnight for DNA to ligate.

### 3. 3. 6      PCR

**PCR**

The enzymes (see Table 3.3) were used according to the manufacturers' specifications; the primers (see Table 3.4) were used in 0.3 µM final concentration. Reactions were run on the

Peltier PTC-200 gradient thermal cycler (MJ Research). Typically, the MgCl$_2$ concentration of 2.5 mM was used. Site directed PCR mutagenesis was performed using QuikChange®II Site-Directed Mutagenesis Kit according to manufacture protocol.

| Step | temperature(°C) | time (m:ss) |
|------|-----------------|-------------|
| 1 | 95 | 0:30 |
| 2 | 95 | 0:30 |
| 3 | 50 | 1:00 |
| 4 | 68 | 6:30 |
| 5 | go to 2 - 19 x | |

Site directed mutagenesis Cbf12

| Step | temperature(°C) | time (m:ss) |
|------|-----------------|-------------|
| 1 | 94 | 0:20 |
| 2 | 95 | 0:30 |
| 3 | 44 | 0:30 |
| 4 | 68 | 1:30 |
| 5 | go to 2 - 2 x | |
| 6 | 94 | 0:20 |
| 7 | 68 | 2:00 |
| 8 | go to 2 - 23 x | |
| 9 | 68 | 5:00 |

Cbf11 and Cbf12 truncated variants synthesis

| MT01 | GCAGGATCCTAAACCTAGTCAGCTGGTAAC |
|------|--------------------------------|
| MT02 | GGATCCCGGGTCAGTTTCCAAAAGCACTTG |
| MT03 | GGATCCACATATGAATTGCCATTGTTTAAGC |
| MT04 | GGATCCCGGGTTAGTGACTTTCCAAAGG |
| mutF | CATTGTTATGCTGAGCCCGGTA**GTGA**TTGAATAAACAAACGC |
| mutR | GCGTTTGTTTATTCAA**TCAC**TACCGGGCTCAGCATAACAATG |

**Table 3. 4** – Table of used primers. mutF and mutR were used for site-directed mutagenesis, mutated nucleotides are in bold. MT01,MT02 for truncated variant of Cbf11 and MT03,MT04 for truncated variant of Cbf12.

## 3. 3. 7        Vectors

Plasmid constructs that were used or prepared and used in this study are listed in (Figure 3.6). General maps of used plasmids are on (Figure 3.5)
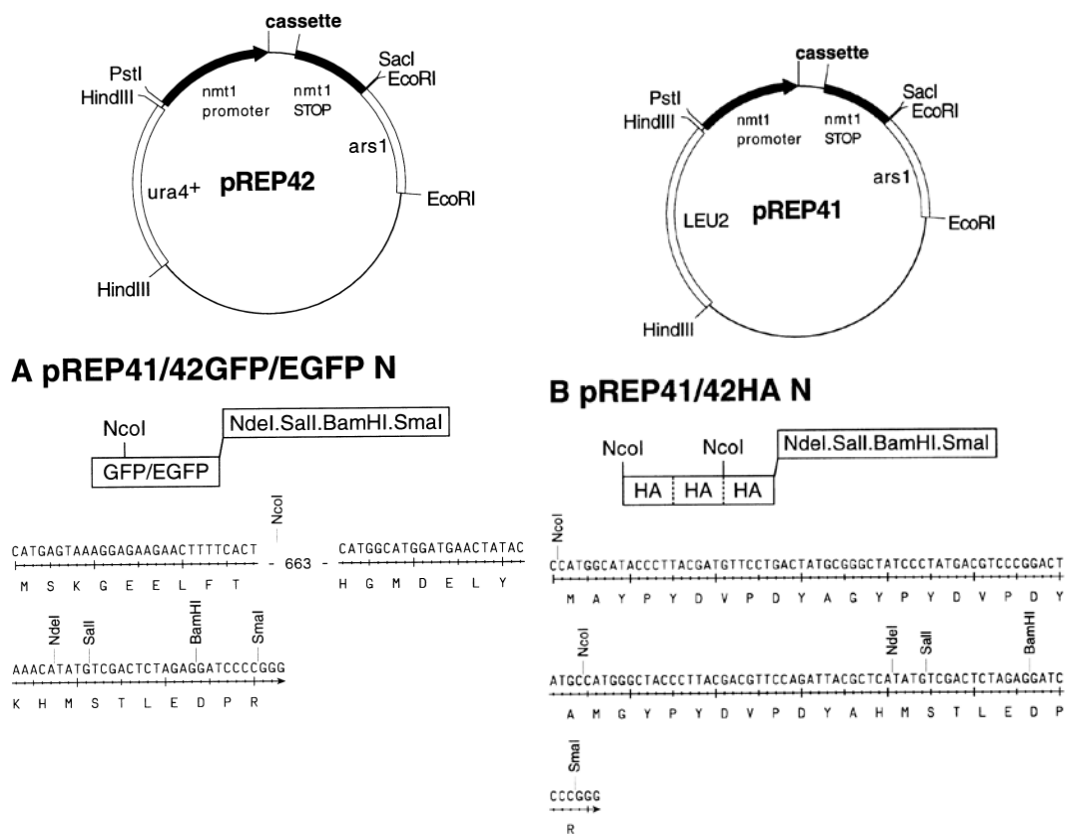


**Figure 3. 3** – pREP41/42 series of plasmids used in the project. (A) pREP42GFPN plasmid used for overexpression of N-terminally GFP-tagged CSL proteins in *S. pombe*. (B) pREP41HAN plasmid used for the overexpression of N-terminally HA-tagged CSL proteins in *S. pombe* (Craven et al., 1998).

| Plasmid name | Inzert | Vector | Complet size/inzert | Cloning site |
|---|---|---|---|---|
| **pMP31** | CBF12 | pREP41HAN | 11751/2893 | NdeI/BglII to NdeI/BamHI |
| **pMP65** | CBF12 $_\Delta$(1-394) | pREP41HAN | 10571/1713 | blunt ligation |
| **pJR07** | CBF11 | pREP41HAN | 10708 / 1850 | NdeI/BamHI |
| **pMP64** | CBF11DBM | pREP41HAN | 10708 / 1850 | NdeI/BamHI |
| **pREP41HAN** | **-** | pREP41HAN | 8858/- | - |
| **pMP34** | CBF12 | pREP42EGFPN | 11896/2893 | NdeI/BglII to NdeI/BamHI |
| **pMP67** | CBF12 $_\Delta$(1-394) | pREP42EGFPN | 10716/1713 | blunt ligation |
| **pJR10** | CBF11 | pREP42EGFPN | 10853 / 1850 | NdeI/BamHI |
| **pMP66** | CBF11DBM | pREP42EGFPN | 10853 / 1850 | NdeI/BamHI |
| **pREP42EGFPN** | - | pREP42EGFPN | 9003/- | - |
| **pMP66** | CBF12 $_\Delta$(1-394) | pREP42MHN | 10246 / 1850 | NdeI/BamHI |
| **pREP42MHN** | - | pREP42MHN | 8396/- | - |
| pFP126 | CBF12 | pUCBglII | - | NdeI/BglII |

**Table 3. 5** – List of plasmids used in experiments. Pslasmids in bold were used in experiments. pFP126 was used for cloning.

## 3. 4   EMAS (Electrophoretic Mobility Shift Assay)

Preparation of native lysates for EMSA is already described in 3. 2. 1. Total protein concentration used for EMSA was 4.23 mg/ml.

### 3. 4. 1        DNA probe preparation and radioactive labeling

DNA oligonucleotide RBP probe (ACAAGGGCC**GTGGGAA**ATTTCCTAAGCCTC) was diluted to concentration 100 μM to be stored. For labeling the probe was diluted to concentration of 5 μM. Diluted probes were heated to 90°C and left to cool down in a room temperature (for about 1 hour).

For radioactive labeling the appropriate mix was prepared:

|  |  |
|---|---|
| dsDNA probe (5 μM) | 2 μl |
| 10x buffer A | 2 μl |
| $\gamma$-$^{32}$P-ATP (4 500Ci/mmol, 10 μCi/ul) | 6 μl |
| MP $H_2O$ | 9 μl |
| T4 polynucleotide kinase | 1 μl |
| (10 U/μl, Fermentas) | |

Reaction mix was shaken, shortly centrifuged and left in 37°C for 45 minutes. Equilibration buffer was added to the mix to total volume of 100 ul. Final mix was purified with NICK Column (G-50 DNA Grade, Amersham) according to manufacture instructions.

**Solution:**

**Equilibration buffer**        10mM Tris-HCl, 1mM EDTA; pH 7.5

                                           pH was modified by adding HCl

### 3. 4. 2        TBE polyacrylamid electrophoresis

The apparatus to make gels (16 x 20 x 0.3 cm) was put together. 40 ml of premix was mixed with 125 µl of 20% APS and 130 µl of TEMED. Final mixture was vortexed and

immediately poured between the glasses. The brush was placed and the mixture was left to polymerize. The samples were prepared according to (Figure 3. 4) then shortly vortexed and left incubated on an ice for 20 minutes. When the acrylamide polymerized between the glasses the brush was removed and the glass part was attached to electrophoresis apparatus. The bottom and top parts of electrophoresis apparatus were filled with 0.5x TBE. Samples were mixed with ¼ of volume of 5x Loading dye, vortexed and shortly centrifuged. Then they were loaded to holes and the apparatus was run under 3 W for 4 hours. After that the glass was dismantled and the gel was sealed in plastic and carefully placed to Kodak storage phosphor screen (Kodak). After 15 hours the screen was visualized using Molecular Imager FX equiped with the Quantity One 4.6.5 software (Biorad).

**Solutions:**

| | |
|---|---|
| **premix of 5% gel** | 62.5 ml of 40% AA/BIS premix (final concentration 5%), 25 ml of 10x TBE (final concentration 0.5x), 25 ml of glycerol (final concentration 5% ), 387.5 ml of MP $H_2O$ |
| **10x TBE** | 54 g of Tris base, 27.5 g of boric acid, 20 ml of 0.5M EDTA, fill up to 500 ml MP $H_2O$ |
| **10x shift buffer** | 250 mM HEPES (pH 7.6), 340 mM KCl, 50 mM $MgCl_2$ |
| **5x Loading Dye** | 70% glycerol v TBE, 0.1% bromphenol blue, 0.1% xylen cyanol |

Reaction mix:

| | |
|---|---|
| Labelled probe (0.5 ng/µl) | 4 µl |
| ssDNA (10 µg/µl) | 2 µl |
| 10x shift buffer | 3.3 µl |

| Sample mix: | |
|---|---|
| Reaction mix (µl) | 9.3 |
| Lysis buffer 1 (µl) | 4 |
| Cell lysate (µl) | 20 |

**Figure 3.4** – Sample mix. Each sample for EMSA is prepared according to the table on the right.

### 3. 4. 3     CBF11 and CBF12 Sequence and design of mutations

CBF11:

**MGDYFAWDFANISGSNTSGSLNLNQLNLDNINNGLHNQEDGAGGRNENSERVGSGSPGSV**
**SMQVLSLFSAVNSALATLEKSEEFPSVVKDEQSIFPAVAKASNSLDELAQNIIPAPSPPG**
**FNRKRKTFDEDSSVEMIRRAISDHLDLLNNCCIGIANLNEDSVHKISLTRSG**KPSQLVTV
SCRHSSVIQKSYGSEKRYLCPPPMVYINGNYSSIFNQSFRTEISIMNDFGQCSQPISEEY
TGQGCMIFRSLHISSLVAAKSKNLRLSLDMFSNVNNQLLSHLVTSSISIVSKPSKKGSKL
KISNITLRSGSVVSLYNRINSQTVRTKYTSIEAGQFCLRGDRWVPLRINLLLPDENGKLK
VCDDVDNPEPIKYGSIVELVDEATGTTSDPLIIRRVEKDHIAEEDGYVNQMHRIVLESAY
PISNVRHLKIAEHSSLAYSNNISVRWFLGATSAQNRNASSEAILPIEWEAVGNLSSNEMT
RVGDSVCWTIVGISHFDCTMMLPFNQNPVPTVTDYPYIEEPPEYLESSRSLQFKIGGYSV
GLQIWLGVHGPLSYSFTAAADTSTMGTVTLGLSQISYDPSCAEQKYPLLFVIPGGIVIIG
KCEILLTSSAFGN

CBF12:

**MSPNVQKRPSSEDIKTQEFYDSTRNIRRVATAIGSINANLESPQLYSLAKSTSLQEPVRI**
**YGDSVSPAISSSKAHSTSSVSPYYSEKNESQALNADGTAFANPSFHSFGLPQEDSQDNTQ**
**TYSTPYTTMNPSNEMHPYPPATFENNYSVLPDHSSQPNAYSFTGSNILPTQSPSLNQMQD**
**YQNLQQNGSSNTTIPSFSSQHDLSQGLTHQPVPNHDEYAFSYPYELQRKPLIPAHPVPSF**
**RPTSALKVNMNSNVPSSDSVRNSSPNQYYASTSKQSIPSQSQNLQPPQKASVLGTVNNYR**
**QYQNSFISLNDYQAAQSNISSPSSRFPTPYSPSVPFGTYQEKEKSYSQDHAELSYYQQSP**
**SMMPPYDRSSVYFQQPLSRTDVPNQSFQQYPTTVDGGSMIPNLYPTSAEQMGLYPQDSQN**
**KDTYPKSLVNRPSSAVCEPARNDSIPMMVYSQPVTIEQRIQYVLS**NCHCLSAFYLCMPSL
CQKSYGTERRYLCPPIVLYLLGTTWLNNVTDNLKISAQTLEDKDNPKFAKNIFYYNADGA
LISPETDIAKSTYQLTNYNENTNFDSFPVWGNALLKTIYYTGQGKNDGFGRSTFLQLSVQ
SKTKYFKLENLRLGVISKPSQKRALMKVSDMSIRHGDCVCLFNRYRAQHNNALFLGTSNV
QRAISKVSLNMKYNSNYFPTTDAPNDAENEGAGLAMANNLWEPFYIFSVDELNKGNNSNP
SDSRSKVLCSNMVIILVSKITGVQSPPLILKKHDNWKVSLSSRAPSEAINCLSKLAFQCH
ETKRFLYIDEKQSSEISFTSGELEYSDPNDPTKATHSVLPWSAMWSIISTQSVRTMFYNE
PIHQNAFHVVPSMPFVKFIRLDENSMFHIYGTGFANDVQIWMAYTRCEVKSINAFKPDTT
LPPDIISDSRFSSRVYACTANLIELICEIPVCMFEPTVELSPILLFQYETLFHSGYKWPL
ESH

The removed N-terminal parts are highlighted in bold and underlined.

# 4. Results

Previous studies of two CSL-homologic proteins Cbf11 and Cbf12 identified in *Schizosaccharomyces pombe* (Prevorovsky et al., 2007) raised the question of function and significance of N-terminal domains of yeast CSL proteins (Prevorovsky et al., 2011). To obtain answers to these questions we decided to create mutated variants of Cbf11 and Cbf12 lacking the N-terminal part. We intended to analyze effects of the N-terminal part removal on DNA-binding properties and cellular localization of truncated protein variants in comparison to wild type proteins. We constructed a variant of Cbf12 protein with a mutation in the DNA-binding core (DBC) to have a protein with non-functioning DBC for comparison with wild type proteins. For better convenience, variants of Cbf proteins carrying DNA-binding mutation are labeled with DBM (DNA-binding mutation) in this diploma thesis. With the new constructs, we tested whether the removal of N-terminal part of Cbf12 or the insertion of DBM would change previously observed phenotypes caused by the wild type Cbf12 overexpression. All experiments described below were performed in at least two biological replicates.
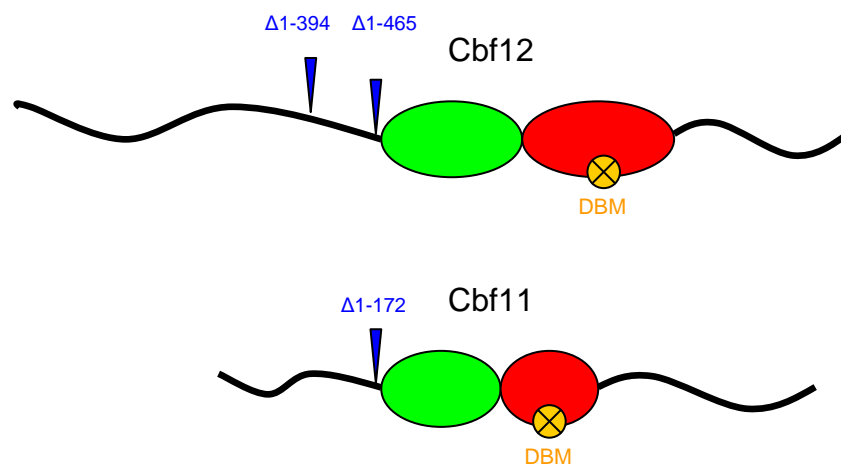


**Figure 4. 0** – Illustration of protein mutations created or used in this study. $\Delta$(1-394), $\Delta$(1-465) and $\Delta$(1-172) illustrates the area where the N-terminal domain was cut off. DBM illustrates the place of DNA-binding mutation.

## 4.1 Construction of DNA-binding mutation of Cbf12

Yeast CSL proteins show significant homology with metazoan CSL proteins in the core parts that are essential for binding to DNA (Prevorovsky et al., 2007). In metazoan CSL proteins, the key residues for the interaction with DNA were identified using mutagenesis

analysis (Chung et al., 1994). We decided to construct a Cbf12 protein variant with directed mutation in DNA-binding region. Based on this study, (Chung et al., 1994) we have chosen the highly conserved Arginine residue, in *S. pombe* protein Cbf12 at the position 644, and replaced it with Histidine residue analogically with the publication.

To perform the site directed mutagenesis we used the QuikChange®II Site-Directed Mutagenesis Kit (Agilent Technologies). This method allowed us to insert a single amino acid substitution R644H in the CBF12 gene. As a template sequence we used the pFP126 plasmid carrying the Cbf12 sequence (Tab. 3. 5 in the section 3). We designed two primers mutR and mutF (see Tab. 3. 4 in the section 3) that carried the desired mutation and that were complementary with template sequence around the mutated nucleotides. Polymerase chain reaction (PCR) extension of these primers with the template plasmid using high fidelity DNA Polymerase PfuUltra (Agilent Technologies) created linear single stranded DNA molecules of the whole template with the inserted mutation. After the PCR reaction, the temperature dropped down and the linear molecules were able to anneal to create desired plasmids with two single strand nicks. To dispose of template plasmids the PCR products were incubated with specific restriction endonuclease DpnI (Agilent Technologies), which recognizes methylated sequence 5´-Gm$^6$ATC-3´. Because the plasmid DNA isolated from most of the bacterial strains is methylated by the Dam methylase (Greier and Modrich, 1979), the template DNA isolated from *E. coli* was methylated, so subsequently it was cut into pieces. New plasmids with the mutated gene sequence were transformed to competent strain of *E. coli* by electroporation and positive transformants were selected on agar media (ŽA) with Ampiciline. Plasmids were isolated by alkaline lysis method from selected colonies. To confirm the presence of mutation in the gene sequence and the gene sequence integrity one of the isolated plasmid was sent to DNA sequencing. Sequencing analysis showed complete gene sequence including the mutated site. Sequenced plasmid carrying the mutation was named MT10 and served for further cloning of CBF12DBM to various expression plasmids.

## 4. 2  *Construction of N-terminal mutations of Cbf11 and Cbf12*

The main focus of our project was to analyze the importance of N-terminal extensions of CSL proteins in *S. pombe.* As described in (Section 2. 4. 2), the partial removal of N-terminal extension in case of Cbf12 has a considerable impact on DNA-binding properties

of this protein *in vitro*. Protein of the full length didn't bind the RBP probe in contrast to the N-terminaly truncated variant $Cbf12_\Delta(1\text{-}394)$ which showed interaction with the probe. N-terminal extensions of fungal CSL proteins are significantly longer as compared to those in metazoan CSL proteins and contain many potential sites of posttranslational modifications (Prevorovsky et al., 2011). To analyze functions of N-terminal domains of Cbf11 and Cbf12 we decided to create mutations, which would remove the whole N-terminal extension leaving only the shorter C-terminal extension and the middle part that makes up the DNA-binding core (see Table 4. 2. 1). We decided to prepare truncated variants which would also carry the DNA-binding mutation (DBM). This way we would have DBM variants of truncated proteins for comparison with truncated variants without DBM (Figure 4. 0).

| | |
|---|---|
| pREP41HAN- $CBF11_\Delta(1\text{-}172)$ | MT01 |
| pREP41HAN- $CBF11_\Delta(1\text{-}172)$DBM | MT06 |
| pREP41HAN- $CBF12_\Delta(1\text{-}465)$ | MT03 |
| pREP41HAN- $CBF12_\Delta(1\text{-}465)$DBM | MT07 |
| pREP41HAN- CBF12DBM | MT09 |
| pREP42GFPN- $CBF11_\Delta(1\text{-}172)$ | MT02 |
| pREP42GFPN- $CBF11_\Delta(1\text{-}172)$DBM | MT04 |
| pREP42GFPN- $CBF12_\Delta(1\text{-}465)$ | MT12 |
| pREP42GFPN- $CBF12_\Delta(1\text{-}465)$DBM | MT14 |
| pREP42GFPN-CBF12DBM | MT15 |

**Table 4. 2. 1** – Table of constructed plasmids with protein variants.

For the design of mutations see section 3. 4. 3 and Figure 4. 0. To create truncated variants of yeast CSL proteins we used PCR with designed primers. These primers consisted of two parts: the first part which was complementary with certain gene sequence, so the resulting product would be the desired sequence from the gene, and the second adaptor part which carried chosen restriction sites (BamHI for CBF11 variants and NdeI/BamHI for CBF12 variants) for further cloning of the sequence. We designed two primers for Cbf11 (MT01, MT02) and two primers for Cbf12 (MT03, MT04) (see Fig 3.4 in the section 3) and we had four template genes on plasmids: CBF11 (pJR10), CBF11DBM (pMP66), CBF12 (pMP31) and CBF12DBM (MT10) (Tab. 3.5 in the section 3). We designed the primers in such a way, that all four reactions would run under the same PCR program (see section 3. 3. 6).

From the PCR reaction we obtained four different mutated sequences corresponding to proteins with the removed N-terminal parts: $CBF11_\Delta(1\text{-}172)$, $CBF11_\Delta(1\text{-}172)DBM$, $CBF12_\Delta(1\text{-}465)$ and $CBF12_\Delta(1\text{-}465)DBM$. Linear PCR products of interest were separated from the template with 1% agarose DNA electrophoresis. Bands with the expected size were cut out and extracted using Nuleospin®Ectract II kit (Clontech). Purified sequences were incorporated into pCR™2.1-TOPO® plasmids using the TOPO®TA Cloning®Kit (Invitrogen). This cloning method uses the natural single nucleotide overhangs of PCR product. DNA polymerase has nontemplate-dependent terminal transferase activity that adds single Adenine (A) to the 3'end of its products. pCR™2.1-TOPO® plasmids in the reaction are linearized with single 3'-thymidine (T) overhangs covalently connected through phosphate group with Topoisomerase I from *Vaccinia* (see Figure 4. 2. 1).

**Figure 4. 2. 1** – Cloning site of pCR™2.1-TOPO® plasmid. Prior to the cloning reaction the plasmid is linearized with Topoisomerase I covalently bound to plasmid's 3'thimidine overhang. When the product of PCR, which has a single 3'overhanging Adenine, comes to the site, its 5'OH group can attack the Phosphate group from the covalent bond between plasmid and Topoisomerase I incorporating the PCR product into the plasmid leaving the Topoisomerase I free. The interaction is supported by the A-T pairing (adopted from Invitrogen).
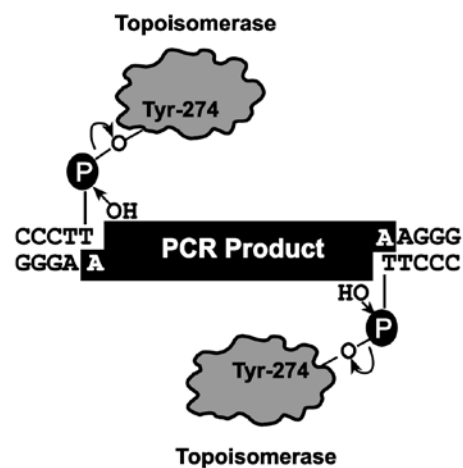


The Phosphate group from covalent connection between 3'-T and Topoisomerase I can be attacked by 5'-OH group of PCR product, which has the complementary 3'-A with plasmids 3'-T (Shuman, 1994), and the PCR product incorporates to now circular pCR™2.1-TOPO® plasmid. We followed a standard procedure according to manufacturer's manual and transformed the final mix to competent cells (TOP10 competent cells, Invitrogen) by chemical transformation. Cells carrying the plasmid were selected on the agar media (ŽA) with an Ampicilline. Plasmids were isolated from chosen colonies. Four chosen plasmids, each with one type of mutated gene, were sequenced and correct plasmids were used for subsequent cloning.

At this point we had all the desired sequences CBF11$_\Delta$(1-172), CBF11$_\Delta$(1-172)DBM, CBF12$_\Delta$(1-465), CBF12$_\Delta$(1-465)DBM and also Cbf12DBM in cloning vectors (TOPO-dd11, TOPOdd11DBM, TOPO-dd12, TOPO-dd12DBM and MT10), but for further use in experiments we had to clone mutated genes into expression vectors. We have chosen vectors pREP41HAN and pREP42GFPN, respectively (see Fig. 3. 3 in the section 3). These vectors enable the episomal expression of N-terminally tagged proteins in *S. pombe* from *nmt*1 promoter. We have already inserted chosen restriction sites to adaptor parts of the primers so at this point we could cut the sequences out of cloning vectors with proper restriction enzymes. In case of CBF12$_\Delta$(1-465) and CBF12$_\Delta$(1-465)DBM we have chosen NdeI and BamHI. In case of CBF11$_\Delta$(1-172) and CBF11$_\Delta$(1-172)DBM we couldn't use NdeI because this site was present in the gene sequence so we decided to use only BamHI and in the case of Cbf12DBM we used NdeI and BglII which is compatible with BamHI but led to the loss of the restriction site. After we cut desired sequences out of cloning vectors, we cut the expression vectors pREP41 and pREP42 with corresponding enzymes and in case of vectors for CBF11$\Delta$(1-172) and CBF11$\Delta$(1-172)DBM we used Shrimp Alkaline Phosphatase (SAP) to remove 5'-phospohate groups to prevent re-ligation of the vector. Then we separated inserts from cloning vectors using 1% agarose DNA electrophoresis. The same procedure was used for the isolation of linearized pREP vectors after the removal of the middle part of linkers. When DNA was separated in the agarose gel, parts containing the desired sequences were cut out and subsequently extracted using Nuleospin®Ectract II kit (Clontech). The quality and concentration of extracted DNA was determined by running 2 µl of the extract solution in 1% agarose DNA electrophoresis. With the determined DNA concentration, we prepared the ligation mix in two variants. One had a molar ratio of insert vs. vector 1:1 and second had the molar ratio 3:1. Control ligations of vectors without insert were included to monitore the effectiveness of vector restriction. The ligation was left in the water bath of 20°C in a fridge overnight. Next day 2 µl of reaction mix were transformed to electrocompetent DH5α strain of *E. coli* by electroporation and grown on the agar media (ŽA) with Ampicilline. Freshly grown colonies were analyzed by Colony PCR using primers (MT01, MT02 resp. MT03, MT04). Plasmids were isolated by alkaline method from positive colonies and restriction analysis was performed to check the plasmid sequence and size (Figure 4. 2. 2, Figure 4. 2. 3, Figure 4. 2. 4). Correct constructs were selected, labeled and listed in the database. After the cloning was successfully finished we had the portfolio of plasmids constructs (Table 4. 2. 1)

51
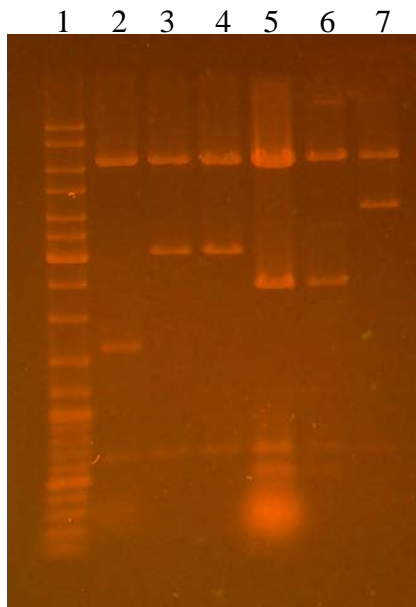
1. GeneRuler™ DNA Ladder Mix, 100-10,000 bp

2. pREP42GFPN

3. pREP42GFPN- CBF12$_\Delta$(1-465)

4. pREP42GFPN- CBF12$_\Delta$(1-465)DBM

5. pREP42GFPN- CBF11$_\Delta$(1-172)

6. pREP42GFPN- CBF11$_\Delta$(1-172)DBM

7. pREP42GFPN-CBF12DBM

**Figure 4. 2. 2** – Restriction analysis of constructed plasmids using MunI. The fragment sizes successively: 2 (6564,1645,632,178), 3 and 4 (6564,3135,632,178), 5 and 6 (6564,2491,632,491,178), 7 (6564,4522,632,178). The botoom smear in the lane 2 and 5 is an uncleaned RNA. Fragments had the expected size.
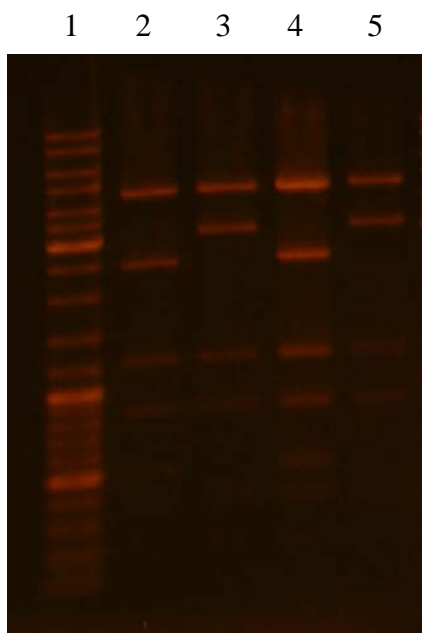


1. GeneRuler™ DNA Ladder Mix, 100-10,000 bp

2. pREP41HAN - CBF11$_\Delta$(1-465)

3. pREP41HAN - CBF12$_\Delta$(1-172)

4. pREP41HAN - CBF11$_\Delta$(1-465)DBM

5. pREP41HAN - CBF12$_\Delta$(1-172) DBM

**Figure 4. 2. 3** – Restriction analysis of constructed plasmids using MunI. The fragment sizes successively: lanes 2 and 4 (4583,2491,1242,850,406,363,178) lanes 3 and 5 (4583,3050,1242,850,178). Fragments had the expected size.
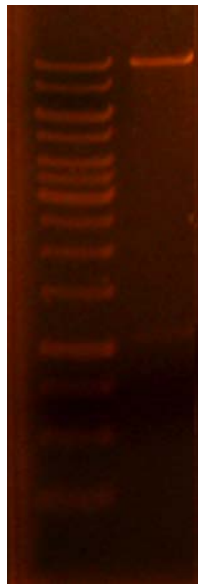
3

1. GeneRuler™ 1 kb DNA Ladder
2. pREP41HAN - CBF12DBM

**Figure 4. 2. 4** – Restriction analysis of constructed plasmids using BamHI. The fragment sizes successively: 2 BamHI (10564,1089).

## 4. 3 The effect of the overexpression of Cbf protein variants on toxicity and adhesivity

We have constructed two sets of plasmids with all mutated gene variants. Plasmids carried different gene variants of CBF11 and CBF12 with N-terminal GFP or HA tag. We also used old plasmid constructs carrying the wild type CBF11 and CBF12, and the CBF11DBM variant, in the study, so we had the whole spectrum of N-terminally HA-tagged yeast Cbf proteins for testing (Table 4. 3. 1).

| | |
|---|---|
| pREP41HAN- CBF11 | pJR08 |
| pREP41HAN- CBF11DBM | pMP64 |
| pREP41HAN- CBF11$_\Delta$(1-172) | MT01 |
| pREP41HAN- CBF11$_\Delta$(1-172)DBM | MT06 |
| pREP41HAN- CBF12 | pMP31 |
| pREP41HAN- CBF12DBM | MT09 |
| pREP41HAN- CBF12$_\Delta$(1-465) | MT03 |
| pREP41HAN- CBF12$_\Delta$(1-465)DBM | MT07 |
| pREP41HAN- CBF12$\Delta$(1-394) | pMP65 |
| pREP41HAN | pREP41HAN |

**Table 4. 3. 1** – Table of N-terminally HA-tagged CBF variants on the pREP41HAN vectors. These plasmids were used in the phenotypical studies and EMSA experiments.

Next step was to analyze an effect of constructed mutated proteins overexpression on adhesivity and to test possible toxicity of protein variants overexpression. We have chosen the strain MP09 with the deletion of both CBF11 and CBF12 from the yeast genome (Δcbf11 Δcbf12) to see the effect of individual yeast CSL protein mutations. All plasmids carrying N-terminally HA-tagged constructs including the empty plasmid as the negative control were transformed into the MP09 strain (Δcbf11 Δcbf12) using the Lithium acetate method. The expression of proteins with the right size (kDa) was verified by Western blotting (see Figure 4. 4. 3 A). Cell culture for phenotpical and EMSA studies were grown in the same time in one cell mass. Toxicity of Cbf protein variants overexpression was tested on EMM minimal media without the amino acids leucin and thiamine. Because the strain MP09 (*h⁻ leu1-32 ura4-D18 ade6-M210 Δcbf11::kanʳ Δcbf12::pCloneNat1⁻*) has adenin, leucin and uracil auxotrophy, the absence of leucin in minimal media secured the preservation of pREP41 plasmid in cells that carry LEU2. The pREP41 series of plasmids has controlled expression under the *nmt*1 promoter. The *nmt*1 promoter is controlled by the concentration of thiamine and the normal expression starts only when thiamine concentration is under 0.5 µMol (Maundrell, 1990; Craven et al., 1998). When cells are shifted from thiamine rich media to thiamine free media the level of intracellular thiamine does not change rapidly because it is mostly depleted by growth and cell division. After shifting the cells with pREP plasmids from thiamine rich media to thiamine free media cells need around 20 hours in 30°C to go through enough cell divisions that the level of thiamine dilutes under the 0.5 µMol limit. We grew strains to OD 0.6 in thiamine rich media and then we shifted them to thiamine free media where they were cultivated for 20 hours keeping them diluted under OD 0.8. Then we created serial dilution in 96-well plate and spotted cells on EMM minimal media without leucin and without thiamine. For the growth control we also spotted cells on the same media but with thiamine where proteins should not be expressed under *nmt*1 promoter. We observed that in case of Cbf12 overexpession we could see slightly slower growth on EMM minimal media and for all of the other strains overexpressing other Cbf12 variants the growth rate was on the level of strain with the control empty plasmid (Figure 4. 3. 1 A). For strains overexpressing variants of Cbf11 we did not see any growth rate difference except for the strain with the overexpression of Cbf11$_\Delta$(1-172) or wild type Cbf11. The strain with the overexpression of Cbf11$_\Delta$(1-172) seemed to grow very slightly faster compared to DBM variants and empty plasmid. But this observation is highly questionable due to a very small difference compared to empty vector control. Significant difference was noted for the overexpression

of wild type Cbf11 which led to faster growth (Figure 4. 3. 1 C) compared to all other strains. This was in agreement with previous observations that the overexpression of Cbf11 can rescue a slower growth of Δcbf11Δcbf12 (Prevorovsky et al., 2009). The same effect was observed for overexpression of wild type Cbf11 on control plates (Figure 4. 3. 1 D). This could be explained by the marginal expression from the *nmt1* promoter which occurs in the media with thiamine (Forsburg, 1993). In general the growth of strains with plasmid on thiamine free media was significantly slower then the growth on media with thiamine. Control plates with strains overexpressing Cbf12 variants showed similar growth rate for all strains (Figure 4. 3. 1 B).
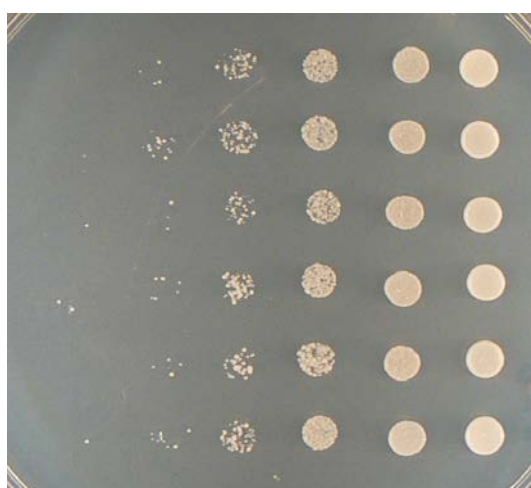
**EMM - leu - thia:**



| ΔCbf11 ΔCbf12 | $10^0$ | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ |
|---|---|---|---|---|---|---|
| 0 | | | | | | |
| Cbf12(Δ1-465) | | | | | | |
| Cbf12(Δ1-465)DBM | | | | | | |
| Cbf12(Δ1-394) | | | | | | |
| Cbf12DBM | | | | | | |
| Cbf12 | | | | | | |

**Figure 4. 3. 1 A** – Growth of strains MP09 (Δcbf11Δcbf12) with plasmids on EMM media withou leucin and thiamine after 3 days in 30°C. The strain with overexpression of Cbf12-HAN shows slightly slower growth (toxicity).

**EMM - leu + thia:**



| ΔCbf11 ΔCbf12 | $10^0$ | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ |
|---|---|---|---|---|---|---|
| 0 | | | | | | |
| Cbf12(Δ1-465) | | | | | | |
| Cbf12(Δ1-465)DBM | | | | | | |
| Cbf12(Δ1-393) | | | | | | |
| Cbf12DBM | | | | | | |
| Cbf12 | | | | | | |

**Figure 4. 3. 1 B** – Growth of strains MP09 (Δcbf11Δcbf12) with plasmids on EMM media withou leucin after 3 days in 30°C. All strains display the same growth rate.

**EMM - leu - thia:**
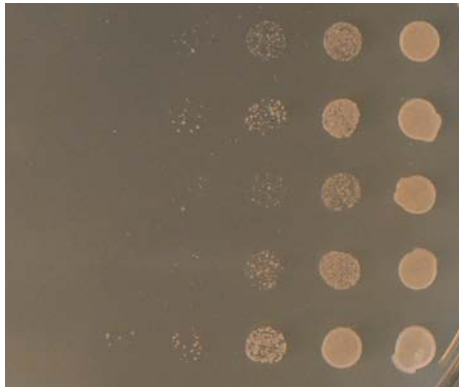
| ΔCbf11 ΔCbf12 | $10^0$ | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ |
|---|---|---|---|---|---|---|
| Cbf11(Δ1-174)DBM | | | | | | |
| Cbf11(Δ1-174) | | | | | | |
| 0 | | | | | | |
| Cbf11DBM | | | | | | |
| Cbf11 | | | | | | |

**Figure 4. 3. 1 C** – Growth of strains MP09 (Δcbf11Δcbf12) with plasmids on EMM media withou leucin and thiamine after 3 days in 30°C. The strain overexpressing Cbf11 shows faster growth.
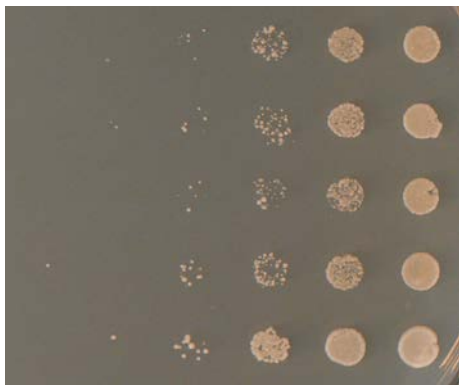
**EMM - leu + thia:**

| ΔCbf11 ΔCbf12 | $10^0$ | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ |
|---|---|---|---|---|---|---|
| Cbf11(Δ1-174)DBM | | | | | | |
| Cbf11(Δ1-174) | | | | | | |
| 0 | | | | | | |
| Cbf11DBM | | | | | | |
| Cbf11 | | | | | | |

**Figure 4. 3. 1 D** – Growth of strains MP09 (Δcbf11Δcbf12) with plasmids on EMM media withou leucin after 3 days in 30°C. The strain CBF11 on plasmid shows faster growth probably due to marginal expression.

When we documented the growth rate after 3 days we let the strains grow for further 4 days and then we performed the washing assay. This test shows the ability of cells to stick to agar plates when exposed to external pressure. In our case the pressure was a normalized stream of tap water for the period of 1 minute. The strength of the stream and temperature was identical for all plates. After the test all plates were documented. As we expected with respect to previous results (Prevorovsky et al., 2009) Cbf12 overexpression led to higher cell adhesivity (Figure 4. 3. 2 A). Interestingly the same effect was observed for Cbf12DBM. Overexpression of all truncated Cbf12 protein variants did not lead to the change in adhesivity compared to the empty plasmid control. For the overexpression of Cbf12 or Cbf12DBM, the increased adhesivity was also documented on control plates with thiamine (Figure 4. 3. 2 B). This was probably due to the marginal expression from the

repressed promoter. This shows that even very low expression of Cbf12 or Cbf12DBM has a possitive effect on adhesivity. For Cbf11 strains, we did not see any change in adhesivity as compared to empty plasmid control. After the assay there were no visible colonies left. Toxicity and adhesivity assays were performed in multiple biological and technical replicates to validate the results.

**EMM - leu - thia:**



| ΔCbf11 ΔCbf12 | 1 | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ |
|---|---|---|---|---|---|---|
| 0 | | | | | | |
| Cbf12(Δ1-465) | | | | | | |
| Cbf12(Δ1-465)DBM | | | | | | |
| Cbf12Δ(1-394) | | | | | | |
| Cbf12DBM | | | | | | |
| Cbf12 | | | | | | |

**Figure 4. 3. 2 A**– Washing assay of strains MP09 (ΔCbf11 ΔCbf12) with plasmids on EMM media withou leucin and thiamine after 7 days in 30°C. The strains with the overexpression of Cbf12-HAN or Cbf12DBM-HAN display a higer adhesivity.

**EMM - leu + thia:**



| ΔCbf11 ΔCbf12 | 1 | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ |
|---|---|---|---|---|---|---|
| 0 | | | | | | |
| Cbf12(Δ1-465) | | | | | | |
| Cbf12(Δ1-465)DBM | | | | | | |
| Cbf12Δ(1-394) | | | | | | |
| Cbf12DBM | | | | | | |
| Cbf12 | | | | | | |

**Figure 4. 3. 2 B** – Washing assay of strains MP09 (ΔCbf11 ΔCbf12) with plasmids on EMM media withou leucin after 7 days in 30°C. The strains with plasmids carrying CBF12-HAN or CBF12DBM-HAN display a higer adhesivity although there is only marginal expression from repressed promoter.
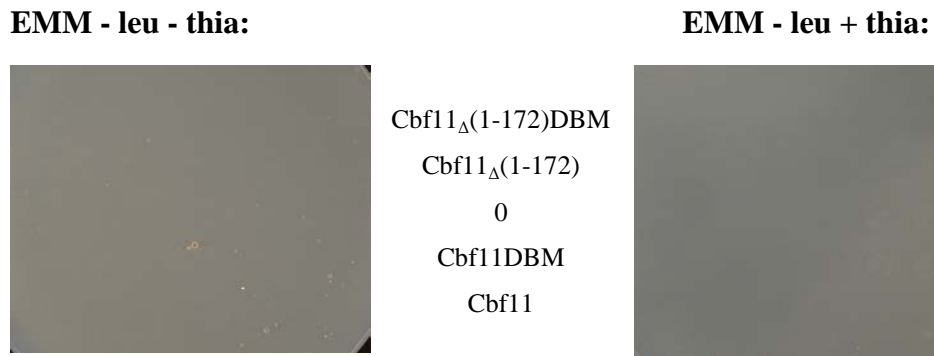
Cbf11$_\Delta$(1-172)DBM

Cbf11$_\Delta$(1-172)

0

Cbf11DBM

Cbf11

**Figure 4. 3. 2 C** – Wash assay of strains overexpressing Cbf11 protein variants on left hand side. Control plate with repressed promoter on right hand side. No visible colonies stayed on the plates.

## 4. 4  DNA-binding properties of Cbf11 and Cbf12

Cbf11 and Cbf12 were identified as putative transcription factors with a sequence conservation of key parts within the DNA-binding core (Prevorovsky et al., 2007). The ability to bind CSL recognition DNA elements in native yeast lysate has already been shown *in vitro* for Cbf11 and Cbf12$\Delta$(1-394). One of these elements was the RBP probe (ACAAGGGCCGTGGGAAATTTCCTAAGCCTC), which is the sequence recognized by the metazoan CSL factors (Prevorovsky et al., 2009; Prevorovsky et al., 2011). Interestingly, the wild type Cbf12 does not seem to bind the RBP probe *in vitro* in contrast to Cbf12$\Delta$(1-394) (Prevorovsky et al., 2011). Because we expected that N-terminal parts of Cbf proteins have influence on DNA-binding we decided to test the ability of proteins carrying new truncated and DNA-binding mutations to bind the RBP probe *in vitro* with the Electrophoretic Mobility Shift Assay (EMSA). EMSA is a common affinity electrophoretic assay used for studies on protein-DNA or protein-RNA interactions. Basic principle (Figure 4. 4. 1) of this method is polyacrylamide electrophoresis of labeled DNA fragments (probes) that contain the expected binding motive for the tested protein (Figure 4. 4. 1). Before the electrophoresis, probes are incubated with the tested protein either in a purified form, or in the whole cell native extract. During the electrophoresis, free probes travel faster than probes, which create a complex with the tested protein. This way in the end a free probe can be distinguished from a probe with the protein bound to it according to the difference in the distance they traveled.
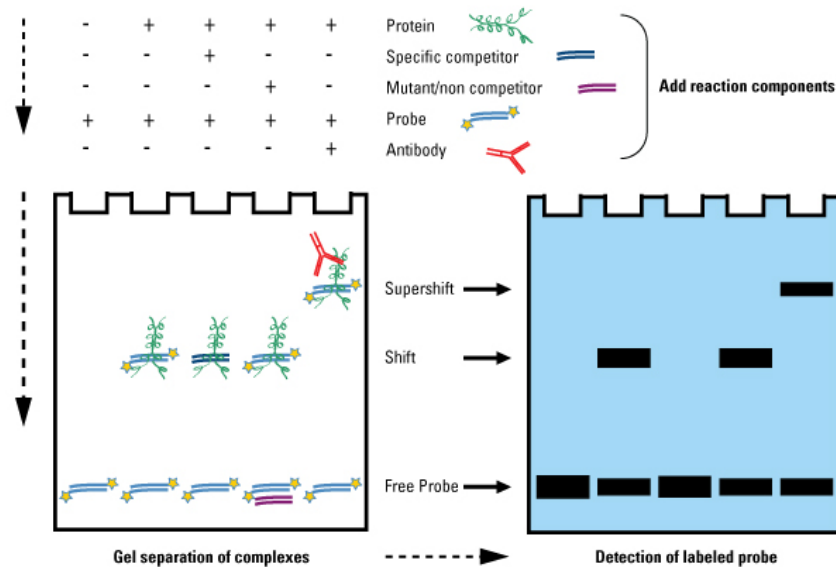
**Figure 4. 4. 1** – Scheme of Electrophoretic Mobility Shift Assay (EMSA). The mix of probe and protein is run in polyacrylamide electrophoresis. The probe that does not interact with the protein travels faster and in the end it is found at the bottom of the gel. The probe with a single protein bound to it travels slower due to the change in size and charge so in the end it appears as a shift on the gel. If another protein (for example antibody) binds to create a complex of proteins on a probe the shift is even stronger and it is usually called supershift (picture adopted from www.piercenet.com).

We used the strains MP09 ($\Delta$cbf11$\Delta$cbf12) from the phenotypical studies which carried plasmids variants of CBF11 and CBF12 with N-terminal HA-tags (Table 4. 3. 1). For the second EMSA run we also included strains transfomed with empty plasmid pREP42MHN or pREP42MHN-CBF12$_\Delta$(1-394) (pMP66) to confirm the position of Cbf12$_\Delta$(1-394) shift. We decided to do this because of problems with expression of HA-tagged Cbf12$_\Delta$(1-394) from pMP65. We grew strains in EMM minimal media without leucin to mid logarithmic phase and then shifted them to EMM minimal media without leucin and thiamine and left to grow for 20 hours keeping the OD no higher than 0.8. Then native lysates were prepared and before each run of EMSA we tested the expression of proteins from plasmid in lysates by Western blotting (Figure 4. 4. 3 A). Unfortunately the expression of Cbf12$_\Delta$(1-394)-HAN from plasmid pMP65 could not be detected on Western blotting. Possible explanation was that plasmid was damaged and the expression of protein was extremely low. Some very weak interaction of Cbf12$_\Delta$(1-394)-HAN with the RBP probe can be observed on EMSA results (section 4. 4) due to the higher sensitivity of radioactive labeled probe compared to imunodetection by Western blotting. Protein concentration in native lysates was measured using the Bio-Rad assay and the total protein concentration of 4.23 mg/ml was used in EMSA experiments. We detected interaction with the RBP probe only

for Cbf11 and very weak interaction for Cbf12$_\Delta$(1-394) and surprisingly for Cbf12 (Figure 4. 4. 2). The shift for Cbf12 was in the same position as the shift for Cbf12$_\Delta$(1-394), which suggested that Cbf12 is proteolytically cleaved within the cell resulting to gain of ability to bind RBP probe. It would be expected that full length Cbf12 would display a shift at a different position. For control strain with MycHis-tagged Cbf12$_\Delta$(1-394) on the plasmid pMP65 we observed a shift at the same position as Cbf12 and also a second shift which is in agrement with previous observations. This shift appears only in lysate with Cbf12$_\Delta$(1-394) and the nature of interaction with the RBP probe leading to different position of the shift has not been identified yeast. For all of the new mutations of Cbf11 and Cbf12 we did not detect any interaction with the RBP probe (Figure 4. 4. 2).
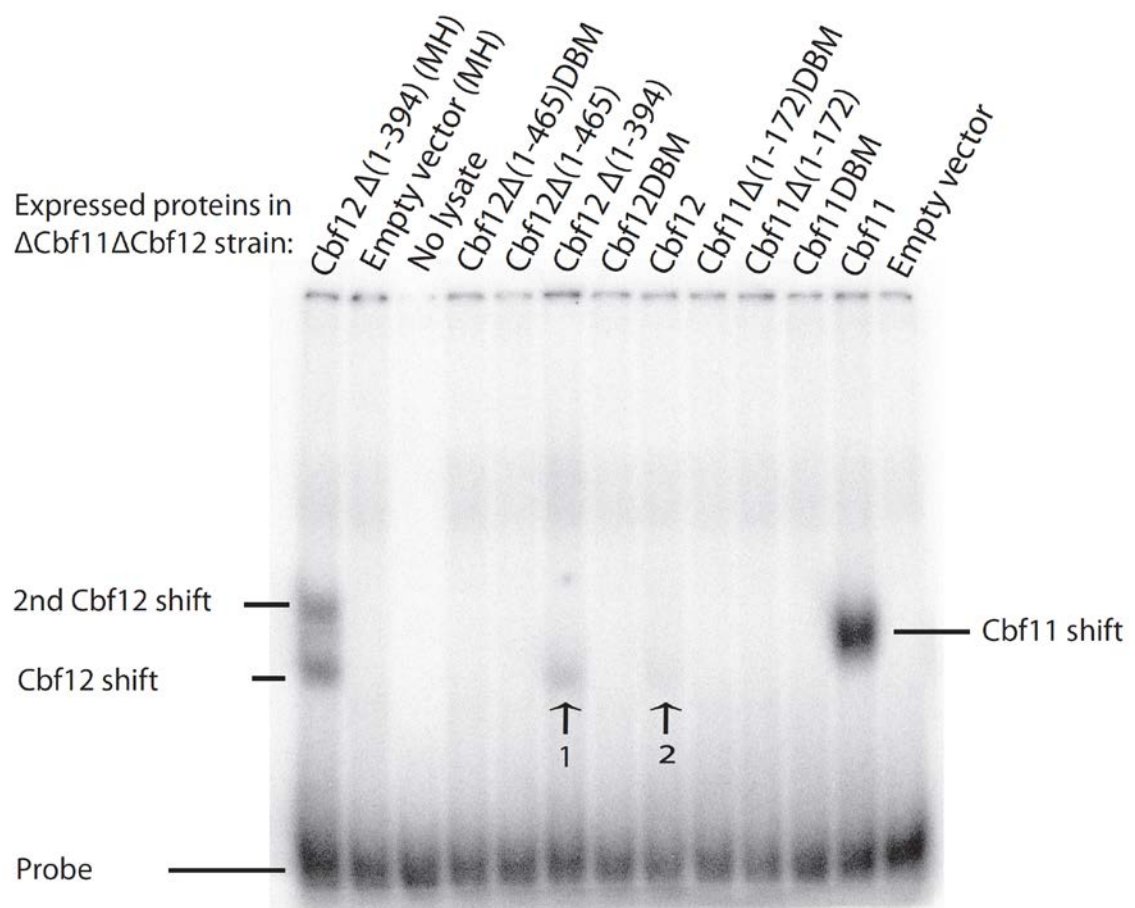


**Figure 4. 4. 2** EMSA DNA-binding assay of Cbf11 or Cbf12 protein variants to the RBP probe. Native lysates of strains MP09 (ΔCbf11 ΔCbf12) with CSL proteins expressed from plasmids. In the first lane there are two strong bands, the lower one is predicted to be N-terminally MycHis-tagged Cbf12 $_\Delta$(1-394) binding to the RBP probe, the upper one is 2$^{nd}$ Cbf12(1-394) shift and the explanation for supershift effect has not been found yet. In the 6$^{th}$ lane, the band for Cbf12 $_\Delta$(1-394)-HAN is visible, and in the 8$^{th}$ lane, the weaker band at the same position is visible for Cbf12-HAN. In the 12$^{th}$ lane, a strong band is visible for Cbf11-HA binding to probe.

This was expected for DBM variants (Cbf11DBM, Cbf11$_\Delta$(1-172)DBM, Cbf12DBM, Cbf12$_\Delta$(1-465)DBM) because of the mutation in DNA-binding domain. But the absence of a shift for N-terminally truncated variants Cbf11$_\Delta$(1-172) and Cbf12$_\Delta$(1-465) was not expected. These findings were correlated with the protein cellular localization (Section 4. 5).
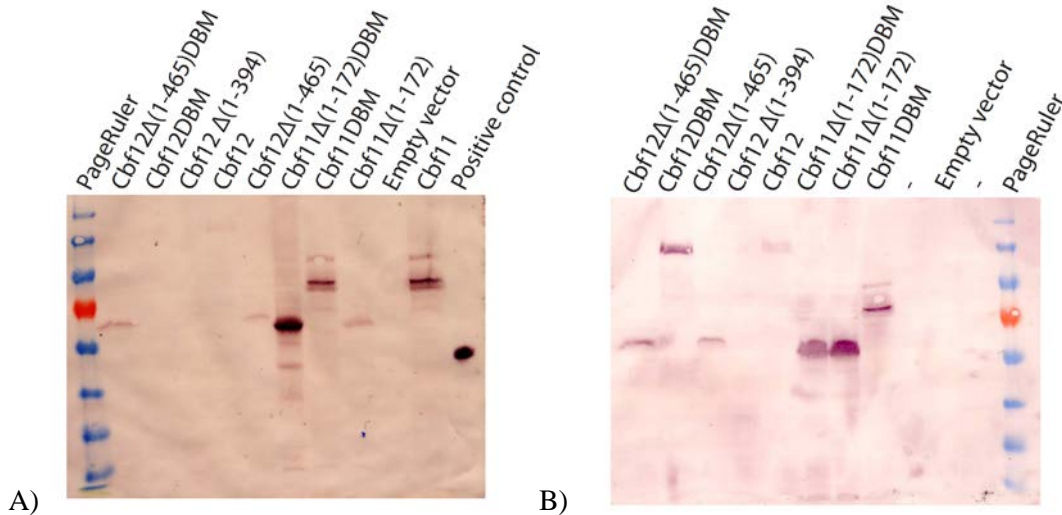


**Figure 4. 4. 3** – Western blotting expression analysis of native lysates used for EMSA. (A) Western blotting of strains MP09 (Δcbf11Δcbf12) with the overexpression of HA-tagged protein variants from plasmids. From left hand side: Marker, Cbf12$_\Delta$(1-465) 60 kDa, Cbf12DBM 117 kDa (weak but visible), Cbf12 $_\Delta$(1-394) 68 kDa "undetectable", Cbf12 117 kDa (weak but visible), Cbf12$_\Delta$(1-465)DBM 60 kDa, Cbf11$_\Delta$(1-172)DBM 57 kDa, Cbf11DBM 72 kDa, Cbf11$_\Delta$(1-172) 57 kDa, Empty vector, Cbf11 72 kDa, Positive control 40 kDa. All proteins were detected except the Cbf12 $_\Delta$(1-394) variant. (B) Western blotting of strains MP03 (ΔCbf12) with the overexpression of HA tagged protein from plasmid. From hand side left: Cbf12$_\Delta$(1-465)DBM 60 kDa, Cbf12DBM 117 kDa, Cbf12$_\Delta$(1-465) 60 kDa, Cbf12$_\Delta$(1-394) 68 kDa "undetectable", Cbf12 117 kDa, Cbf11$_\Delta$(1-172)DBM 57 kDa, Cbf11$_\Delta$(1-172) 57 kDa, Cbf11 72 kDa, -, Empty vector, -., Marker. All proteins were detected except the Cbf12 $_\Delta$(1-394) variant.

Based on previous unpublished results showing an influence of Cbf11DBM overexpression on amount of endogenic WT Cbf11 binding to the RBP probe (M. Ptáčková), we decided to test the influence of constructed mutated proteins on the binding properties of endogenic Cbf11. pREP41 plasmids with HA-tagged genes with mutations were transformed to the strain MP03 (Δcbf12) which had the genomic CBF12 deleted. Strains were grown in EMM minimal media without leucin to mid logarithmic phase and then shifted to EMM minimal media without leucin and thiamine and left to grow for 20 hours keeping the OD no higher than 0.8. Then we prepared native lysates and the expression of proteins from plasmid was tested by western-blotting for each lysate (Figure 4. 4. 3 B). From EMSA results we observed that the overexpression of Cbf11DBM reduces the signal of endogenic Cbf11

bound to the RBP probe as expected from previous results. Surprisingly, we saw the same effect only slightly weaker for Cbf11$_\Delta$(1-172) and Cbf11$_\Delta$(1-172)DBM (Figure 4. 4. 5). In lanes with Cbf12 or Cbf12$_\Delta$(1-394) a shift of these variants at the approximately same position was present (Figure 4. 4. 4). This shift is most likely the shift of Cbf12$_\Delta$(1-394) and proteolytically cleaved wild type Cbf12. The the increase in shift signal compare to shifts of Cbf12$_\Delta$(1-394) and proteolytically cleaved wild type Cbf12 in strains MP09 ($\Delta$Cbf11 $\Delta$Cbf12) shows a possible inprovement in binding ability of Cbf12 in the presence of Cbf11.
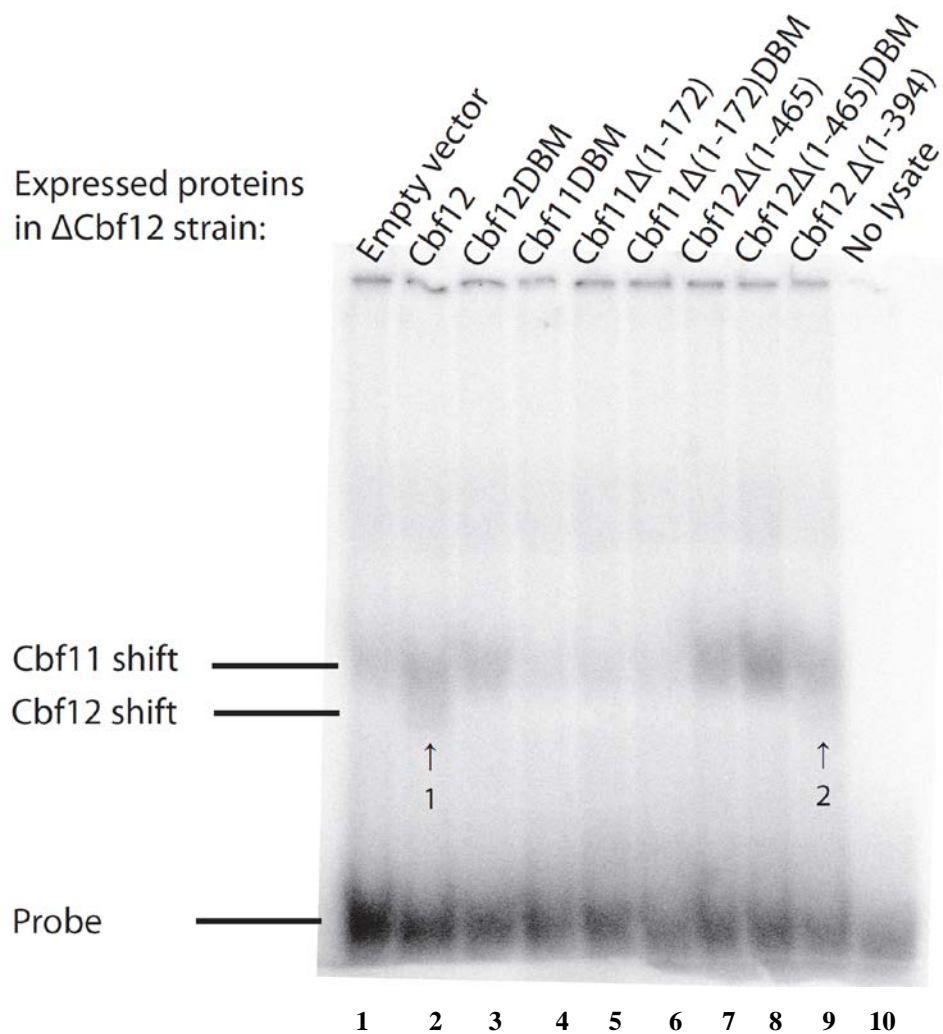


**Figure 4. 4. 4** –EMSA of the strain MP03 ($\Delta$cbf12) with the overexpression of HA tagged Cbf11 and Cbf12 variants from plasmid. In the 1st lane, there is the band for endogenic Cbf11 binding shift. In the 2nd lane, there is the band for endogenic Cbf11 and lower band for Cbf12 expressed from plasmid. In the 3rd lane, only endogenic Cbf11 binds, Cbf12DBM does not bind. In the 4th, 5th and 6th lane, the signal of endogenic Cbf11 is significantly weaker. This points to the ability of Cbf11DBM, Cbf11$\Delta$(1-172) and Cbf11$\Delta$(1-172)DBM to reduce the amount of endogenic Cbf11 bound to the RBP probe. In the 7th and 8th lane, only the shift of endogenic Cbf11 is present. In the 9th lane, there are again two bands, the top one for endogenic Cbf11 and the lower one for Cbf12 $\Delta$(1-394). 10th lane does not contain any lysate only the probe.

**EMSA of ΔCbf12 strain overexpressiong HA-tagged Cbf protein variants**
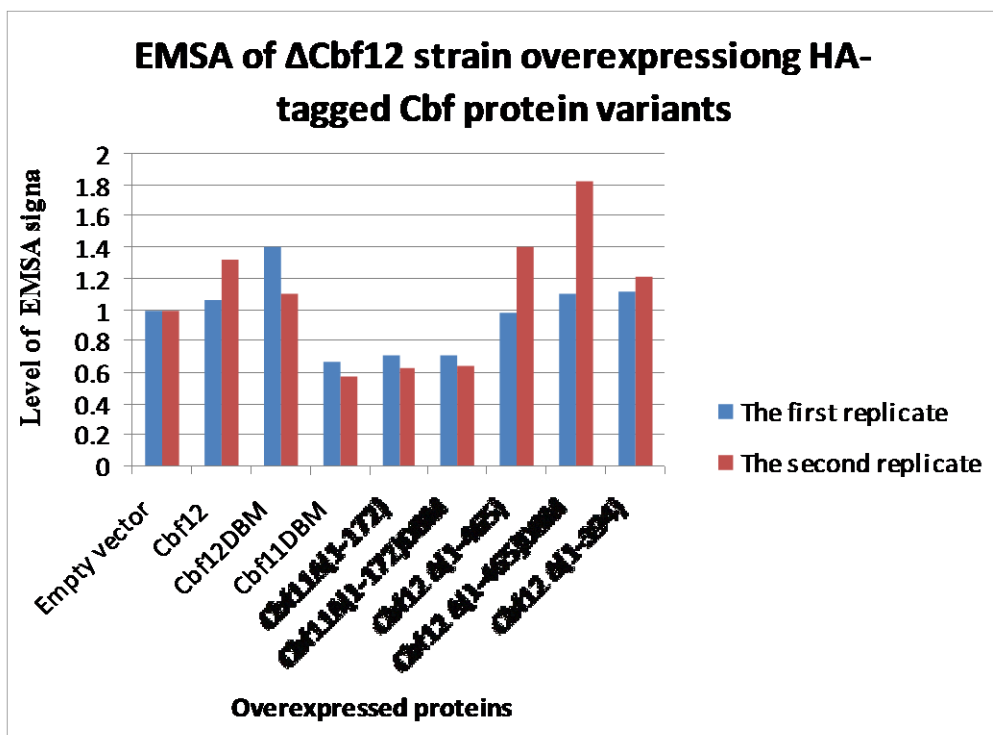
**Figure 4. 4. 5** – The evaluation of signals for the EMSA experiment from (Fig. 4. 4. 3). The decrease in signal of endogenic Cbf11 in strains with the overexpression of Cbf11DBM or Cbf11Δ(1-172) or Cbf11Δ(1-172)DBM was observed in both repetitions.



**Figure 4. 4. 6** – Second biological replicate of EMSA DNA-binding assay of strains MP09 (ΔCbf11 ΔCbf12) with CSL proteins overexpressed from plasmids. As in the first replicate the only visible shifts are: strong Cbf11 shift and very week shifts of Cbf12 and Cbf12$_\Delta$(1-394) marked with (*).

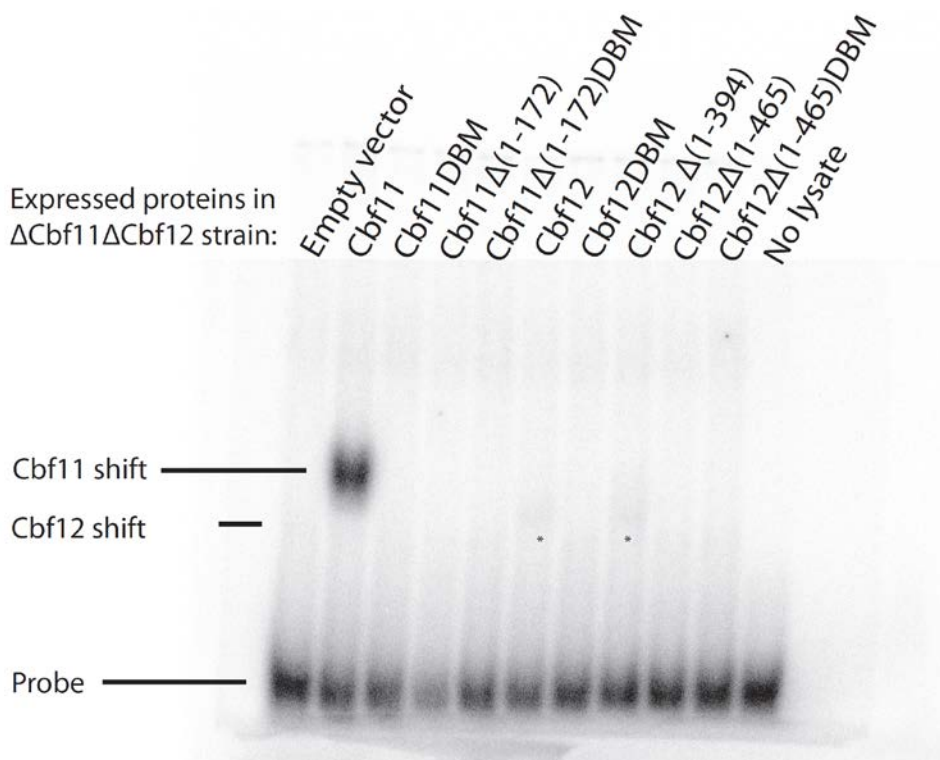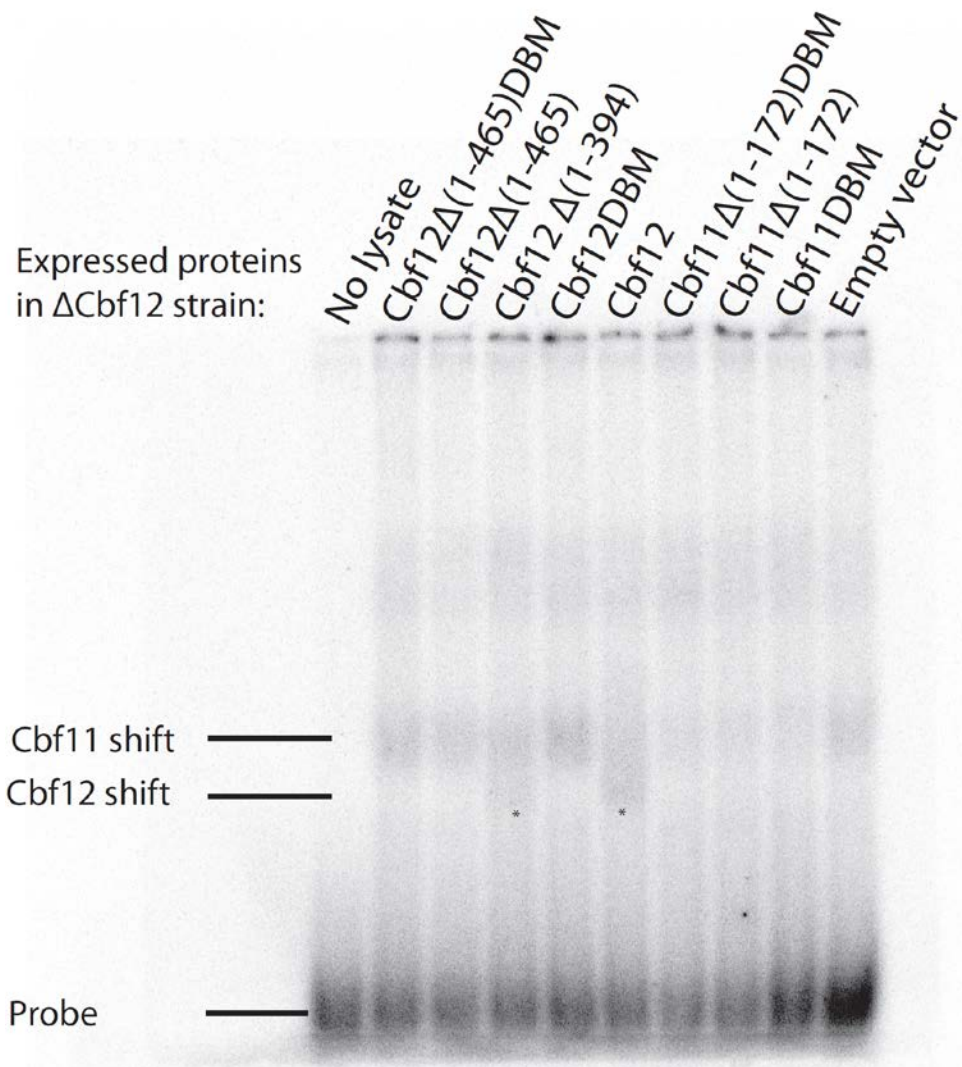**Figure 4. 4. 7** – Second biological replicate of EMSA DNA-binding assay of strains MP03 (ΔCbf12) with CSL proteins expressed from plasmids. As in the first replicate the decrease in a signal is visible in lanes with Cbf11DBM, Cbf11Δ(1-172) and Cbf11Δ(1-172)DBM. Shift for Cbf12 and Cbf12$_\Delta$(1-394) is also visible, it is marked with (*).

## 4. 5 *Cellular localization of Cbf protein constructs using GFP tag*

Along with EMSA studies we wanted to determine the cellular localization of the truncated variants of Cbf11 and Cbf12 and compare it to the wild type Cbf proteins. For this reason, a spectrum of CBF11 and CBF12 gene variants in pREP42GFPN plasmids was constructed (Table 4. 2. 1) as described earlier and previously constructed plasmids with GFP-tagged CBF genes were included (Table 3. 6 in section 3). All plasmids were transformed to strain MP09 (Δcbf11Δcbf12) using lithium acetate method. We used this strain to corelate cellular localization with EMSA results. Strains were grown in EMM minimal media without uracil to mid-exponential phase and then shifted to EMM minimal media without uracil and thiamine. Then the strains were grown for 20 hours for proteins to be expressed

from plasmids and samples were taken for preparation of cell denaturated extracts to control the protein expression by Western blotting (Figure 4. 5. 1). The rest of the cell mass was used to prepare microscopic slide. We used the type of slide with living cells where the culture is covered by solid agarose layer to be immobilized. Slides were examined using the inverted microscope Olympus IX81. For strains overexpressing Cbf11, Cbf11DBM, Cbf12, Cbf12DBM and Cbf12$_\Delta$(1-394) we observed diffused nuclear localization of proteins and much weaker cytoplasmic localization (Figure 4. 5. 2). For Cbf11$_\Delta$(1-172) and Cbf11$_\Delta$(1-172)DBM strains the signal had an equal strength for nucleus and cytoplasm (Figure 4. 5. 2). For the Cbf12 truncated variants Cbf12$_\Delta$(1-465) and Cbf12$_\Delta$(1-465)DBM the signal seemed to be weaker or equal in the nucleus as compared to the cytoplasm where the signal was on the level of Cbf11 truncated variants (Figure 4. 5. 2). In all slides some cells were either dead or exhibited a pathological phenotype but this was expected for the strain MP09 (Δcbf11Δcbf12) that displays *cut* phenotypes and problems with cell division (Prevorovsky et al., 2009). Controlled strains with empty vector or without any vector did not show any signal under the same microscope setup as the previous strains with Cbf11 and Cbf12 variants overexpression.



**Figure 4. 5. 1** - Western blotting of strains MP09 (Δcbf11Δcbf12) with overexpressed GFP-tagged variants of Cbf proteins. On the left hand side: Marker, Cbf11 (100 kDa), Cbf11Δ(1-172)DBM 85 kDa Cbf12 (147 kDa, very weak signal), Cbf12$_\Delta$(1-465) (90 kDa), Empty vector, Cbf11DBM (100 kDa), Cbf11Δ(1-172)DBM 85 kDa, Cbf12DBM (147 kDa), Cbf12$_\Delta$(1-465)DBM (90 kDa), Cbf12Δ(1-394) (98 kDa). All variants were detected with the right size.
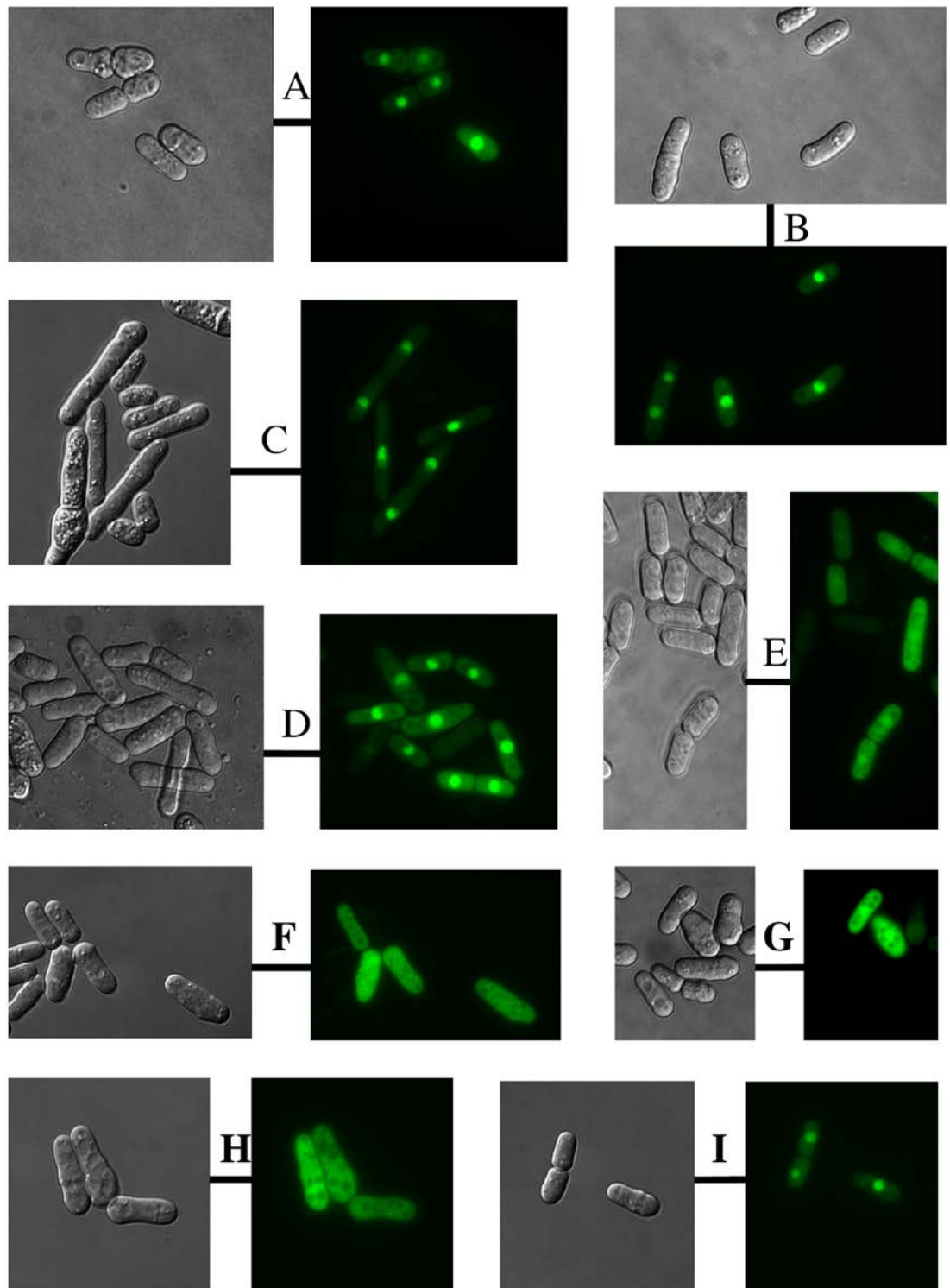
**Figure 4. 5. 2** – Localization of overexpressed GFP-tagged Cbf protein variants in the strain MP09 (Δcbf11Δcbf12). (A) The Cbf11 shows diffused nuclear localization with a very weak cellular localization which is probably the result of the overexpression. Very similar patern of localization was observed for (B) Cbf11DBM, (C) Cbf12, (D) Cbf12DBM and (I) Cbf12 $_\Delta$(1-394). For (E) Cbf11Δ(1-172) and (G) Cbf11Δ(1-172)DBM the signal in the nucleus is equal to the signal in the cytoplasm. For (F) Cbf12$_\Delta$(1-465) and (H) Cbf12$_\Delta$(1-465)DBM the signal is even more shifted into the cytoplasm. In all strains unidentified membrane bodies with no signal were observed. These bodies could be vacuoles.

# 5. Discussion

## *5. 1 CSL proteins in S. pombe*

Most of the knowledge about the CSL family of transcription factors and understanding the mechanisms behind their functions come from studies on metazoan models primarily related to the Notch signaling pathway (Artavanis-Tsakonas et al., 1999; Bray, 2006). In recent years, new examples of Notch-independent functions of CSL proteins have been found in various metazoan organisms (Morel and Schweisgut, 2000; Raharjo and Gaudet, 2007; Ito et al., 2007; Barolo et al., 2000; Beres et al., 2006; Kaspar a Klein, 2006; Koelzer a Klein, 2003; Masui et al., 2007). In our laboratory, a new group of CSL proteins was identified in fungal organisms belonging to ascomyceta, basidiomyceta and zygomycota, which all lack the parts of the Notch pathway upstream from CSL proteins (Prevorovsky et al., 2011). According to bioinformatics analysis, yeast CSL proteins might share the ancestral function to regulate gene expression although the signal processes and interacting network are probably different from the one in metazoan organisms (Prevorovsky et al., 2007; Prevorovsky et al., 2009). After the discovery of fungal CSL proteins, two members of this group from *Schizosaccharomyces pombe* Cbf11 and Cbf12 were studied using many available approaches including phenotypical studies on mutant strains with the deletion of one or both of the CSL proteins. These studies indicated that Cbf11 and Cbf12 are antagonistic regulators of some cellular processes such as adhesion, production of unidentified extracellular material that contains hydrophobic and reflective compound, coordination of colony morphogenesis, cell/nuclear division and ploidy maintenance (Prevorovsky et al., 2009). Metazoan CSL proteins are transcription factors with ability to directly bind DNA. The ability to bind the general CSL recognition sequences was shown for the wild type Cbf11 in contrast to the wild type Cbf12, where the DNA-binding could not be detected (Prevorovsky et al., 2009). Subsequently the ability to bind the CSL recognition sequence was shown for N-terminally truncated variant $Cbf12_\Delta(1\text{-}394)$. The N-terminal domain is not part of the conserved DNA-binding core. The observation of influence of N-terminal domain on DNA-binding, together with a deeper analysis of N-terminal domains of fungal CSL proteins, pointed to possible functions and importance of these long terminal domains, which also represent one of the characteristics of fungal CSL proteins (Prevorovsky et al., 2011). It is expected that these N-terminal "tails" originated in the last common ancestor of fungi and animals and it is likely that without a specific

function they would have disappeared over time (Warringer and Blomberg, 2006). The fact that they can be found in all fungal CSL proteins argues for their function significance. These long N-terminal proteins are also absent in metazoan CSL proteins. In this project we intended to study the effect of removing complete N-terminal domains of both CSL proteins in *S. pombe* on their DNA-binding properties and cellular localization. DNA-binding mutation of Cbf12 was constructed to test the specificity of Cbf12 DNA-binding and to analyze a change in strong Cbf12 phenotypes (adhesivity and toxicity) with introducing the DNA-binding mutation of Cbf12.

## 5. 2 DNA-binding properties of truncated variants of yeast CSL proteins

We tested the DNA-binding properties of truncated variants of fission yeast CSL proteins using electrophoretic mobility shift assay EMSA. This method uses the difference of DNA or RNA probe mobility in electric field when there are interacting proteins bound to it compared to a free probe. For Cbf11, which showed very strong interaction with the CSL recognition probes in the WT protein, we expected that the removal of N-terminal domain, which is not part of the DNA-binding core, would not influence interaction with DNA. The opposite case was Cbf12 where the removal of smaller part of N-terminal domain enabled the interaction with DNA *in vitr*o. We intended to create a protein with even longer portion of N-terminal part removed, possibly leading to a stronger ability to bind CSL recognition DNA elements. To our surprise none of the new truncated variants of Cbf11 or Cbf12 was able to bind the chosen RBP probe. It is most likely due to the fact that the N-terminal domain we cut off contains a part that is crucial for the ability of the protein to bind DNA. One possible explanation is that the N-terminal domain mediates an interaction with an indispensable binding partner that assists the DNA-binding. However, this explanation has many counter-arguments. The CSL proteins in Metazoa can bind DNA-recognition elements on their own (Tun, 1994). This was also shown for Cbf11 in bacterial lysate (Prevorovsky et al., 2009). The bacterial lysate with Cbf11 would have to contain such binding partner with sufficient homology. Also *in vitro* EMSA experimets are far from the actual conditions inside cells. Another possibility that we investigated is that the removal of such a long N-terminal part of Cbf11 and Cbf12 interrupted the fold of DNA-binding core of these proteins. The fold of the entire protein does not seem to be disturbed in a way

that would make it a "junk" fold and instant target of proteasomal degradation, because the truncated proteins are localized across the whole cell equally as seen on the GFP localization results and the Western blotting also showed equal or even higher signal compared to wild type Cbf proteins.  But the fact that all experiments are performed with overexpressed proteins still leaves the possibility that the proteasomal apparatus is simply not capable of an efficient degradation of overexpressed unfolded proteins. An expression from a weaker promoter or endogenic locus would exclude this possibility in future experiments. Although the cut N-terminal sequence is not conserved between fungal or metazoal species, it can still play a role in the constitution of DNA-binding core in yeast CSL proteins. We analyzed the the fold at the part of the sequence where the cut was designed using the metazoan crystallographic data of CSL protein Lag-1 from *C. elegans* (figure 5. 1). The area of cut is by α-helix (α2) of C-terminal Rel homology region and the change leavs out a second α-helix (α1) (Kovall and Hendrickson, 2004, Prevorovsky et al., 2007)). On closer inspection of α-helixes (α1) and (α2) we found that surrounding structures, especially certain β-structure, are close enough (2.7 – 3.1 A) to interact with each other in CSL protein Lag-1 from *C. elegans* but this sequence is not conserved between yeast and metazoan CSL proteins.
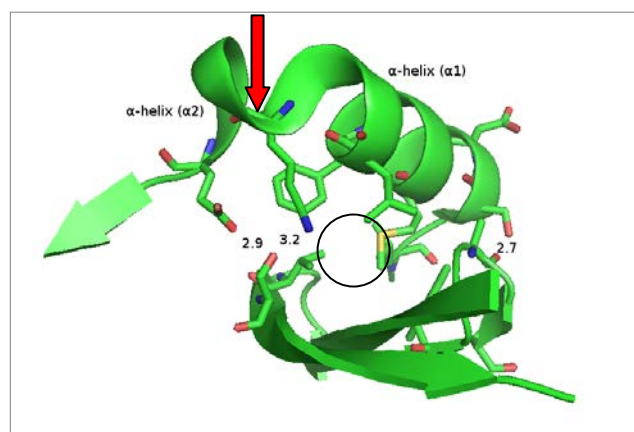


**Figure 5. 1** – 3D structure of α-helix (α1) interactions in *C. elegans* CSL protein Lag-1. Displayed numbers show distance (Angstroms) between potential residues creating hydrogen bonds. Black circle displays potential hydrophobic pocket. Red arrow points to the place of cut in mutated sequences.

Considering our last discovery that new truncated yeast CSL proteins lost the ability to bind RBP probe supports the possibility that interrupting the structure of this sequence could damage the structure of DNA-binding core enough to inhibit the DNA-binding ability. To test this hypothesis, CSL truncated variants which would contain the critical

sequence would have to be constructed and analogically tested by EMSA on CSL recognition elements. This finding would contribute to the knowledge of the yeast CSL structure folding but until the structural resemblance between metazoan and yeast CSL proteins is determined the significance of such discovery would only relate to yeast CSL proteins.

## 5. 3 Interaction between mutated variants of Cbf11 and endogenic Cbf11

Previous unpublished results of EMSA experiments, where we saw a decrease in EMSA signal of endogenic Cbf11 bound to RBP probe when Cbf11DBM was overexpressed in the strain, indicated that there is a possible interaction between Cbf11DBM and wild type Cbf11. We did not have any further results that would specify the nature of this interaction. We decided to repeat the experiment in the $\Delta$Cbf12 strain, where out of two fission yeast Cbf proteins only the endogenic Cbf11 is present, to verify previous results and include the test of interaction between wild type Cbf11 and all of the new variants of fission yeast Cbf proteins. Previous results for Cbf11DBM were repeated as we saw a decrease in signal of endogenic Cbf11 when the Cbf11DBM was overexpressed in the strain. Interestingly, the same effect was observed for Cbf11$_\Delta$(1-172) and Cbf11$_\Delta$(1-172)DBM. This shows that all three Cbf11 protein variants (Cbf11DBM, Cbf11$_\Delta$(1-172) and Cbf11$_\Delta$(1-172)DBM) which are not able to bind DNA in vitro effect the ability of wild type Cbf11 to bind the DNA-recognition element. One of the possible explanations is that the overexpression of non-functioning variants of Cbf11 proteins leads to a repression of endogenic Cbf11 level resulting in decreased level of endogenic wild type Cbf11. Further experiments including Cbf11 tagged knockin strains are planned to explain this phenomenon. In these experiments we will be able to monitore the level of expression of endogenic Cbf11 and we will be able to rule out the posibility of a decreased level of endogenic Cbf11 expression. Other possibility is that the interaction betwen wild type Cbf11 and variants that do not bind DNA probe is present at the protein level. For example, there can be an unidentified binding cofactor or complex that assists Cbf11 with distinctive DNA-binding. When mutated Cbf11 variants are overexpressed this cofactor/complex can be sequestered by protein variants which do not have the ability to bind DNA. This would cause a shortage of such cofactor/complex availability for endogenic Cbf11 and the signal of endogenic Cbf11 bound to DNA probe would be reduced as we see on the EMSA results.

But opposing this explanation such binding cofactor or cofactor complex has not been identified and although no experiments with purified Cbf11 have been conducted yet some previous experiments favor the possibility that Cbf11 can bind recognition DNA elements in a direct way (Prevorovsky et al., 2009). Another possible explanation of the decrease in ability of endogenic Cbf11 to bind DNA probe could be competition for binding site, which does not seem possible because none of the interacting variants showed ability to bind DNA probe. The cause of significant decrease in binding to the RBP probe could have been a restriction of endogenic Cbf11 from interacting with DNA. This could be explained by a hypothtical requirement of Cbf11 dimerisation to bind DNA and by interacting with the mutated variants endogenic Cbf11 would be sequestered in non-functioning complexes. Further experiments with monitoring the level of endogenic Cbf11 expression are planed along with experiments testing the possibility of dimerization among Cbf proteins.

## 5. 4 DNA-binding properties of Cbf12 and Cbf12DBM

Cbf12 has one of the longest N-terminal extensions among the fungal CSL protein with many potential sites of postranslational modifications (Prevorovsky et al., 2011). In previous published experiments, the interaction of Cbf12 with CSL recognition element RBP could not be detected (Prevorovsky et al., 2011). The DNA-binding of Cbf12 was only observed when truncated variant of Cbf12$_\Delta$(1-394) was tested. In agreement with older results, we observed that Cbf12$_\Delta$(1-394) bind to the RBP probe in $\Delta$Cbf11$\Delta$Cbf12 strain. We also saw a weak shift of the strain with overexpression of Cbf12 at the same position as the shift for Cbf12$_\Delta$(1-394) suggesting, that Cbf12 can undergo proteolytic cleavage to gain an ability to bind DNA. Specificity of Cbf12 interaction with RBP probe was proved by the absence of shift in the strain overexpressing Cbf12DBM. If the interaction would be non-specific the DNA-binding mutation of Cbf12 would bind to the RBP probe too, which was not the case. The utilization of a protein cleavage for the control of thir function has been documented for Acinetobacter protein UmuDAb which is cleaved to be activated after DNA damage (Hare et al., 2012). Also protein Stat6, which is by contrast cleaved in nucleus to function as negative regulator of transcription for target genes in mouse mast cells, uses protolitic modification for regulation of protein function (Suzuki et al., 2002). Proteolytic processing of proteins can also regulate their cellular localization, for example Nuclear homologue of Ca(2+)/calmodulin-dependent protein kinase phosphatase CaMKP-

N changes its localization from nuclear to cytoplasmic after the cleavage of N-terminal portion (Sueyoshi et al., 2012). Even stronger signal of DNA-binding of overexpressed Cbf12 or Cbf12$_\Delta$(1-394) was observed on EMSA in $\Delta$Cbf12 strains possibly suggesting that Cbf11 potentiate each ability of Cbf12 to bind RBP probe. Analogically we observed that signal of shift was also stronger for endogenic wild type Cbf11 when variants of Cbf12 were coexpressed which would suggest that DNA-binding of Cbf11 is potentiated by Cbf12 variants and that Cbf11 and Cbf12 potentiate each other in ability to bind DNA. But because the conditions of in vitro EMSA assay are far from normal physiologic setting in the cell and the level of protein overexpression in native lysate for EMSA assays was not equal these results must be assessed carefully. Further proves are required to confirm such statement.

## 5. 5 Effect of Cbf12 on adhesivity

We tested effects of Cbf12DBM and new truncated variants on adhesivity, because old results showed a positive regulation of adhesivity by Cbf12. We saw, as expected, a strong positive effect of Cbf12 overexpression on adhesivity and to our surprise we observed the same effect for Cbf12DBM overexpression which have been shown to lose ability to directly bind the RBP probe. These results were even observed on media where the promoter for the overexpression was shut off so the expression was very low suggesting that low levels of protein expression are sufficient enough for increased adhesivity to be manifested. This result mean that the positive function of Cbf12 on adhesivity could be independent of direct DNA-binding function of Cbf12. The case could be that Cbf12 does not bind DNA directly. Instead it uses different proteins as DNA-binding mediator but such a mediator has not been identified yet and the conserved motives of DNA-binding domain of CSL proteins suggest that Cbf12 binds directly to DNA. Because the EMSA experiments were performed only with the RBP probe, which is the metazoan CSL responsive element and still has not been shown to bind Cbf12 *in vivo*, the case could be that Cbf12DBM does not bind the RBP probe but it binds a different responsive element in *S. pombe in vivo* to affect pathways regulating adhesivity. The effect on adhesivity can also be indirect and Cbf12 can only serve as a component of the signaling pathway regulating adhesivity independently from its DNA-binding function. One of the possibilities is that the N-terminal part that is expected to be cleaved off could play some role in adhesivity

regulation. This is supported by the fact that on Western blotting analysis of N-terminally tagged Cbf12 the cleaved N-terminal part of the protein is detected in large amounts in some cases. If the N-terminal part did not have any function it would probably be degraded soon after proteolytic processing of Cbf12 although the degradation could not be fast enough in case of overexpression. The accumulation of Cbf12 N-terminal parts could be due to the different affectivity of Western blotting for proteins of different sizes in system with overexpression. We also tested possible toxicity of the overexpression of Cbf protein variants but we did not observe toxicity in any case. Only slightly slower rate of growt was observed in case of Cbf12 overexpression.

## 5. 6 Influence of N-terminal domain of yeast CSL proteins to cellular localization

When we analyzed the protein subcellular localization for all Cbf protein variants in *S. pombe* using N-terminal GFP tag we noticed an interesting fact. Wild type variants of Cbf11 and Cbf12 have a preferably diffused nuclear localization and the same has been observed for Cbf11DBM and Cbf12DBM and the truncated variant $Cbf12_\Delta(1\text{-}394)$. But $Cbf11_\Delta(1\text{-}172)$ and $Cbf11_\Delta(1\text{-}172)DBM$ show very even distribution throughout the cell excluding some unidentified membrane bodies, possibly vacuolas. The change in distribution compared to the full length protein is very distinct. For $Cbf12_\Delta(1\text{-}465)$ and $Cbf12_\Delta(1\text{-}465)DBM$, the distribution was mostly in cytoplasm and it seemed that the nucleus has a lower amount of signal which appeared as a darker round in the middle of the cell. It seems that removing the N-terminal part of Cbf proteins disturbs their natural localization inside the nucleus. In eukaryotic cells the nuclear membrane separates the genome from the rest of the cell. This membrane double layer contains nuclear pore complexes (NPCs) that create connection between the cytoplasm and the nuclear parts of cell. Smaller proteins up to 60 kDa can diffuse through nuclear pores but larger proteins require Nuclear Localization Sequence (NLS) for Nuclear import into the nucleus (Ding et al., 2010; Perez-Terzic et al., 1997). The change of cellular localization could be due to the loss of N-terminal parts containing NLS. For Cbf12, the NLS would have to lay in 72 amino acid long sequence that makes up the difference between the DNA-binding variant Cbf12Δ(1-394) which is located in the nucleus and Cbf12Δ(1-465) which displays mixed cytoplasmic and nuclear localization. For Cbf11 the possible position of NLS could be

anywhere at the N-terminal region that was cut off. The loss of a NLS would explain the strong increase of cytoplasmic localization. The signal in the nucleus can be caused by high levels of overexpressed protein. This would also be supported by the lower level of Cbf12$_\Delta$(1-465) and Cbf12$_\Delta$(1-465)DBM in the nucleus compare to truncated variants of Cbf11 because Cbf12 protein variants are larger which makes their diffusion to the nucleus more difficult. Overexpression of proteins can cause a shift in their cellular localization, for example the overexpression of Regucalcin in rat kidney proximal tubular epithelial NRK52E cells increases its nuclear localization (Nakagawa and Yamaguchi, 2006). The NLS of Cbf11 and Cbf12 could also be damaged making it significantly weaker or another weak NLS which could substitute the main NLS could be present in another part of Cbf proteins. The N-terminal parts of fission yeast Cbf proteins could also be important for interaction with another protein carrying the NLS. This interaction would make the nuclear localization of Cbf proteins possible. This would also explain the mixed cytoplasmic and nuclear localization because the interaction could be only weakened by the removal of N-terminal parts. When Cbf11 and Cbf12 truncated variants are overexpressed some portion of the protein can still create complex and be transported to nucleus. Analogical situation was shown for Metazoal CSL protein CBF1 where muations at amino acid 233 or 249 restricted the protein to the cytoplasm insteade of standard localization in the nucleus. It was shown that this effect could be rescued by the overexpression of SMRT. It was also shown that SMRT interact with SKIP through its C-terminal part. Overexpression of mutated protein that had this C-terminal part cut off could not rescue the restriction of mutated CBF1 in cytoplasm. This showed that the trimeric complex of CBF1-SMRT-SKIP is required for the nuclear localization of CBF1. Certain mutations among the proteins that form the complex disturbed the interaction between proteins leading to change in their cellular localization (Zhou and Hayward, 2001).

The change of cellular localization of truncated variants of CSL proteins in fission yeast could point to possible localization of the NLS sequence. With the current situation among bioinformatic tools for NLS prediction, experimentally traced NLS contribute to knowledge of possible NLS forms which can help to improve bioinformatic NLS prediction.

# 6.  Conclusions

- In this study we constructed N-terminally truncated variants of fission yeast CSL genes Cbf11 and Cbf12 and the DNA-binding mutation of Cbf12. Subsequently, DNA binding properties of all new protein variants were analyzed *in vitro* along with their cellular localization. We analyzed the influence of these new protein variants on adhesivity and toxicity phenotype.

- We have shown that the removal of complete N-terminal parts of yeast CSL proteins leads to the loss of DNA-binding abilities. For Cbf12DBM, we did not observe any interaction with the RBP probe as expected.

- Removal of N-terminal part of fission yeast CSL proteins leads to the change in their subcellular localization. While the wild type Cbf proteins display a strong nuclear localization, new truncated protein variants showed cytoplasmic localization with equal or slightly weaker signal in the nucleus compared to the cytoplasm.

- We also confirmed an influence of overexpressed Cbf11DBM, which is unable to bind DNA, on endogenic Cbf11, manifested by a decrease in DNA-binding of endogenic Cbf11. We have shown that the new truncated variants of Cbf11 display the same effect. Some of the EMSA results indicated a possibility that Cbf11 and Cbf12 potentiate each other in the DNA-binding ability.

- We confirmed previous results showing that Cbf12 is a positive regulator of adhesivity. The same effect on adhesivity had also the variant of Cbf12 with the DNA-binding mutation.. This indicates that the effect of Cbf12 on adhesivity could be independent of the DNA-binding activity.

# 7. References

1. Ansieau, S., Strobl, L. J. & Leutz, A. Activation of the Notch-regulated transcription factor CBF1/RBP-Jkappa through the 13SE1A oncoprotein. *Genes & development* **15**, 380–5 (2001).

2. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. *Science (New York, N.Y.)* **284**, 770–6 (1999).

3. Barolo, S. *et al.* A notch-independent activity of suppressor of hairless is required for normal mechanoreceptor physiology. *Cell* **103**, 957–69 (2000).

4. Barolo, S., Stone, T., Bang, A. G. & Posakony, J. W. Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes & development* **16**, 1964–76 (2002).

5. Beres, T. M. *et al.* PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. *Molecular and cellular biology* **26**, 117–30 (2006).

6. Bocchetta, M., Miele, L., Pass, H. I. & Carbone, M. Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. *Oncogene* **22**, 81–9 (2003).

7. Bolós, V., Grego-Bessa, J. & de la Pompa, J. L. Notch signaling in development and cancer. *Endocrine reviews* **28**, 339–63 (2007).

8. Bray, S. J. Notch signalling: a simple pathway becomes complex. *Nature reviews. Molecular cell biology* **7**, 678–89 (2006).

9. Brou, C. *et al.* A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Molecular cell* **5**, 207–16 (2000).

10. Chen, L., Glover, J. N., Hogan, P. G., Rao, A. & Harrison, S. C. Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. *Nature* **392**, 42–8 (1998).

11. Craven, R. A. *et al.* Vectors for the expression of tagged proteins in Schizosaccharomyces pombe. *Gene* **221**, 59–68 (1998).

12. Decottignies, A., Sanchez-Perez, I. & Nurse, P. Schizosaccharomyces pombe essential genes: a pilot study. *Genome research* **13**, 399–406 (2003).

13. Ding, Q., Zhao, L., Guo, H. & Zheng, A. C. The nucleocytoplasmic transport of viral proteins. *Virologica Sinica* **25**, 79–85 (2010).

14. Dontu, G. *et al.* Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast cancer research : BCR* **6**, R605–15 (2004).

15. Dou, S. *et al.* The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. *Molecular and cellular biology* **14**, 3310–9 (1994).

16. Egel, R. (Ed.) (2004) The Molecular Biology of *Schizosaccharomyces pombe* - Genetics, Genomics and Beyond. Springer-Verlag Berlin Heidelberg New York.

17. Fischer, A. & Gessler, M. Delta-Notch--and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic acids research* **35**, 4583–96 (2007).

18. Folk, P., Půta, F. & Skruzný, M. Transcriptional coregulator SNW/SKIP: the concealed tie of dissimilar pathways. *Cellular and molecular life sciences : CMLS* **61**, 629–40 (2004).

19. Forsburg, S. L. Comparison of Schizosaccharomyces pombe expression systems. *Nucleic acids research* **21**, 2955–6 (1993).

20. Fryer, C. J., White, J. B. & Jones, K. A. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Molecular cell* **16**, 509–20 (2004).

21. Fuchs, K. P. *et al.* Mutational analysis of the J recombination signal sequence binding protein (RBP-J)/Epstein-Barr virus nuclear antigen 2 (EBNA2) and RBP-J/Notch interaction. *European journal of biochemistry / FEBS* **268**, 4639–46 (2001).

22. Fujitani, Y. *et al.* Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. *Development (Cambridge, England)* **133**, 4439–50 (2006).

23. Gaiano, N. & Fishell, G. The role of notch in promoting glial and neural stem cell fates. *Annual review of neuroscience* **25**, 471–90 (2002).

24. Geier, G. E. & Modrich, P. Recognition sequence of the dam methylase of Escherichia coli K12 and mode of cleavage of Dpn I endonuclease. *The Journal of biological chemistry* **254**, 1408–13 (1979).

25. Ghai, V. & Gaudet, J. The CSL transcription factor LAG-1 directly represses hlh-6 expression in C. elegans. *Developmental biology* **322**, 334–44 (2008).

26. Ghosh, G., van Duyne, G., Ghosh, S. & Sigler, P. B. Structure of NF-kappa B p50 homodimer bound to a kappa B site. *Nature* **373**, 303–10 (1995).

27. Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V. E. & Johnson, J. E. Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. *Development (Cambridge, England)* **132**, 5461–9 (2005).

28. Grallert, A., Grallert, B., Ribar, B. & Sipiczki, M. Coordination of initiation of nuclear division and initiation of cell division in Schizosaccharomyces pombe: genetic interactions of mutations. *Journal of bacteriology* **180**, 892–900 (1998).

29. Graves, B. J. *et al.* Structure of interleukin 1 alpha at 2.7-A resolution. *Biochemistry* **29**, 2679–84 (1990).

30. Guldal, C. G. & Broach, J. Assay for adhesion and agar invasion in S. cerevisiae. *Journal of visualized experiments : JoVE* 64 (2006).

31. Hare, J. M., Adhikari, S., Lambert, K. V., Hare, A. E. & Grice, A. N. The Acinetobacter regulatory UmuDAb protein cleaves in response to DNA damage with chimeric LexA/UmuD characteristics. *FEMS microbiology letters* (2012).

32. Hedges, S. B. The origin and evolution of model organisms. *Nature reviews. Genetics* **3**, 838–49 (2002).

33. Heitzler, P. & Simpson, P. The choice of cell fate in the epidermis of Drosophila. *Cell* **64**, 1083–92 (1991).

34. Henkel, T., Ling, P. D., Hayward, S. D. & Peterson, M. G. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. *Science (New York, N.Y.)* **265**, 92–5 (1994).

35. Hori, K. *et al.* A nonclassical bHLH Rbpj transcription factor complex is required for specification of GABAergic neurons independent of Notch signaling. *Genes & development* **22**, 166–78 (2008).

36. Hoveyda, N. *et al.* Neonatal diabetes mellitus and cerebellar hypoplasia/agenesis: report of a new recessive syndrome. *Journal of medical genetics* **36**, 700–4 (1999).

37. Hsieh, J. J. & Hayward, S. D. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. *Science (New York, N.Y.)* **268**, 560–3 (1995).

38. Hsieh, J. J., Zhou, S., Chen, L., Young, D. B. & Hayward, S. D. CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 23–8 (1999).

39. Iso, T., Kedes, L. & Hamamori, Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *Journal of cellular physiology* **194**, 237–55 (2003).

40. Ito, M., Katada, T., Miyatani, S. & Kinoshita, T. XSu(H)2 is an essential factor for gene expression and morphogenesis of the Xenopus gastrula embryo. *The International journal of developmental biology* **51**, 27–36 (2007).

41. Jaleco, A. C. *et al.* Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *The Journal of experimental medicine* **194**, 991–1002 (2001).

42. Jarriault, S. & Greenwald, I. Evidence for functional redundancy between C. elegans ADAM proteins SUP-17/Kuzbanian and ADM-4/TACE. *Developmental biology* **287**, 1–10 (2005).

43. Johnson, S. E., Ilagan, M. X. G., Kopan, R. & Barrick, D. Thermodynamic analysis of the CSL x Notch interaction: distribution of binding energy of the Notch RAM region to the CSL beta-trefoil domain and the mode of competition with the viral transactivator EBNA2. *The Journal of biological chemistry* **285**, 6681–92 (2010).

44. Kao, H. Y. *et al.* A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes & development* **12**, 2269–77 (1998).

45. Kaspar, M. & Klein, T. Functional analysis of murine CBF1 during Drosophila development. *Developmental dynamics : an official publication of the American Association of Anatomists* **235**, 918–27 (2006).

46. Kawaichi, M. *et al.* Genomic organization of mouse J kappa recombination signal binding protein (RBP-J kappa) gene. *The Journal of biological chemistry* **267**, 4016–22 (1992).

47. Koelzer, S. & Klein, T. A Notch-independent function of Suppressor of Hairless during the development of the bristle sensory organ precursor cell of Drosophila. *Development (Cambridge, England)* **130**, 1973–88 (2003).

48. Kosman, D., Ip, Y. T., Levine, M. & Arora, K. Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo. *Science (New York, N.Y.)* **254**, 118–22 (1991).

49. Kovall, R. A. More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene* **27**, 5099–109 (2008).

50. Kovall, R. A. & Hendrickson, W. A. Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. *The EMBO journal* **23**, 3441–51 (2004).

51. Kriwacki, R. W., Hengst, L., Tennant, L., Reed, S. I. & Wright, P. E. Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 11504–9 (1996).

52. Kurooka, H. & Honjo, T. Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. *The Journal of biological chemistry* **275**, 17211–20 (2000).

53. Lai, E. C. Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. *EMBO reports* **3**, 840–5 (2002).

54. Laky, K. & Fowlkes, B. J. Notch signaling in CD4 and CD8 T cell development. *Current opinion in immunology* **20**, 197–202 (2008).

55. Lam, L. T. & Bresnick, E. H. Identity of the beta-globin locus control region binding protein HS2NF5 as the mammalian homolog of the notch-regulated transcription factor suppressor of hairless. *The Journal of biological chemistry* **273**, 24223–31 (1998).

56. Lee, S. H., Wang, X. & DeJong, J. Functional interactions between an atypical NF-kappaB site from the rat CYP2B1 promoter and the transcriptional repressor RBP-Jkappa/CBF1. *Nucleic acids research* **28**, 2091–8 (2000).

57. Ling, P. D. & Hayward, S. D. Contribution of conserved amino acids in mediating the interaction between EBNA2 and CBF1/RBPJk. *Journal of virology* **69**, 1944–50 (1995).

58. Logeat, F. *et al.* The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8108–12 (1998).

59. Masui, T., Long, Q., Beres, T. M., Magnuson, M. A. & MacDonald, R. J. Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. *Genes & development* **21**, 2629–43 (2007).

60. Maundrell, K. nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. *The Journal of biological chemistry* **265**, 10857–64 (1990).

61. McDonnell, A. V. *et al.* Fold recognition and accurate sequence-structure alignment of sequences directing beta-sheet proteins. *Proteins* **63**, 976–85 (2006).

62. Morel, V. & Schweisguth, F. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. *Genes & development* **14**, 377–88 (2000).

63. Mumm, J. S. & Kopan, R. Notch signaling: from the outside in. *Developmental biology* **228**, 151–65 (2000).

64. Mumm, J. S. *et al.* A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Molecular cell* **5**, 197–206 (2000).

65. Murtaugh, L. C., Stanger, B. Z., Kwan, K. M. & Melton, D. A. Notch signaling controls multiple steps of pancreatic differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14920–5 (2003).

66. Murzin, A. G., Lesk, A. M. & Chothia, C. beta-Trefoil fold. Patterns of structure and sequence in the Kunitz inhibitors interleukins-1 beta and 1 alpha and fibroblast growth factors. *Journal of molecular biology* **223**, 531–43 (1992).

67. Nakagawa, T. & Yamaguchi, M. Overexpression of regucalcin enhances its nuclear localization and suppresses L-type Ca2+ channel and calcium-sensing receptor mRNA expressions in cloned normal rat kidney proximal tubular epithelial NRK52E cells. *Journal of cellular biochemistry* **99**, 1064–77 (2006).

68. Nambu, J. R., Lewis, J. O., Wharton, K. A. & Crews, S. T. The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**, 1157–67 (1991).

69. Ohashi, S. *et al.* NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network. *Gastroenterology* **139**, 2113–23 (2010).

70. Olave, I., Reinberg, D. & Vales, L. D. The mammalian transcriptional repressor RBP (CBF1) targets TFIID and TFIIA to prevent activated transcription. *Genes & development* **12**, 1621–37 (1998).

71. Oswald, F., Liptay, S., Adler, G. & Schmid, R. M. NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-Jkappa. *Molecular and cellular biology* **18**, 2077–88 (1998).

72. Oswald, F. *et al.* SHARP is a novel component of the Notch/RBP-Jkappa signalling pathway. *The EMBO journal* **21**, 5417–26 (2002).

73. Palomero, T. *et al.* NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 18261–6 (2006).

74. Perez-Terzic, C., Jaconi, M. & Clapham, D. E. Nuclear calcium and the regulation of the nuclear pore complex. *BioEssays : news and reviews in molecular, cellular and developmental biology* **19**, 787–92 (1997).

75. Petcherski, A. G. & Kimble, J. Mastermind is a putative activator for Notch. *Current biology : CB* **10**, R471–3 (2000).

76. Převorovský, M. *et al.* N-termini of fungal CSL transcription factors are disordered, enriched in regulatory motifs and inhibit DNA binding in fission yeast. *PloS one* **6**, e23650 (2011).

77. Prevorovský, M. *et al.* Cbf11 and Cbf12, the fission yeast CSL proteins, play opposing roles in cell adhesion and coordination of cell and nuclear division. *Experimental cell research* **315**, 1533–47 (2009).

78. Prevorovský, M., Půta, F. & Folk, P. Fungal CSL transcription factors. *BMC genomics* **8**, 233 (2007).

79. Raharjo, I. & Gaudet, J. Gland-specific expression of C. elegans hlh-6 requires the combinatorial action of three distinct promoter elements. *Developmental biology* **302**, 295–308 (2007).

80. Rangarajan, A. *et al.* Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *The EMBO journal* **20**, 3427–36 (2001).

81. Rebay, I. *et al.* Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687–99 (1991).

82. Ronchini, C. & Capobianco, A. J. Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). *Molecular and cellular biology* **21**, 5925–34 (2001).

83. Rose, S. D., Swift, G. H., Peyton, M. J., Hammer, R. E. & MacDonald, R. J. The role of PTF1-P48 in pancreatic acinar gene expression. *The Journal of biological chemistry* **276**, 44018–26 (2001).

84. Sellick, G. S. *et al.* Mutations in PTF1A cause pancreatic and cerebellar agenesis. *Nature genetics* **36**, 1301–5 (2004).

85. Shirakata, Y., Shuman, J. D. & Coligan, J. E. Purification of a novel MHC class I element binding activity from thymus nuclear extracts reveals that thymic RBP-Jkappa/CBF1 binds to NF-kappaB-like elements. *Journal of immunology (Baltimore, Md. : 1950)* **156**, 4672–9 (1996).

86. Sipiczki, M., Grallert, B. & Miklos, I. Mycelial and syncytial growth in Schizosaccharomyces pombe induced by novel septation mutations. *Journal of cell science* **104 ( Pt 2**, 485–93 (1993).

87. Sueyoshi, N. *et al.* Functional processing of nuclear Ca2+/calmodulin-dependent protein kinase phosphatase (CaMKP-N): evidence for a critical role of proteolytic processing in the regulation of its catalytic activity, subcellular localization and substrate targeting in vivo. *Archives of biochemistry and biophysics* **517**, 43–52 (2012).

88. Suzuki, K. *et al.* Proteolytic processing of Stat6 signaling in mast cells as a negative regulatory mechanism. *The Journal of experimental medicine* **196**, 27–38 (2002).

89. Talora, C., Sgroi, D. C., Crum, C. P. & Dotto, G. P. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes & development* **16**, 2252–63 (2002).

90. Tamura, K. *et al.* Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). *Current biology : CB* **5**, 1416–23 (1995).

91. Taniguchi, Y., Furukawa, T., Tun, T., Han, H. & Honjo, T. LIM protein KyoT2 negatively regulates transcription by association with the RBP-J DNA-binding protein. *Molecular and cellular biology* **18**, 644–54 (1998).

92. Thiagalingam, S. *et al.* Histone deacetylases: unique players in shaping the epigenetic histone code. *Annals of the New York Academy of Sciences* **983**, 84–100 (2003).

93. Thomas, J. B., Crews, S. T. & Goodman, C. S. Molecular genetics of the single-minded locus: a gene involved in the development of the Drosophila nervous system. *Cell* **52**, 133–41 (1988).

94. Tun, T. *et al.* Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. *Nucleic acids research* **22**, 965–71 (1994).

95. Van De Bor, V., Walther, R. & Giangrande, A. Some fly sensory organs are gliogenic and require glide/gcm in a precursor that divides symmetrically and produces glial cells. *Development (Cambridge, England)* **127**, 3735–43 (2000).

96. Wallberg, A. E., Pedersen, K., Lendahl, U. & Roeder, R. G. p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains in vitro. *Molecular and cellular biology* **22**, 7812–9 (2002).

97. Warringer, J. & Blomberg, A. Evolutionary constraints on yeast protein size. *BMC evolutionary biology* **6**, 61 (2006).

98. Weijzen, S., Zlobin, A., Braid, M., Miele, L. & Kast, W. M. HPV16 E6 and E7 oncoproteins regulate Notch-1 expression and cooperate to induce transformation. *Journal of cellular physiology* **194**, 356–62 (2003).

99. Wilson, J. J. & Kovall, R. A. Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. *Cell* **124**, 985–96 (2006).

100. Wood, V. *et al.* The genome sequence of Schizosaccharomyces pombe. *Nature* **415**, 871–80 (2002).

101. Xie, H. *et al.* Functional anthology of intrinsic disorder. 3. Ligands, post-translational modifications, and diseases associated with intrinsically disordered proteins. *Journal of proteome research* **6**, 1917–32 (2007).

102. Zhou, S. *et al.* SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function. *Molecular and cellular biology* **20**, 2400–10 (2000).

103. Zhou, S. & Hayward, S. D. Nuclear localization of CBF1 is regulated by interactions with the SMRT corepressor complex. *Molecular and cellular biology* **21**, 6222–32 (2001).