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MEMBERS OF THE CREB/ATF AND AP1 FAMILY OF TRANSCRIPTION FACTORS ARE INVOLVED IN THE REGULATION OF *SOX18* GENE EXPRESSION

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Abstract – The SOX18 transcription factor plays an important role in endothelial cell specification, angiogenesis and atherogenesis. By profiling transcription factor interactions (TranSignal™ TF Protein Array) we identified several transcription factors implicated in angiogenesis that have the ability to bind to the *SOX18* optimal promoter region *in vitro*. In this report we focused our attention on distinct transcription factors identified by the array as belonging to AP-1 and CREB/ATF protein families. In particular, we analyzed the effects of CREB, JunB, c-Jun and ATF3 on *SOX18* gene expression. Functional analysis revealed that CREB acts as a repressor, while JunB, c-Jun and ATF3 act as activators of *SOX18* promoter activity. Our findings indicate that a transcriptional network that includes CREB, JunB, c-Jun and ATF3 could be involved in angiogenesis-related transcriptional regulation of the *SOX18* gene.

Key words: SOX18, CREB/ATF, AP-1, angiogenesis

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INTRODUCTION

SOX (sex-determining region Y box) genes constitute a large family of diverse and well-conserved genes that encode for transcription factors implicated in the control of various developmental processes (Pevny and Lovell-Badge, 1997; Wegner, 1999). They are characterized by the presence of a DNA-binding HMG box domain (Pevny and Lovell-Badge, 1997; Wegner, 1999) and classified into ten groups (A – J), based on HMG box homology and intron-exon structure (Bowles et al., 2000). The *Sox18/SOX18* gene, together with the Sox7/*SOX7* and *Sox17/SOX17* genes, belongs to the *SOX* group F (Bowles et al., 2000). It has been shown that *Sox*F family members have a role in vascular development and postnatal neovascularization (Cermenati et al., 2008; Matsui et al., 2006). Moreover, the functional importance of SOX18 proteins in vascular development is revealed by the vascular defects caused by *Sox18*/*SOX18* mutations in mice and humans. Mutations in *Sox18* underlie the mutant phenotype of *ragged* mutant mouse (Downes and Koopman, 2001) and mutations in human *SOX18* are associated with the hypotrichosis-lymphedema-telangiectasia syndrome (Irrthum et al., 2003). Murine *Sox18* is demonstrated to be involved in the induction of angiogenesis during wound healing and tissue repair (Darby et al., 2001) and *SOX18* is shown to play a role in atherosclerosis in humans (Garcia-Ramirez et al., 2005). Furthermore, it has been demonstrated that interfering with SOX18 function inhibits blood vessel formation and subsequent tumor growth (Young et al., 2006).

So far, our major goal has been to investigate the transcriptional regulation of the human *SOX18* gene. We have characterized the *SOX18* promoter and demonstrated that the ubiquitous transcription factors Sp3 (specificity protein 3), ZBP-89

(zinc finger binding protein 89), NF-Y (nuclear factor Y) and EGR1 (early growth response protein 1) are involved in the regulation of its expression in the HeLa tumor cell line and in the endothelial cell line EA.hy926 (Petrovic et al., 2010a; Petrovic et al., 2009; Petrovic and Stevanovic, 2007). Furthermore, we have demonstrated that the angiogenic factor VEGF and the pro-inflammatory cytokine TNF increase the SOX18 protein level in human umbilical vein endothelial cells (HUVEC) (Petrovic et al., 2010b). Also, we showed that non steroidal anti-inflammatory drugs have an inhibitory effect on SOX18 in endothelial cells (Petrovic et al., 2010b). Thus, our aim has been to gain additional insights into the complex mechanisms involved in the regulation of human *SOX18* gene expression that will improve our understanding of its role in physiological and pathophysiological processes.

In this study we have extended our search for transcription factors involved in the regulation of the human *SOX18* gene expression. By transcription factor (TF) interaction profiling (TranSignal™ TF Protein Array) we have identified several TFs implicated in angiogenesis that have the ability to bind to the *SOX18* optimal promoter region *in vitro*. Since *in silico* analysis revealed the presence of a cAMP response element (CRE) within the *SOX18* optimal promoter, we focused our attention on the functional analysis of TFs (identified by the TranSignal™ TF Protein Array), that bind to CRE/CRE-like elements (Berhane and Boggaram, 2001; Hai and Curran, 1991). In particular, we have analyzed the effects of CREB (cAMP response element-binding), ATF3 (activating transcription factor 3), c-Jun and JunB. These TFs are members of the basic region/leucine zipper (bZIP) family of transcription factors Hai and Curran, 1991). The bZIP factors contain a basic domain required for interactions with DNA and an adjacent leucine zipper domain that facilitates dimerization between family members (Busch and Sassone-Corsi, 1990).

Here we report that overexpression of the *SOX18* promoter activity in HeLa cells is downregulated by CREB and upregulated by ATF3, c-Jun and JunB. These findings indicate that CREB/ATF and AP-1 transcription factors might play important roles in the transcriptional regulation of *SOX18* gene expression.

MATERIALS AND METHODS

TranSignal™ TF Protein Array IV

DNA-protein interactions were examined by TranSignal™ TF Protein Array IV (Panomics). This array enables the simultaneous interaction profiling of the promoter of interest with 42 TFs immobilized on an array membrane. Briefly, we generated a 5′ biotinylated DNA probe that encompasses the *SOX18* promoter region (-700 to +147 bp) in order to assess which TFs arrayed on the membrane have the potential to bind to the analyzed region. This region, encompassing the optimal *SOX18* promoter (-726 to +166 bp) (Petrovic et al., 2010a; Petrovic and Stevanovic, 2007), was amplified using primers containing artificial *Xho*I and *Hin*dIII restriction sites:

Forward: 5′TACTCGAGAGCCAGCAAGCCACTGAG3′ and

Reverse: 5′CTAAGCTTAACGGAGCGCGGGAGCGC3′, (restriction sites underlined).

The obtained PCR product was digested with the corresponding enzymes. The *SOX18* promoter fragment, 847 bp in size, was labeled at the 5′ end at the *Xho*I site with biotin using Bio-16-dUTP (Roche) and the Klenow fragment (Amersham). Hybridization, washing and detection of signals was performed according to the manufacturer`s instructions. The specificity of binding was confirmed by a competition assay using 10-times molar excess of the unlabeled *SOX18* DNA probe.

In silico analysis

MatInspector Release professional 7.2.2 (http://www.genomatix.de/) was used to analyze the putative

transcription factor binding sites within the *SOX18* promoter region.

Site-directed mutagenesis

Site-directed mutagenesis was performed by PCR, according to the protocol of the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene), using an 892pCAT6 construct as a template (Petrovic and Stevanovic, 2007). By using oligonucleotide SOX-18CREBmut - 5'CTCAAAGTCCCTGGTGCT**C-GA**GGTTACACATGG3', a triple exchange variant (*wt*: T**GAC**G; *mut*: T**CGA**G) was introduced into the CRE binding half-site (Fig. 2A). Following mutagenesis, the mutated construct was sequenced to confirm the presence of the required mutation and also to verify that no additional mutations were introduced by PCR.

Transient transfection assays

HeLa cells were transfected using the calcium-phosphate precipitation method, as previously described (Petrovic et al., 2010a). Briefly, 1.2 x 10^6 cells were seeded in a 10 cm dish and transfected with 10 μg of *SOX18* promoter constructs 892pCAT6/892pCAT6mut, together with 3 μg of pCH110 vector (Amersham Pharmacia Biotech) and 4 μg of pBluescript (Stratagene). In the co-transfection assays, 2 μg of either an empty expression vector (pcDNA3) or the corresponding CREB expression vector was used. For ATF3, c-Jun and JunB, 10 μg of corresponding expression vectors were used (pCDNA3 for c-Jun and JunB and pTarget for ATF3 expression vector). Extracts for β-galactosidase (β-gal) and chloramphenicol acetyltransferase (CAT) assays were prepared 48 h after transfection. β-gal and CAT assays were performed as described (Kovacevic Grujicic et al., 2005). The normalized CAT activities were evaluated as a percentage of the selected promoter construct which was set as 100% activity. Mean values of relative CAT activities were compared with Student's *t* test using SPSS10.0 software. A *p* value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Identification of TFs binding to SOX18 promoter

The TranSignal™ TF Protein Array IV provides screening of transcription factor abilities to bind to and potentially regulate the expression of a gene of interest. This methodology enables the simultaneous profiling of interactions between the promoter of interest and 42 TFs immobilized on array membranes.

In order to investigate direct interactions between the various transcription factors and the promoter region of the human *SOX18* gene, we performed a TF Protein Array using a DNA probe derived from the *SOX18* promoter spanning the region -700 to +147 bp relative to the transcription start point (*tsp*). In this study, we selected the TranSignal™ TF Protein Array IV that contains, among others, transcription factors implicated in angiogenesis. We tested their ability to bind to the fragment encompassing the *SOX18* promoter region. We detected the binding of numerous TFs to the *SOX18* optimal promoter region. The specificity of the binding was confirmed by a competition assay using a 10-times molar excess of the unlabeled DNA probe. Out of 42 TFs tested, 22 TFs displayed the ability to specifically bind to the *SOX18* promoter region (data not shown). Factors identified by this array belong to EGR (Early growth response), ETS (E-twenty six), PPAR (peroxisome proliferator-activated receptor), AP-1 (activator protein 1) and CREB/ATF family of proteins. We have previously shown that EGR1 transcription factor binds to the minimal promoter region of the human *SOX18* gene and acts as a potent activator of both *SOX18* promoter activity and its endogenous expression in HeLa and EA.hy926 cells (Petrovic et al., 2010a).

Accordingly, we focused our attention on the distinct bZIP TFs, identified by the TF protein array, that belong to CREB/ATF and AP-1 protein families (Fig. 1), which are implicated in the process of angiogenesis (Gerald et al., 2004; Nawa et al., 2002; Schmidt et al., 2007; Schorpp-Kistner et al., 1999; Zhang et al., 2004). In particular, we investigated the

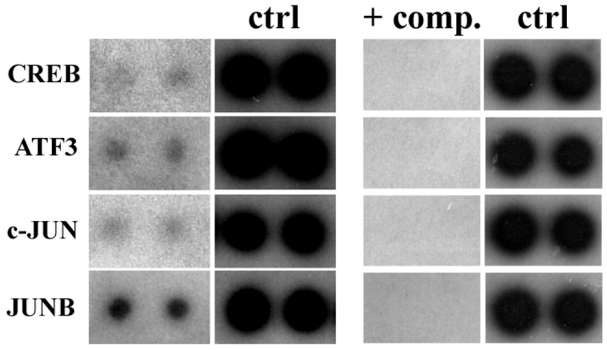

Fig. 1. DNA-protein interactions between the *SOX18* promoter region (-700 to +147) and CREB, ATF3, c-Jun and JunB transcription factors. TranSignal™ TF Protein Array IV membrane was incubated with a 5' biotinylated *SOX18* promoter fragment in the absence or presence of 10 times molar excess of unlabeled probe (+ comp). The proteins on the array are spotted in duplicate. HRP markers (spotted in duplicate) that served as a positive control of detection are designated as ctrl.

effect of CREB, ATF3 and AP-1 family members (c-Jun and JunB) on *SOX18* promoter activity.

Functional analysis of the CRE binding motif within SOX18 promoter

MatInspector analysis of the *SOX18* optimal promoter region revealed one putative CRE binding half-site positioned at -477 to -473 bp, relative to *tsp* (Fig. 2A). It is interesting that the octamer sequence motif encompassing this CRE binding half-site and 3 downstream nucleotides is almost identical (7 out of 8 nucleotides are identical) to the previously described CRE element in the *c-fos* promoter (Fisch et al., 1987). Thus, we assume that this motif represents a functional regulatory element, since a plethora of deviations from the consensus sequence for CRE/ATF has been reported (Benbrook and Jones, 1994; Yin et al., 2008).

In order to functionally characterize this putative regulatory element, we performed site-directed mutagenesis of the CRE half-site within the *SOX18* promoter region as described in Materials and Methods (Fig. 2B). The ability of the mutant reporter construct and its *wild type* counterpart to drive the expression of the reporter gene was analyzed in the HeLa cell line. Mutations in the CRE half-site resulted in approximately 1.4 fold increase of *SOX18* promoter activity (Fig. 2B). Thus, the functional analysis of the *SOX18* promoter suggests that the analyzed binding motif represents a negative control element.

Effect of CREB overexpression on SOX18 promoter activity

It has been shown that CREB binds to both CRE half-sites (TGACG) as well as to full palindromic motifs with different affinities (Mayr and Montminy, 2001; Nichols et al., 1992). Since CREB is able to repress several target genes (Lamph et al., 1990; Lemaigre et al., 1993; Melnikova et al., 2010; Ofir et al., 1991) and we demonstrated that mutations in the CRE half-site resulted in an up-regulation of *SOX18* promoter activity, our further goal was to test the effect of ectopically expressed CREB on *SOX18* promoter activity. Cotransfection experiments in HeLa cells revealed that overexpression of CREB leads to a considerable reduction of the 892pCAT6 construct activity, by approximately 60% (Fig. 3). Accordingly, we hypothesize that CREB acts as a repressor of *SOX18* promoter activity, at least in part, through the CRE motif analyzed in this study.

Several lines of evidence implicate CREB in angiogenesis. This transcription factor dramatically affects cellular functions such as enhanced growth, increased angiogenesis and decreased apoptosis, which define the fate of a growing tumor (Abramovitch et al., 2004). Also, the expression of CCN1, which is known to be involved in tumorigenesis and angiogenesis, is activated by CREB-mediated transcriptional activation (Meyuhas et al., 2008). In addition, Jin et al. have implicated CREB in mediating VEGFR1 (Vascular endothelial growth factor receptor 1) expression *in vivo* (Jin et al., 2009). The results presented here further suggest that CREB might exert its role in angiogenesis, at least in part, by controlling *SOX18* gene expression.

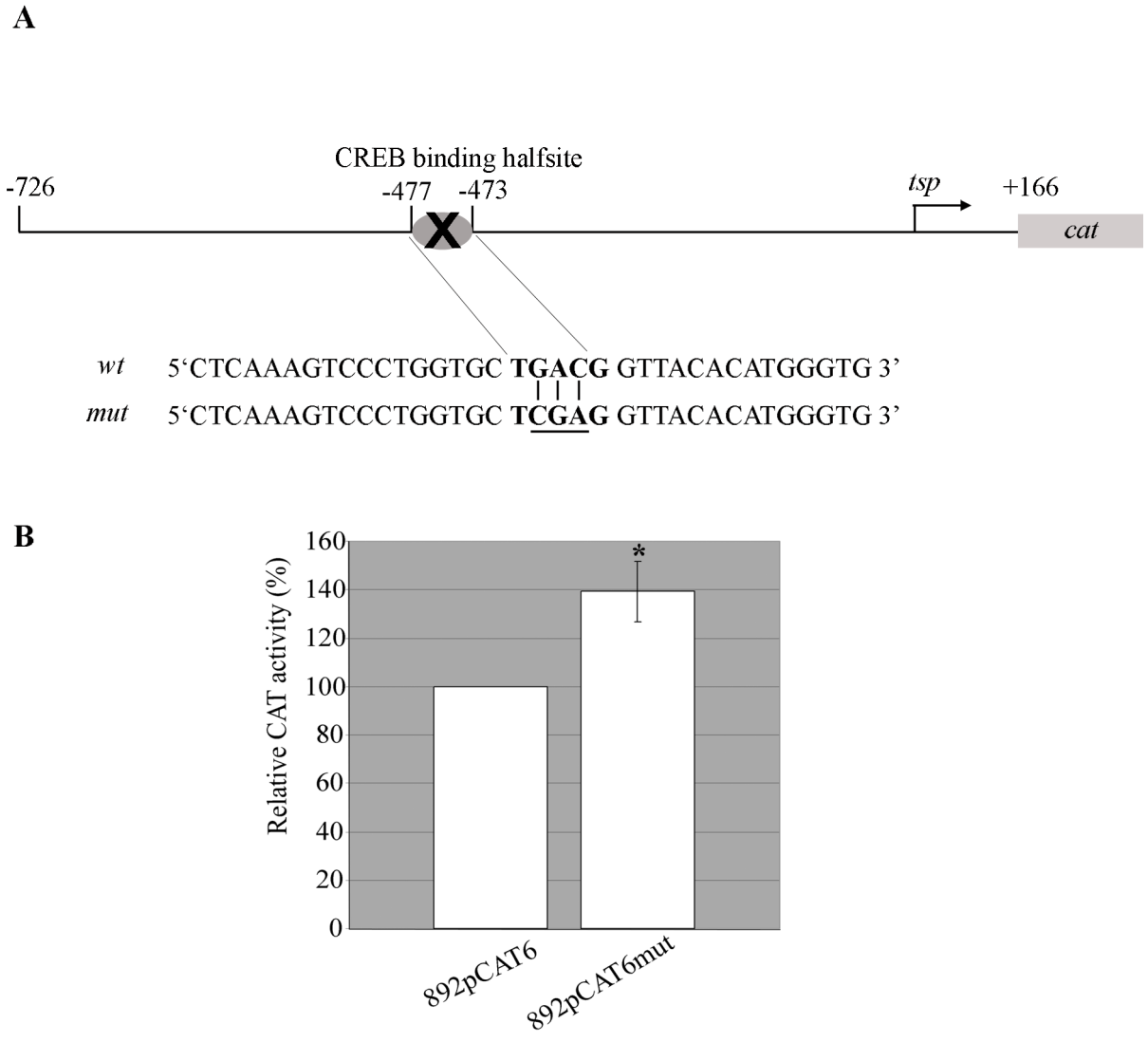

Fig. 2. The effect of CRE binding half-site mutation on *SOX18* promoter activity. A) Schematic illustration of the 892pCAT6mut construct. The positions of the putative CRE binding half-site, relative to *tsp*, are indicated. The CRE binding half-site is presented in bold; mutated nucleotides are underlined. B) Functional analysis of the mutant construct 892pCAT6mut. HeLa cells were transfected with either *wild type* (892pCAT6) or CRE mutated (892pCAT6mut) *SOX18* promoter-reporter construct and analyzed for promoter activity as a function of CAT activity. Normalized CAT activity was calculated as the percentage of the 892pCAT6 reporter construct activity which was set as 100%. The data are presented as the means ± S.E.M. of at least three independent experiments. Mean values of relative CAT activities were compared with Student's *t*-test. Value of $p<0.05$ is presented by *.

Effects of overexpression of JunB, and c-Jun ATF3 on SOX18 promoter activity

Proteins of the bZIP family bind as dimers to their cognate DNA binding sequences (Hai and Curran, 1991). The sequence of each bZIP domain also governs whether these proteins form homodimers or heterodimers (Alberini, 2009). The specificity of function for these proteins is determined primarily by the variable combinations between bZIP proteins,

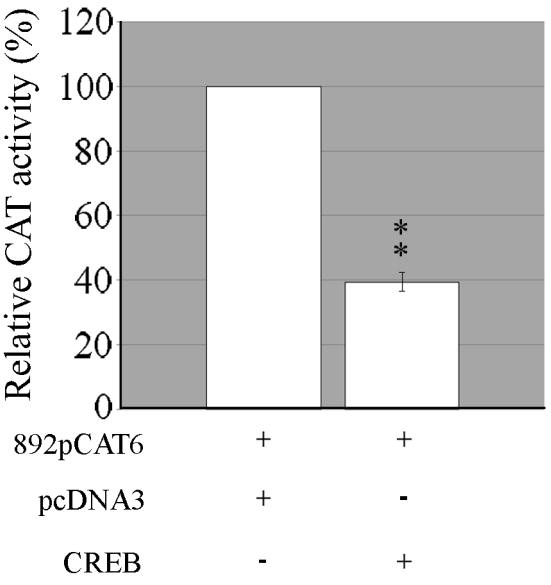

Fig. 3. The effect of CREB overexpression on *SOX18* promoter activity. HeLa cells were transiently co-transfected with the *SOX18* promoter-reporter construct (892pCAT6), together with either the empty vector pcDNA3 or the CREB expression vector. Normalized CAT activity was calculated as the percentage of 892pCAT6 activity in cells co-transfected with empty vector pcDNA3, which was set as 100%. The data are presented as the means ± S.E.M. of three independent experiments. Mean values of relative CAT activities were compared with Student's *t*-test. Value of p<0.01 is presented by **.

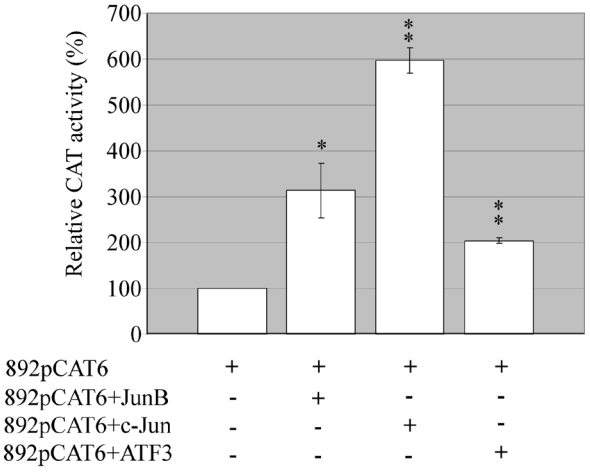

Fig. 4. The effects of JunB, c-Jun and ATF3 overexpression on *SOX18* promoter activity. HeLa cells were transiently cotransfected with *SOX18* promoter-reporter construct (892pCAT6), together with either pcDNA3 JunB, pcDNA3 c-Jun or pTarget ATF3 expression vectors. Normalized CAT activity was calculated as the percentage of the 892pCAT6 activity in cells co-transfected with corresponding empty vector which was set as 100%. The data are presented as the means ± S.E.M. of at least three independent experiments. Mean values of relative CAT activities were compared with Student's *t*-test. Value of p<0.05 is presented by * and values of p<0.01 are presented by **.

as well as by interactions with other TFs and co-factors (Hai and Curran, 1991; Hsu et al., 1991; van Dam and Castellazzi, 2001). In this report, the bZIP proteins JunB, c-Jun and ATF3 that were identified by TF Protein Array, were subjected to further functional analysis.

We have investigated the individual effects of JunB, c-Jun and ATF3 overexpression on *SOX18* promoter activity in HeLa cells. Co-transfection experiments revealed that JunB, c-Jun and ATF3 act as transcriptional activators, upregulating *SOX18* promoter activity by 3, 6 and 2- fold, respectively (Fig. 4). It is interesting to point out that increasing evidence implicates CREB/ATF and AP-1 family members in angiogenic responses and programs (Gerald et al., 2004; Schorpp-Kistner et al., 1999; Zhang et al., 2004). For example, Id1, one of the ATF3 target genes, regulates angiogenesis by changing the expression levels of thrombospondin 1 and VEGF (Benezra et al., 2001; Kang et al., 2003; Ling et al., 2005; Volpert et al., 2002). Similar to *SOX18*, ATF3 expression has also been detected in atherosclerotic lesions (Garcia-Ramirez et al., 2005; Nawa et al., 2002). It has also been reported that JunB has a critical role in vascular development and tumor angiogenesis by regulating VEGF transcription in response to mitogens and hypoxia (Schmidt et al., 2007). In addition, DNAzyme-mediated suppression of c-Jun inhibits the corneal neovascularization stimulated by VEGF and also significantly reduces tumor growth in an *in vivo* mouse model (Zhang et al., 2004).

Taken together, the presented results indicate that a transcriptional network including CREB, ATF3, c-Jun and JunB could be involved in the angiogenesis-related transcriptional regulation of *SOX18* gene expression. It should be pointed out that CREB/

ATF and AP-1 factors act in response to a variety of stimuli, including cytokines, growth factors, stress, mechanical injury and ischemia/hypoxia, as also shown for SOX18 itself (Cai et al., 2000; Chi et al., 2006; Darby et al., 2001; Hess et al., 2004; Leonard et al., 2008; Nawa et al., 2002; Young et al., 2006). Accordingly, it would be interesting to further explore the interplay of these TFs in *SOX18* gene regulation under various stress conditions.

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