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Yeast one-hybrid screen of a thymus epithelial library identifies ZBTB7A as a regulator of thymic insulin expression

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Abstract

Insulin self-tolerance is, to a large extent, assured by the expression of small quantities of insulin by medullary thymic epithelial cells (mTECs). Regulation of thymic insulin expression differs from that in pancreas and its therapeutic manipulation could play an important role in the prevention of type 1 diabetes (T1D). Knowledge of the transcriptional regulators involved in the mTEC nuclear environment is essential for the development of such therapeutics. The yeast one-hybrid (Y1H) approach was used in order to identify such mTEC-specific nuclear proteins. We used a target composed of the human insulin gene promoter joined to the upstream class III VNTR allele, which is associated with both protection from T1D and higher thymic insulin expression, and a cDNA library from our insulin-producing mouse mTEC line. The Y1H screening allowed the identification of eleven proteins. An *in vitro* assay was used to confirm and quantify protein-DNA binding to the human insulin gene promoter alone or joined to a class I or class III VNTR allele, and identified the transcription factors ZBTB7A, JUN and EWSR1 as strong interacting partners. All three proteins could induce insulin expression in transfected HEK-293 cells, but ZBTB7A provided the most robust results especially in the presence of AIRE, with an additional 11-fold increase of the insulin mRNA levels from a co-transfected reporter driven by the class III VNTR allele. Thus, ZBTB7A is identified as a strong candidate for regulation of thymic insulin expression.

Keywords

insulin expression; thymus; transcription factors; type 1 diabetes; yeast one-hybrid

1. Introduction

Type 1 diabetes (T1D) is a complex trait with a strong genetic component (Polychronakos and Li, 2011). So far, more than 40 T1D susceptibility loci have been identified (Ounissi-Benkalha and Polychronakos, 2008). After the major histocompatibility complex (MHC) region (Aly et al., 2006; Anjos and Polychronakos, 2004), the second strongest effect comes from a 4.1-kb region on chromosome 11 and maps to a variable number of tandem repeats

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CONFLICT OF INTEREST

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(VNTR) located 596 bp upstream of the insulin gene (Ounissi-Benkalha and Polychronakos, 2008; Bennett et al. 1995). It consists of tandem repeats of 14–15 bp bearing the consensus sequence unit ACAGGGGTGTGGGG (Bell et al., 1982; Vafiadis et al., 2001). The class I allele is the shortest and predisposes to T1D (26 to 63 repeats) whereas the long class III allele (140 to 210 repeats) provides a co-dominant protective effect, reducing the risk of T1D of 2- to 4-fold compared to class I/I homozygotes (Anjos and Polychronakos, 2004).

As previously demonstrated in our laboratory, the impact of the VNTR on the disease is related to the expression of insulin in the thymus (Vafiadis et al., 1997), where levels of insulin mRNA were 2 to 3 times higher from chromosomes carrying class III alleles compared to class I, showing that VNTR alleles only marginally influence insulin mRNA levels in the pancreas. It was therefore hypothesized that insulin expression in the thymus could be implicated in the establishment of immune central tolerance. These results were validated by several other studies and insulin was confirmed to be one of the major autoantigens involved in the disease (Durinovic-Belló et al., 2010; Fan et al., 2009; Kent et al., 2005; Nakayama et al. 2005; Taubert et al., 2007). This observation inspired studies that have demonstrated a more general mechanism through which specific autoreactive T cells undergo negative selection due to thymic expression of tissue-restricted antigens (TRAs) (Derbinski et al., 2001). Human and animal-model studies have shown association between thymic insulin expression and autoreactivity toward insulin as lower levels of insulin in the thymus have been correlated to the presence of autoreactive insulin-specific T lymphocytes (Durinovic-Belló et al., 2010; Chentoufi and Polychronakos, 2002; Durinovic-Belló et al., 2005) and accelerated diabetes in the non-obese diabetic (NOD) mouse (Thébault-Baumont et al., 2003).

The selection for self-tolerant T cells takes place in the thymus and requires AIRE, the autoimmune regulator, responsible of the expression of thousands of TRAs in a specialized subset of medullary thymic epithelial cells (mTECs) (Derbinski et al., 2001; Palumbo et al., 2006). By a mechanism that is still poorly understood, AIRE induces the expression of TRAs within the mTECs, which present the antigens for the purpose of negative selection (Anderson et al., 2005; Irla et al., 2008). The AIRE-dependance of thymic insulin expression has been directly demonstrated in the mouse (Anderson et al., 2002) and indirectly in the human (Ahonen et al., 1990).

Although insulin expression in the thymus seems to depend on both AIRE and the VNTR (Taubert et al., 2007; Cai et al., 2011), the transcriptional regulation of thymic gene expression is still poorly understood. In order to address this question, we probed an insulin-producing mTEC line that was previously generated in our laboratory (Palumbo et al., 2006). We used the yeast one-hybrid (Y1H) approach to identify factors that had the capacity to regulate insulin expression by binding to a genomic sequence composed of the human insulin gene promoter joined to the class III VNTR allele. Candidates thus identified were functionally evaluated.

2. Materials and methods

2.1 Yeast one-hybrid screening

The Y1H screening was performed using the Matchmaker™ One-Hybrid Library Construction & Screening Kit from Clontech, as recommended by the manufacturer. The yeast strain Y187, which is unable to synthetize histidine, was used for the assay. The vector containing the DNA target, or bait, was assembled by transferring the bait to vector pHIS2.1 (supplied with the kit). The bait was composed of the class III VNTR allele (E1 clone (Vafiadis et al., 2001)) joined to the minimal promoter of the human insulin gene, which reached nucleotide -188 (with regard to the ATG) at its 3′ end. The cDNA library used for

the screening was generated using the Advantage 2 PCR Kit and the vector pGADT7-Rec2, both from Clontech, using RNA from our insulin-producing mTEC line (Palumbo et al., 2006). For the one-hybrid library screening, both the bait and the cDNA library were cotransformed in competent yeast cells and the resulting cells were plated on the appropriate selective medium (synthetically defined (SD) medium containing leucine, tryptophan and histidine). Positive colonies were restreaked on the selective medium to remove contaminating plasmids and, by yeast colony PCR, we amplified the genes responsible for the Y1H interactions. For these amplifications, we used the Expand High Fidelity^{PLUS} PCR System from Roche in combination with the primers Matchmaker ADLD from Clontech. PCR products were sequenced for identification of the interacting proteins.

2.2 Protein-DNA binding assay

The Protein-DNA Binding Assay Kit from Clontech was used in this step. Clones to be tested were first transfected in HEK-293 cells and two days later, a whole cell extract was prepared as recommended by the manufacturer. Of this extract, 75 μg of protein (measures by the BCA™ Protein Assay Kit from PIERCE) was mixed with either blocking buffer (for the background reference) or one of the three biotinylated DNA targets. Protein-DNA mixes were then added to wells coated with streptavidin and subsequently washed and revealed using the appropriate substrates. ProLabel activity was quantified using the VICTOR plate reader from PerkinElmer. Luminescence was measured every 15 minutes and results were determined by taking the mean signal:background ratio at 45 and 60 minutes. Every assay, starting from the transfection step, was carried out in triplicates.

2.3 Cloning of the interacting genes and controls

Overall, 19 genes were cloned in frame with the ProLabel tag from the vector pProLabel-C (Clontech). Those included the 11 mouse genes from the Y1H screening, 5 human orthologues of these genes (*Ddx1*, *Zfp90*, *Jun*, *Zbtb7a* and *Ewsr1*), and *AIRE* and *Pdx-1* as positive controls, both mouse and human. The Expand High Fidelity^{PLUS} PCR System from Roche or the Platinum® Pfx DNA Polymerase from Invitrogen was used for PCR amplifications according to the target. All 19 genes were cloned, transformed and sequenced using standard methods.

2.4 Transient transfections

HEK-293 cells were used for transfections related to the protein-DNA binding assay and to the assay for the modulation of insulin expression as well. The CalPhos™ Mammalian Transfection Kit form Clontech was used to this end. Between 2 and 7 μg of the pProLabel constructs were used for each reaction (depending on the ProLabel tag expression (data not shown)) whereas different amounts of plasmids were used to study the modulation of insulin expression (depending on the type of vector).

2.5 Generation of the DNA targets

Three different DNA targets were generated to carry out the protein-DNA binding assay: the human insulin gene promoter alone, with the first 50 bases of exon 1 (oligo ins389; 389 bp), or joined to the class I (oligo I; 1863 bp) or class III VNTR allele (oligo III; 3393 bp). For the protein-DNA binding assay, the three DNA targets were labeled with biotin using the Biotin 3′ End DNA Labeling Kit from PIERCE, as recommended by the manufacturer. Following each reaction, labeling efficiency was assessed and quantified by slot blot and chemiluminescence using the kit controls.

2.6 Modulation of insulin expression

Modulation of insulin expression by human JUN, EWSR1 and ZBTB7A was assessed in HEK-293 cells, with or without AIRE in triplicate transfections. Constructs containing the promoter regions were different for this assay as they were bearing the complete insulin gene including the introns. Vectors pENTRY-noVNTR, -classI (814 allele) or -classIII (E1 allele) were thus used as reporters for this assay and insulin mRNA levels were measured by quantitative PCR, using the TaqMan® Gene Expression Assay from Applied Biosystems.

3. Results

3.1 Identification of the putative regulators

The Y1H system allows the identification of novel genes encoding proteins that bind to a specific DNA target, in this case, factors regulating insulin expression in the thymus by binding to the human insulin gene promoter or the class III VNTR allele. Following the Y1H screening, 152 clones stemming from our mouse mTEC cDNA library were obtained. Of these, 11 were selected for further analyses because they fulfilled two important criteria: i) they were previously shown to be nuclear proteins, and ii) they were previously shown to display functions related to the immune system, transcription or T1D. These clones are part of different categories of proteins and as expected, most of them are transcription factors (Table 1).

3.2 Validation of the interactions

In order to narrow down the number of library proteins, an *in vitro* assay was used to validate and quantify protein-DNA interactions (Clontech). Human and mouse versions of the transcription factor Pdx-1 were used as controls since they are known to specifically bind the insulin gene promoter. Thus, the 11 previously identified library proteins were submitted to this analysis using three different DNA targets: the human insulin gene promoter alone (oligo ins389), or joined to the class I (oligo I) or class III VNTR allele (oligo III). Results from this analysis are displayed in Figure 1. A signal was considered positive when a signal:background ratio of 5 was reached (Clontech) and five clones fulfilled this criterion: Ddx1, Zbtb7a, Zfp90, Jun and Ewsr1 (Figure 1a). It is noteworthy that both positive controls generated a very strong signal demonstrating that mouse proteins have the ability to recognize and bind to a specific human DNA sequence.

The five mouse proteins that generated the best signal had their human counterpart cloned in frame with the ProLabel tag in vector pProLabel-C and the same assay was repeated with the same three DNA targets (Figure 1b). Human AIRE was tested as well in this experiment in order to study interactions with this important immune factor. Three transcription factors reached the threshold ratio of 5 and came out of this analysis: JUN, EWSR1 and ZBTB7A. It is noteworthy that AIRE provided a signal above the determined threshold but the significance of this binding remains elusive for the moment. Although AIRE is involved in the expression of several TRAs such as insulin (Derbinski et al., 2001), this result does not necessarily imply a direct binding of AIRE to our DNA targets.

3.3 ZBTB7A synergistically modulates insulin expression

Human JUN, EWSR1 and ZBTB7A were then further analyzed in order to determine their capacity to modulate insulin expression in a human cellular system. To this end, HEK-293 cells were used because they were previously shown to act as a very effective model in similar studies (Ferguson et al., 2009). The three different reporter vectors were co-transfected with one of the three clones, with or without AIRE, and insulin mRNA levels were determined by quantitative PCR (Figure 2). All three clones had the capacity to induce insulin expression from the reporter and this expression was amplified in the presence of

AIRE. However, ZBTB7A showed the strongest induction of expression especially in the presence of AIRE, which increased insulin expression by at least 10-fold regardless of the reporter (statistically significant, determined by two-way ANOVA; $p<0.01$), suggesting that ZBTB7A could play a crucial role in insulin expression in the thymus. In addition, insulin levels reached with ZBTB7A were higher for the class III VNTR allele compared to the class I allele, suggesting a direct role in the genetic effect.

4. Discussion

Because of the divergent patterns of insulin expression in the pancreas and in the thymus (Vafiadis et al., 1997; Levi and Polychronakos, 2009), we started with the assumption that the transcriptional regulators involved in thymic insulin expression differ between the two organs. The three transcription factors that came out of the Y1H screening and were shown capable of modulating insulin expression within a non-thymus cellular system with or without AIRE, may be involved in determining these differences. ZBTB7A is of particular interest as it drastically increases insulin expression in that system especially in the presence of AIRE, with a 12-fold increase of the insulin mRNA levels in conjunction with the class III VNTR allele. Although the actual Y1H screening assessed a mouse cDNA library against a human genomic DNA sequence, it was likely that positive interactions would be identified since we knew transcription from the human insulin promoter could be activated in the mouse (Ounissi-Benkalha and Polychronakos, unpublished). Moreover, it was also known that transcription factors are usually very well conserved among species and so are their binding domains (Lowry and Atchley, 2000). Even though the insulin VNTR is primate-specific, it is unlikely that new transcription factors have evolved between mouse and human to interact with them.

Although some characterization remains to be done, our results strongly suggest that this factor could play a prominent role in the prevention of the autoimmunity observed in T1D. The strong synergy between ZBTB7A and AIRE, as well as the enhanced expression with the class III vs. class I construct suggest a specific role in thymic insulin expression. This is in agreement with a recent study by Cai *et al.* in which it was demonstrated that VNTRs and AIRE could synergistically modulate insulin expression in mTECs (Cai et al., 2011) and supported by the preferential expression pattern of ZBTB7A in the thymus (data not shown), suggesting that this transcription factor is one of the key elements involved in protection from T1D.

Four years ago, Ferguson *et al.* isolated an engineered zinc finger protein (ZFP) from a library based on its ability to activate transcription of the endogenous insulin gene (Ferguson et al., 2009). ZBTB7A is also a ZFP, consistent with such functions in the thymus. Even though the engineered ZFP identified in this study was shown to act through the VNTRs and that we identified a related Zbtb7a binding domain within the insulin gene minimal promoter, we cannot exclude that ZBTB7A interacts with the VNTRs given the results obtained following the *in vitro* binding assay (Figure 1).

Transcription factors displaying the ability to induce thymic expression of a specific TRA have already been identified and this is the case of IRF8 (Giraud et al., 2007). This protein is responsible for the expression of CHRNA1, a gene encoding the alpha-subunit of the muscle acetylcholine receptor, the main autoimmune target in myasthenia gravis. More recently, MafA was identified as a transcription factor that has the ability to modulate insulin expression in the thymus (Noso et al., 2010). Although MafA is also involved in pancreatic insulin expression, the authors stated that MafA was sufficient to induce thymic expression even though it is known to be a weak transactivator (Nishizawa et al., 2003). The promoter activity of MafA, which was proportional to the *Ins2* levels in their mTEC line, was

inversely proportional to the length of the VNTRs, which contradicts the trend usually observed (Cai et al., 2011). Moreover, expression of TRAs, including *Ins2*, in mTECs has been shown to depend on transcriptional regulators and transcriptional start sites different from those used in peripheral cells (Villaseñor et al., 2008).

5. Conclusions

Although Zbtb7a is usually known to be involved in cellular differentiation or oncogenic transformation, we showed here that it plays a role in thymic expression of insulin and maybe other TRAs. Given this feature of the protein, it is highly likely that Zbtb7a influences susceptibility to T1D and that it could be used in various therapeutic approaches for prevention of the disease.

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Abbreviations

T1D	type 1 diabetes
MHC	major histocompatibility complex
VNTR	variable number of tandem repeats
TRA	tissue-restricted antigen
AIRE	autoimmune regulator
mTEC	medullary thymic epithelial cell
Y1H	yeast one-hybrid
SNP	single nucleotide polymorphism
ZFP	zinc finger protein

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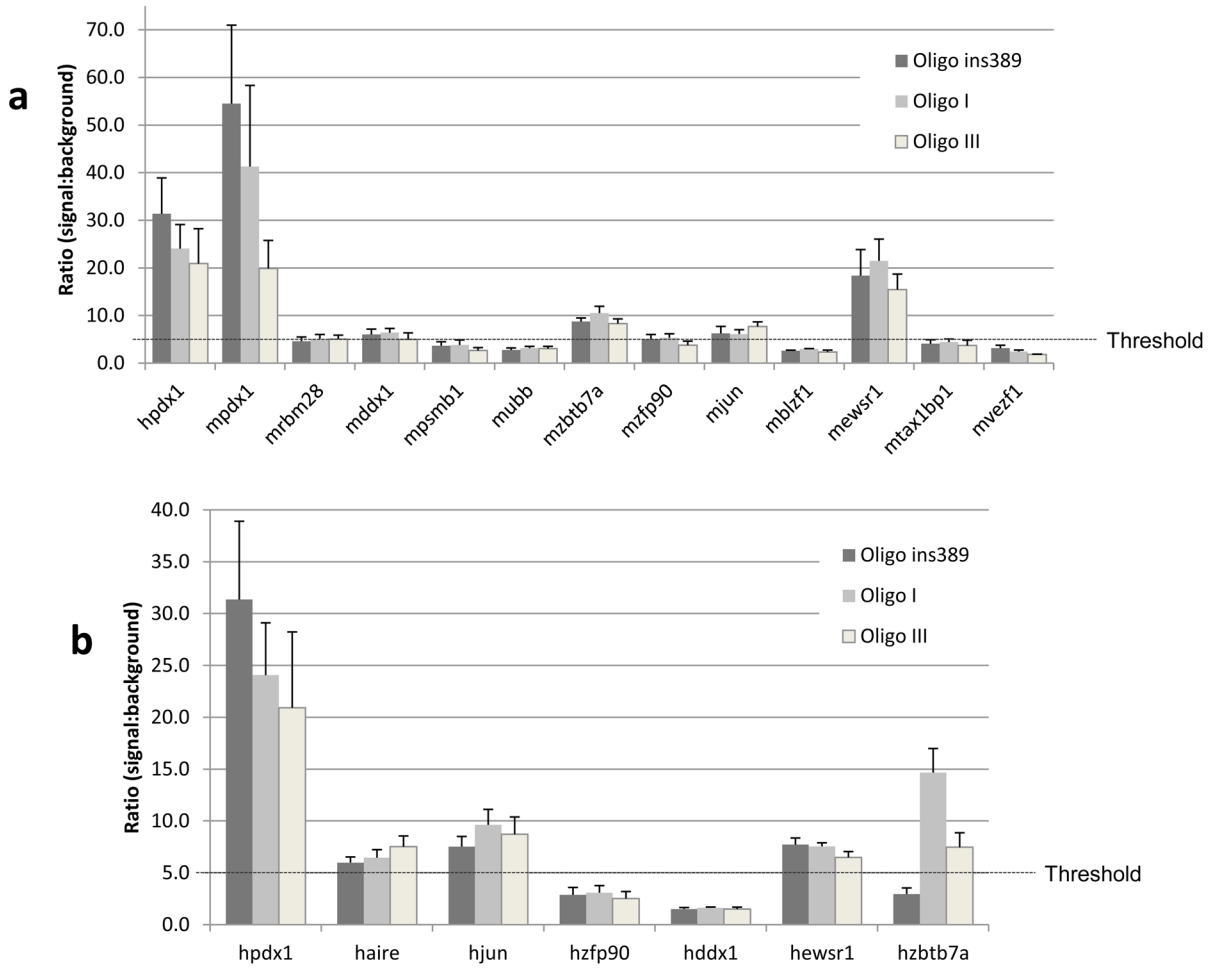

Figure 1.

Results of the protein-DNA binding assay. **(a)** Experiment involving the 11 mouse library proteins from the Y1H experiment. Mouse and human Pdx-1 were used as controls. For each protein of interest, four reactions were made: one without a DNA target, in order to establish the background level, and three reactions involving the different DNA targets (oligo ins389, oligo I and oligo III). Results are presented as a ratio of the signal generated by a protein-DNA interaction divided by the background signal. A signal was considered positive when a signal:background ratio of 5 was reached (Clontech). **(b)** Experiment involving the human orthologues of the five best library proteins from the previous assay. Human Pdx-1 was used as control. Legend is displayed in the upper right corner of each graph and every experiment was carried out in triplicates. Error bars represent standard deviation. The letter *m* or *h* before the name of the gene respectively means mouse or human.

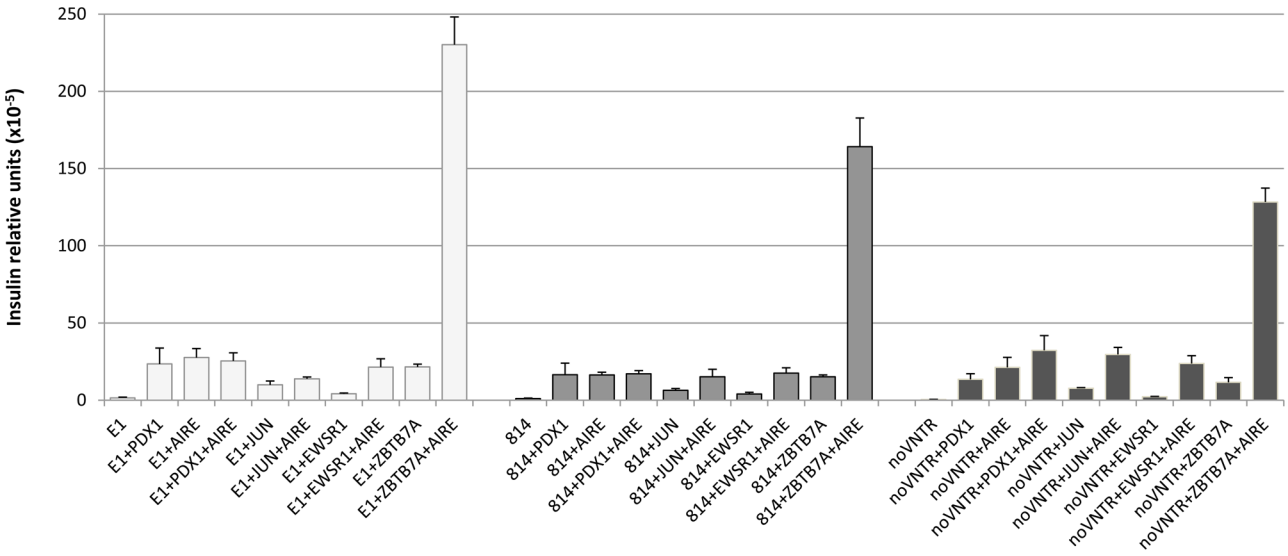

Figure 2.

Insulin expression induced by transfection of the candidate factors in HEK-293 cells. Results are displayed in insulin relative units ($x10^{-5}$) as mRNA levels were normalized with both GAPDH and AIRE. Cells were transfected with one of the three reporters (E1, 814 or noVNTR), in combination with human Jun, Ewsr1 or Zbtb7a, with or without AIRE. As controls, reporters were transfected alone, with human Pdx-1, with human AIRE or with both Pdx-1 and AIRE. Experiments were carried out in triplicates. Error bars represent standard deviation. Statistics determined by a two-way ANOVA. Reporters: E1 (class III), white; 814 (class I), grey and noVNTR, dark grey.

Table I

List of the eleven proteins selected following the Y1H screening.

Proteins	Category	Putative Functions
RBM28	RNA binding	Associated to endocrinopathy syndrome (Nousbeck et al., 2008)
DDX1	Helicase	Interacts with RelA, a subunit of NF-kappaB, and enhances its transactivation (Ishaq et al., 2009)
TAX1BP1	Immune signalling	Negative regulator of TNF-α and involved in NF-kappaB signalling (Iha et al., 2008)
PSMB1	Adaptor protein	Located in the IDDM8 region, associated with susceptibility to rheumatoid arthritis (Hinks et al., 2006)
UBB	Adaptor protein	Binds to UBASH3A (T1D risk factor) (Concannon et al., 2008)
EWSR1	Transcription factor	Can interact with many transcription factors (Law et al., 2006) and binds G-quadruplex structures (Takahama et al., 2011)
VEZF1	Transcription factor	Vascular zinc finger transcription factor, transcription activator in some systems (Gowher et al., 2008)
ZBTB7A	Transcription factor	Zinc finger transcription factor, can act as a transcription activator or repressor (Jeon et al., 2008)
ZFP90	Transcription factor	Zinc finger transcription factor with a powerful transcriptional repression activity (Thiel et al., 2001)
JUN	Transcription factor	Involved in thymocyte development (Riera-Sans and Behrens, 2007)
BLZF1	Transcription factor	Basic leucine zipper transcription factor that can interact with AP-1 (Tong et al., 1999)