Functional Characterization of ZBTB20 and the Role of ZBTB20-Dependent Transcription Regulation in Autism Spectrum Disorders and Intellectual Disability

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FUNCTIONAL CHARACTERIZATION OF ZBTB20 AND THE ROLE OF ZBTB20-DEPENDENT TRANSCRIPTION REGULATION IN AUTISM SPECTRUM DISORDERS AND INTELLECTUAL DISABILITY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Genetics

by
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Autism spectrum disorders (ASD) and intellectual disability (ID) are the two most frequently reported, often co-morbid, neurodevelopmental disorders that affect children all over the world. Previously, a genetic association of the ZBTB20 gene, located at chromosome 3q13.2, with ASD and ID was identified. The gene is highly expressed in developing brain and encodes two isoforms of 668- (short isoform) and 741- (long isoform) amino acid proteins that belong to the BTB (broad complex, tramtrack, bric-a-brac) – zinc finger family of transcription factors.

The human ZBTB20 protein was functionally characterized using various molecular and cellular approaches, to elucidate its contribution to ASD and ID. ZBTB20 is primarily localized in the nucleus, the short and long isoforms of ZBTB20 form homodimers and heterodimers. The N-terminal region of ZBTB20 that includes the BTB domain appears to be critical for dimerization. A ZBTB20 yeast two-hybrid screen was performed using human fetal brain cDNA library, to determine its function in neuronal cells. Several brain-expressed proteins that interact with ZBTB20, including the E2 SUMO conjugating enzyme UBC9 were identified. Moreover, ZBTB20 contains two putative conserved SUMOylation (ΨKXE) motifs. The ZBTB20 protein undergoes SUMOylation, binds SUMO1, and the N-terminal region of ZBTB20 is critical for its interaction with UBC9. The
ZBTB20 K330 residue in SUMOylation motif 1 is the likely target for *in vivo* SUMOylation. Altering residue K330 had no effect on ZBTB20 subcellular localization, stability or repression activity, but interfered with its dimerization.

Consistent with studies in mice, ZBTB20 functions as a transcriptional repressor. Overexpression of wild type ZBTB20, but not the ASD/ID-associated ZBTB20 mutants in HEK293H cells resulted in significantly reduced expression of transcription factor genes *MEF2C, TBR1* and *FEZF2*, previously shown to be associated with ASD and ID. These results suggest a potential contribution of a ZBTB20-dependent transcription regulation mechanism in neurodevelopmental disorders such as ASD and ID.
DEDICATION

To my husband, my parents and my little sister.
ACKNOWLEDGEMENTS

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PREFACE

The goal of this study is to functionally characterize the BTB (Broad complex, Tramtrack, Bric-a-Brac)-zinc finger protein, ZBTB20, and understand its role in the development of autism spectrum disorder (ASD) and intellectual disability (ID).

The first chapter provides a detailed literature review of the human ZBTB20. To understand the physiological functions of human ZBTB20, the known functions of murine Zbtb20 protein are described. The ZBTB20 gene defects in neurodevelopmental disorders including autism spectrum disorder and intellectual disability are reviewed. Finally, the post-translational modification SUMOylation and the biological functions regulated by it are explained.

The second chapter describes the intracellular localization of human ZBTB20 protein. The homotypic and heterotypic interaction of human ZBTB20 protein was determined. This study also identified eight novel ZBTB20 interacting proteins in the human fetal brain cDNA library using the yeast two-hybrid screen.

The third chapter describes the interaction of ZBTB20 with one of the ZBTB20 interacting proteins identified in the yeast two-hybrid screen, the E2 SUMO conjugating enzyme UBC9. Two putative SUMOylation sites were identified in
the human ZBTB20 protein. Further, SUMOylation of ZBTB20 and its effects on the function of ZBTB20 were studied.

The fourth chapter describes the ZBTB20-dependent transcription regulation of neuronal transcription factor genes, which are negatively correlated in the ZBTB20 coexpressed gene list. We also examined the effect of ZBTB20 long or short isoform overexpression on the expression of ASD and ID associated transcription factor genes in HEK293H cells.

The fifth chapter describes the siRNA-mediated knockdown of human ZBTB20 in two human cell lines, human embryonic kidney HEK293H cells and the human neuronal progenitor ReNcell VM cells. Finally, using ZBTB20 overexpression as well as ZBTB20 knockdown in mammalian cells, the role of ZBTB20-dependent transcription regulation in ASD and ID can be determined.
CHAPTER 1

LITERATURE REVIEW

Introduction

Zinc finger and BTB domain containing 20 (ZBTB20)

The human ZBTB20 gene is located on chromosome 3, at position 3q13.2. The gene spans approximately 880 kb of genomic DNA and has 10 exons (Figure 1.1). The ZBTB20 gene was previously known as ZNF288 (Harboe et al., 2000), DPZF (Zhang et al., 2001) and HOF (Mitchelmore et al., 2002).

The human ZBTB20 gene was first localized to chromosome 3q13.2 using radiation hybrid mapping and fluorescence in situ hybridization (FISH) (Harboe et al., 2000). It was also identified in the human dendritic cell cDNA library (Zhang et al., 2001). The ZBTB20 gene encodes a protein containing the BTB/POZ (Broad Complex, Tramtrack, Bric a Brac/ Poxvirus and zinc finger) domain at the N-terminal and the C2H2 Kruppel type zinc fingers at the C-terminal domain. The gene encodes two protein isoforms by alternative splicing, a long isoform (741 amino acids) and a short isoform (668 amino acids) (Figure 1.1).
Figure 1.1: Schematic diagram showing the location of the ZBTB20 gene on chromosome 3q13.2, ZBTB20 transcripts and protein isoforms.
Figure 1.1: Schematic diagram showing the location of the \textit{ZBTB20} gene on chromosome 3q13.2, \textit{ZBTB20} transcripts and protein isoforms

\textbf{A.} Ideogram of chromosome 3 showing the location of the \textit{ZBTB20} gene at chromosomal position 3q13.2. The \textit{ZBTB20} gene forms two major transcripts, \textit{ZBTB20} short isoform transcript (NM_015642) and \textit{ZBTB20} long isoform transcript (NM_001164342). The short isoform transcript has exons 1-10; green shaded region indicates the short isoform translated region; arrow indicates the translation initiation in the \textit{ZBTB20} short isoform transcript. The long isoform transcript has exons 7A, 7B, 8, 9 and 10; brown shaded region indicates the long isoform translated region; arrow indicates the translation initiation in the \textit{ZBTB20} long isoform transcript. 

\textbf{B.} Schematic representation of both the \textit{ZBTB20} short (668 amino acids) and long (741 amino acids) isoform proteins. The BTB domain (BTB) and the C2H2 Kruppel type zinc fingers (ZnF) are shown. A region specific to the long isoform (1-73 amino acids) is indicated in orange.
The ZBTB20 protein was extensively studied in the mouse model system. The murine Zbtb20 gene was first isolated from mouse oligodendrocyte cDNA library and designated HOF\textsuperscript{L} and HOF\textsuperscript{s}, for the long and short isoforms respectively (Mitchelmore et al., 2002). This study demonstrated that both the isoforms of Zbtb20 were highly expressed in hippocampal neurons, cerebellar granule cells, as well as in differentiated and undifferentiated glial cells in the mouse brain.

**ZBTB20 belongs to the POK family of transcription factors**

ZBTB20 protein belongs to the BTB/POZ and Kruppel type zinc finger family (POK) of transcription factors. Members of the POK family play critical roles in various biological processes including differentiation, oncogenesis and development (Kelly and Daniel, 2006; Lee and Maeda, 2012). ZBTB20 has a unique N-terminal BTB domain that was first identified as a conserved sequence in the *Drosophila* proteins Broad complex, Tramtrack and Bric-a-Brac (Albagli et al., 1995). It was also identified in a group of poxvirus proteins and was therefore also named the Poxvirus and Zinc finger domain (Bardwell and Treisman, 1994). The BTB domain is a highly conserved domain of approximately 100 amino acids. The BTB domain of the human ZBTB20 protein is 106 amino acids in length (Zhang et al., 2001).
Towards the C-terminal domain, ZBTB20 has five C2H2 Kruppel type zinc finger domains. The C2H2 Kruppel type zinc finger domain is one of the most common types of DNA-binding domains, approximately 25- to 30- amino acids in length. More than 600 human genes encode the tandemly repeated C2H2 zinc finger protein family (Collins, 1998; Kelly and Daniel, 2006), characterized by the consensus sequence CX$_2$-4CX$_3$FX$_5$LX$_2$HX$_3$-4 (where X represents any amino acid) (Zhang et al., 2001). The conserved cysteine (C) and histidine residues (H) in the C2H2 zinc fingers tetrahedrally bind the Zn$^{2+}$ ion, which helps in attaching the zinc fingers to the target DNA sequence.

Both the BTB/POZ domain and the C2H2 Kruppel type Zinc finger domain are conserved protein domains. The BTB domain is a protein-protein interaction motif, which has been shown to mediate both homotypic as well as heterotypic interactions. PLZF (Promyelocytic Leukemia Zinc Finger) and BCL-6 (B-cell lymphoma-6), which belong to the POK family of transcription factors form homodimers via their BTB domains (Ahmad et al., 1998; Hoatlin et al., 1999; Li et al., 1999). The BTB domain was also shown to be involved in heterotypic interactions (Daniel and Reynolds, 1999; Davies et al., 1999; Okabe et al., 1998) as well as interaction with other non-BTB containing proteins (Geyer et al., 2003; Krek, 2003). The BTB domain containing proteins were identified as substrate specific adaptors for Cullin 3 (Cul3), a RING type E3 ubiquitin ligase (Geyer et
al., 2003; Krek, 2003) using the yeast two-hybrid screen (Xu et al., 2003) and mass-spectrometric analysis (Pintard et al., 2003).

The BTB/POZ domain interacts with co-repressors. Some BTB domain containing transcription factors such as PLZF, BCL-6 etc. interact with components of the histone deacetylase (HDAC) complex (Dhordain et al., 1997; Huynh and Bardwell, 1998). The HDACs mediate removal of the acetyl group from histone lysine tails, causing closed chromatin conformation, leading to transcriptional repression. However, other BTB containing transcription factors, like Hypermethylated in cancer 1, HIC-1, repress transcription in an HDAC-independent manner (Deltour et al., 2002). Thus, understanding the functional roles of the BTB/POZ and C2H2 Kruppel type zinc fingers in ZBTB20 could elucidate its possible physiological roles in neuronal development and other biological processes.

**The physiological roles of mouse Zbtb20**

Various functions have been assigned to the mouse Zbtb20 protein (Figure 1.2). The Zbtb20 knockout mouse model as well as the targeted deletion of the Zbtb20 gene in specific tissues in mice has provided insight into the functioning of the Zbtb20 protein. Moreover, Zbtb20 overexpression studies done in mice
complemented the functions identified by Zbtb20 knockdown in cell lines or Zbtb20 knockout mouse models as described in the sections below.

**Figure 1.2: Schematic diagram of the known functional roles of the mouse Zbtb20 protein**

**Role of mouse Zbtb20 in postnatal survival**

To determine the *in vivo* function of the Zbtb20 protein, knockout mice were generated (Sutherland et al., 2009). The Zbtb20 knockout mice showed growth retardation as well as reduced body weight and axial growth. These mice also showed postnatal lethality; none of these mice survived more than 12 weeks of age. The Zbtb20 knockout mice showed abnormal glucose homeostasis, hypoglycemia and metabolic dysfunction. These mice had reduced energy stores, especially glycogen, along with adipose tissue defects, although they did
not display any feeding defects (Sutherland et al., 2009). One of the unique phenotypes of the \textit{Zbtb20} knockout mice was liver dysfunction. Further, transcript-profiling analysis was performed in the liver of \textit{Zbtb20} knockout mice to identify the pathways involved. Growth, glucose metabolism and detoxification were some of the pathways affected in \textit{Zbtb20} knockout mice.

**Role of mouse \textit{Zbtb20} in glucose homeostasis**

\textit{Zbtb20} knockout mice displayed abnormal glucose homeostasis. Fructose-1,6-bisphosphatase (\textit{Fbp1}) is a gluconeogenic enzyme involved in insulin secretion and glucose metabolism in mouse β-cells. Previous studies (Kebede et al., 2008; Zhang et al., 2010) showed that \textit{Fbp1} regulates glucose-stimulated insulin secretion in β-cells. Since \textit{Zbtb20} is highly expressed in the wild type pancreatic islets, Zhang and coworkers (Zhang et al., 2012) generated β-cells specific \textit{Zbtb20} knockout mice. They showed that Zbtb20 regulates glucose-stimulated insulin secretion by directly binding the \textit{Fbp1} promoter and repressing \textit{Fbp1} gene expression. Thus \textit{Fbp1} gene expression was found to be upregulated when Zbtb20 protein expression was ablated. They further showed that the reduced expression of the \textit{Zbtb20} gene in β-cells resulted in decreased glucose utilization, reduced insulin secretion and impaired glucose-stimulated insulin secretion. Thus Zbtb20 plays a vital role in glucose homeostasis by regulating the transcription of the \textit{Fbp1} gene.
Role of mouse Zbtb20 in toll-like receptor triggered innate immunity

As previously mentioned, ZBTB20 is highly expressed in human dendritic cells (Zhang et al., 2001). Besides dendritic cells, ZBTB20 is also abundantly expressed in immune cells including myeloid cells, macrophages, monocytes, B cells and T cells (Zhang et al., 2001). To study the role of Zbtb20 in immune cells, myeloid cell-specific Zbtb20 knockout mice were generated (Liu et al., 2013). Interestingly, these mice were found to be resistant to endotoxin shock. The myeloid cell-specific Zbtb20 knockdown mice showed reduced levels of toll-like receptor (TLR) triggered inflammatory responses, determined by challenging the mice with various TLR ligands and bacterial infections. Thus these mice showed impaired cytokine production in TLR-triggered Zbtb20 deficient myeloid cells.

Liu and coworkers (2013) demonstrated that the murine Zbtb20 protein represses the inhibitory gene IκBα by binding directly to IκBα gene promoter. This led to reduced IκBα gene transcription as well as IκBα protein production. IκBα protein has been shown to impair the activity of NF-κB protein. As the murine Zbtb20 transcription factor represses IκBα protein, it results in NF-κB activation. It was found that enhanced NF-κB activation, led to increased TLR-triggered cytokine production. Thus by selectively repressing IκBα gene, murine
Zbtb20 causes NF-κB activation which results in increased toll-like receptor-triggered innate immune response.

**Role of mouse Zbtb20 as a transcriptional repressor**

Mouse studies involving targeted knockout of the Zbtb20 gene in various cell types have shown that the murine Zbtb20 protein functions as a transcriptional repressor. Zbtb20 knockout experiments using a Cre/loxP recombination approach was performed in liver (Xie et al., 2008), β-cells of the pancreas (Zhang et al., 2012) and myeloid cells (Liu et al., 2013) associated with innate immune response in mice. These Zbtb20 knockout experiments have led to the identification of the Zbtb20-regulated target genes. The promoter regions directly bound to the Zbtb20 transcription factor were identified using chromatin immunoprecipitation (ChIP) analysis followed by sequencing and electrophoretic mobility shift assays (EMSA). Ablation of murine Zbtb20 specifically in the liver resulted in the de-repression of alpha-fetoprotein (Afp) gene transcription. Using ChIP and EMSA, Xie and coworkers (2008) showed that Zbtb20 directly binds the -108/-53 region of the Afp gene promoter region. This was the first evidence supporting the role of Zbtb20 as a transcriptional repressor.

As previously discussed, Zbtb20 was knocked-out in the β-cells using Cre/loxP technology or knocked-down in MIN6 (pancreatic β-cell line), using mouse
Zbtb20 gene specific siRNAs. Reduced expression of Zbtb20 resulted in increased Fbp-1 gene expression accompanied by hyperglycemia and impaired glucose-stimulated insulin secretion. Using ChIP assays in mouse pancreatic islets as well as in MIN6 cells, Zbtb20 was shown to directly bind the Fbp1 promoter approximately 1 kb upstream of the transcriptional start site (Zhang et al., 2012).

Another example of the role of Zbtb20 as a transcriptional repressor came from myeloid cell-specific Zbtb20 knockout mice, generated using the Cre/loxP approach (Liu et al., 2013). Using ChIP analysis accompanied by high throughput DNA sequencing (ChIP-Seq) in toll-like receptor-triggered as well as untreated macrophages, Liu and coworkers (2013) demonstrated that Zbtb20 directly bound to the IκBα gene promoter and reduced its expression. Disruption of the murine Zbtb20 gene and ablation of its expression showed, the Zbtb20 transcription factor to act as a transcriptional repressor.

Role of mouse Zbtb20 in hippocampal development

Murine Zbtb20 protein was shown to play a significant role in hippocampal neurogenesis. The hippocampus is part of the limbic system involved in learning, memory and behavior. It is located within the temporal lobes of the brain, at the medial-temporal edge of the neocortex. Hippocampus formation refers to the
hippocampus proper (which consists of CA1, CA2, CA3 and CA4 fields, also called as the Cornu Ammonis), dentate gyrus and the subiculum.

The murine Zbtb20 gene expression specifically coincides with early differentiating hippocampal CA1 and CA3 pyramidal neurons and granule cells of the dentate gyrus (Mitchelmore et al., 2002). Murine Zbtb20 is expressed in hippocampal primordium as early as mouse embryonic day, E12.5 and is later expressed specifically in the developing hippocampal progenitor cells and postmitotic neurons. This was determined by in situ hybridization using anti-Zbtb20 antibody in the developing mouse hippocampus (Xie et al., 2010).

**Effect of mouse Zbtb20 knockout on hippocampal neurogenesis and memory in mice**

To determine the physiological role of Zbtb20 in hippocampal development, the hippocampus of Zbtb20 knockout mice were analyzed (Xie et al., 2010). These mice showed severe morphological defects in CA1, CA3 and dentate gyrus regions of the hippocampus. Xie and coworkers (2010) showed that Zbtb20 knockout mice resulted in a reduction in size of the hippocampus. They also showed that the smaller hippocampus in the Zbtb20 knockout mice, as compared to the wild type mice, was due to increased apoptosis of hippocampal neurons.
Besides cytoarchitectural changes observed in the Zbtb20 knockout mice, Xie and coworkers (2010) demonstrated differential gene expression in the hippocampus of Zbtb20 knockout mice as compared to the wild type mice. They showed that expression of the neocortical layer-specific genes was markedly increased, while expression of hippocampal-specific genes was reduced, in the hippocampal CA1 field of Zbtb20 knockout mice. Thus the targeted deletion of Zbtb20 led to the transformation of the CA1 field to a neocortex-like structure. These studies done by the Zhang laboratory (Xie et al., 2010) demonstrated that murine Zbtb20 is involved in the specification of hippocampal CA1 pyramidal neuron identity.

Since the hippocampus is associated with learning and memory, the effect of Zbtb20 knockout on hippocampus-dependent memory formation was also analyzed. CA1 specific Zbtb20 knockout mice were generated with the Zbtb20 knockout restricted to the mature CA1 pyramidal cells of the hippocampus (Ren et al., 2012). Using various behavioral tests, Ren and coworkers demonstrated that these CA1-Zbtb20 knockout mice had impaired retention of spatial memory and reduced long-term potentiation (LTP). Thus the targeted deletion of Zbtb20 in mice resulted in impaired hippocampal neurogenesis and memory formation.
Ectopic expression of murine Zbtb20 gene results in hippocampus-like corticoneurogenesis

To study the role of Zbtb20 in hippocampal development, both long and short isoforms of the Zbtb20 gene were ectopically expressed in non-hippocampal immature pyramidal neurons (Nielsen et al., 2007). Zbtb20 was specifically overexpressed in the subiculum, retrosplenial and the cortical regions. This resulted in hippocampus-like corticoneurogenesis (Nielsen et al., 2007) and pyramidal cell layers expressing markers of CA1 projection neurons (Nielsen et al., 2010). In order to determine the behavioral defects associated with Zbtb20 transgenic mice, Nielsen and coworkers (Nielsen et al., 2007) performed behavioral tests like visual cliff tests (Fox, 1965) and platform maize tests (Pompl et al., 1999) to measure visual and spatial memory. These studies showed that the Zbtb20 overexpressed mice had impaired spatial and visual memory cues as compared to wild type mice. These findings suggested Zbtb20 overexpression is associated with hippocampus-like pyramidal neuron morphogenesis and behavioral abnormalities associated with memory impairments.
**ZBTB20 gene defects in neurodevelopmental disorders**

In order to identify potential candidate genes for neurodevelopmental disorders like autism spectrum disorders (ASD) and intellectual disability (ID), Srivastava and coworkers (Rimsky et al., manuscript in preparation) characterized a translocation breakpoint in a 10-year-old female patient with developmental delay and mild autism. The patient had a *de novo* translocation t(3;12)(q13.2;p11.2) which resulted in the physical disruption of the *ZBTB20* gene at the 3q breakpoint and the SRY (sex determining region Y)-box5 gene, SOX5 at the 12p breakpoint. One chromosomal copy of the *ZBTB20* gene was disrupted at its 5'UTR, identified in the patient’s lymphoblasts using appropriate FISH probes. Moreover, the *ZBTB20* disruption resulted in significantly reduced expression of both the *ZBTB20* gene and protein as compared to the two control individuals tested (Rimsky et al., manuscript in preparation). Haploinsufficiency of the *ZBTB20* gene could be one of the causative factors, responsible for the phenotype in the patient with the t(3;12) translocation.

Additional *ZBTB20* gene variants were identified in a cohort of unrelated patients with ASD and/or ID. Two missense *ZBTB20* gene variants c.137C>G, p.P46R and c.1037G>T, p.G346V, shown in Figure 1.3, were identified in two unrelated families by Rimsky and coworkers (Koul et al., 2013; Rimsky et al., manuscript in preparation) but found to be absent in a significantly large number of control
individuals. The ZBTB20 p.P46R variant was identified in one male patient and his twin sister, both affected with pervasive developmental disorders (PDD). The missense ZBTB20 mutation p.G346V was identified in an African American female patient with ASD, severe ID and no verbal abilities. Functional studies were performed by overexpressing both the ZBTB20 gene variants in cultured cortical pyramidal neurons, followed by in-depth analysis of the dendritic spine morphology and arborization. Ectopic expression of the ZBTB20 p.P46R variant resulted in abnormal spine enlargement while overexpression of the ZBTB20 p.G346V variant caused increased basal dendritic arborization in the cultured cortical neurons. Thus, both ZBTB20 variants, P46R in the long isoform and G346V in the short isoform, are potentially associated with ASD and/or ID and appear to play a role in dendritic spine morphology as well as dendritic arborization (Koul et al., 2013; Rimsky et al., manuscript in preparation).

The Srivastava laboratory in collaboration with other groups has also identified a 720 kb intergenic deletion of ZBTB20 (Figure 1.4) in a patient diagnosed with borderline intelligence, attention deficit, impulsivity and frequent mood swings (Koul et al., 2013; Rimsky et al., manuscript in preparation). The de novo deletion was identified using array comparative genomic hybridization (aCGH) and confirmed using a FISH technique with the appropriate probes. The de novo intragenic deletion included most of the ZBTB20 transcribed region with the exception of the last four exons as shown in Figure 1.4. Recently, several
microdeletions in the 3q13 region encompassing the ZBTB20 gene have also been identified (Molin et al., 2012; Vuillaume et al., 2013; Wisniowiecka-Kowalnik et al., 2013).

**Figure 1.3: The position of the ASD and ID associated variants in the ZBTB20 long and short isoforms**

Schematic diagram showing the ZBTB20 long isoform (741 amino acids) and the short isoform (668 amino acids) proteins. The ZBTB20 long isoform variant (P46R and G419V) and the ZBTB20 short isoform variant (G346V) is indicated. The BTB domain (BTB) at the N-terminus and the C2H2 Kruppel-type zinc finger domains (ZnF) at the C-terminus are shown in both the ZBTB20 isoforms. A region specific to the long isoform ZBTB20 (1-73 amino acids) is indicated in orange.
Figure 1.4: Schematic diagram showing the location of the deletions, duplications and translocation breakpoint region involving the ZBTB20 gene

Ideogram of chromosome 3, showing the location of the ZBTB20 gene and neighboring genes (DRD3 and GAP43) in the 3q13.2 region. The deletions (Red), duplications (Blue) and the breakpoint region (BPR, brown) involving the ZBTB20 gene are indicated with the related studies. The shortest region of overlap (SRO) common to the deletions shared by more than 15 people is also indicated.
**ZBTB20 gene is a candidate gene associated with the 3q13.31 microdeletion and the reciprocal microduplication syndrome**

Copy number variations (CNVs) in the proximal long arm of chromosome 3 are rare and have been found to be associated with variable phenotypes. Recently both deletions and duplications in the 3q13 region, encompassing the *ZBTB20* gene have been identified (Molin et al., 2012). The common phenotypes associated with CNVs in this region include developmental delay, increased postnatal growth, characteristic facial features and behavioral abnormalities. Among all the genes in this region, *ZBTB20* is a strong candidate gene associated with autism spectrum disorders, intellectual disability and developmental delay.

Molin and coworkers (2012) characterized all the 13 previously described deletions in the 3q12.3q21.3 region of different sizes as well as identified 15 novel microscopic or submicroscopic deletions in the 3q12.3q21.3 region using various microarray platforms. A deletion map consisting of all the 28 identified deletions in the 3q13 region led to the delineation of 3q13.31 as the shortest region of overlapping deletion (SRO). 24 patients out of the 28 patients analyzed shared an approximately 0.6 Mb of SRO as shown in Figure 1.4. This led to the identification of a newly recognized 3q13.31 microdeletion syndrome.
characterized by developmental delay, muscular hypotonia, a high arched palate, distinct facial features including short philtrum and protruding lips.

Recently, additional cases of the 3q13.31 microdeletion syndrome have been identified. Wisniowiecka-Kowalnik and coworkers (Wisniowiecka-Kowalnik et al., 2013) reported a de novo ~4.5-Mb deletion in 3q13.2q13.31 in a 6 year old boy with atypical autism, severe developmental delay and intellectual disability (Figure 1.4). Further, Vuillaume and coworkers (2013) presented a case of 3q13.31 microdeletion in a 16-year old girl sharing clinical features commonly observed in patients with the 3q13.31 microdeletion syndrome. Further, reciprocal microduplications (Karavitakis et al., 2013; Vuillaume et al., 2013) to the 3q13.31 microdeletion syndrome have been identified (Figure 1.4). These patients with 3q13 microduplication share many overlapping dysmorphic features in common with the 3q13.31 microdeletion syndrome patients. Karavitakis and coworkers (2013) identified a 3.671 Mb duplication at 3q13.2q13.31 in a newborn male with clinical features overlapping with 3q13.31 microdeletion syndrome including dysmorphic features, developmental delay and multiple congenital abnormalities. Vuillaume and coworkers (2013) also identified a 2.76 Mb microduplication by aCGH in two brothers and their father. The phenotype shared by the brothers resembled the phenotype of the 3q13.31 microdeletion syndrome, including severe intellectual disabilities, developmental delay, behavioral abnormalities and obesity. Both these 3q13.31 reciprocal
microduplication cases identify ZBTB20 as well as the neighboring gene DRD3 as the possible candidate genes associated with developmental delay.

Thus, the deletions and duplications described above suggest the ZBTB20 gene as well as the neighboring genes including DRD3 in the 3q13 chromosomal region as strong candidate genes for developmental delay. Interestingly, both the loss and gain in dosage of the ZBTB20 as well as neighboring genes result in overlapping symptoms. It is possible that these copy number variations result in a dosage imbalance, which can cause failure of neuronal homeostasis, leading to these shared clinical phenotypes. Altered gene dosage and impaired neuronal homeostasis have been widely implicated in autism spectrum disorders (Toro et al., 2010) as well as other neurodevelopmental disorders (Ramocki and Zoghbi, 2008).

**SUMOylation- A post-translational modification**

SUMOylation is the covalent attachment of Small Ubiquitin-related MOdifier (SUMO) proteins to a target protein, in a dynamic and reversible manner. SUMOylation regulates protein function in diverse biological processes of the cell (Seeler and Dejean, 2003). The first SUMOylated protein to be identified was the Ran GTPase-activating protein (RanGAP1) localized exclusively in the cytoplasm.
(Mahajan et al., 1997; Matunis et al., 1996). Geiss-Friedlander and Melchior (2007) showed that SUMOylation of RanGAP1 was responsible for its localization to the cytoplasmic fibers of the nuclear pore complex. SUMOylation also regulates target protein activity, stability and interaction with other proteins (Geiss-Friedlander and Melchior, 2007).

SUMO proteins belong to the ubiquitin-related protein family. Although SUMO and ubiquitin share only 18% amino acid identity, they are similar in structure and protein folds as determined by nuclear magnetic resonance (NMR) studies (Bayer et al., 1998). Like ubiquitin, SUMO proteins are present universally in the eukaryotic kingdom. While Saccharomyces, Caenorhabditis and Drosophila have one SUMO; humans have four SUMO paralogues, SUMO1, SUMO2, SUMO3 and SUMO4. SUMO2 and SUMO3 are very similar and have 97% sequence identity. However, there is only 47% sequence identity between mature SUMO1 and SUMO2 or SUMO3. SUMO4 has been identified only in humans in the kidney, lymph node and spleen, though its function is still largely unknown (Geiss-Friedlander and Melchior, 2007).

**The SUMOylation cascade**

The SUMOylation cascade is similar to the ubiquitination pathway and involves three classes of distinct enzymes, illustrated in Figure 1.5. The SUMO protein is
expressed in an immature form and carries a variable stretch of amino acids, after the invariant glycine-glycine motif at its C-terminus. The nascent SUMO is proteolytically cleaved by SUMO-specific proteases (SENP) to expose the glycine-glycine motif at its C-terminus. Mature SUMO is then activated, in an ATP-dependent manner by E1 SUMO-activating enzyme (SAE1/SAE2 heterodimer). The SUMO E1 activating enzyme attaches to the SUMO by a thioester bond, formed between the C-terminal glycine of SUMO and the cysteine residue of the SAE1 enzyme. SUMO is then transferred to the catalytic cysteine of the Ubiquitin-Conjugating Enzyme E21 (UBE2I) also referred to as UBC9 by a thioester bond facilitated by target-specific SUMO-E3 ligases. Finally this leads to the formation of an isopeptide bond between the C-terminal glycine residue of SUMO and the lysine residue of the target protein. However, the SUMO-deconjugating enzymes make SUMOylation a highly reversible and dynamic process (Geiss-Friedlander and Melchior, 2007).
SUMO protein is proteolytically cleaved by the SUMO-specific protease to expose the C-terminal di-glycine (GG) motif. Mature SUMO then enters the SUMO-conjugation cycle. It is activated by E1 activating enzyme (SAE1/SAE2) in the presence of ATP, resulting in the formation of a thioester bond between the catalytic cysteine (C) of SAE2 and the glycine (G) of SUMO. SUMO is then conjugated to the E2 conjugating enzyme (UBC9) by a thioester bond formed between the catalytic cysteine (C) of UBC9 and the glycine (G) of SUMO. UBC9 catalyzes the formation of an isopeptide bond between the lysine (K) of the target and the glycine of SUMO, usually in presence of an E3 ligase. The SUMO modification is reversible due to the SUMO specific-protease, which disrupts the isopeptide bond and releases the SUMO. XX stands for variable stretch of amino acids. This figure is redrawn with modifications from Flotho and Melchior, 2013.
The SUMO consensus sequence

At least 50% of the SUMO target proteins have a SUMO consensus motif, \( \psi \text{KXE/D} \) where \( \psi \) represents large hydrophobic amino acid residues (leucine, isoleucine or valine), K, E and D are lysine, glutamic acid and aspartic acid respectively while X represents any amino acid. These SUMO consensus motifs are directly recognized by SUMO conjugating enzyme UBC9. Some of the additional SUMO consensus motifs are: inverted consensus motif, \((\text{E/D})\text{XK}\psi\); hydrophobic consensus motif, \(\psi\psi\psi\text{KXE}\); phosphorylation-dependent SUMOylation motif (PDSM), \(\psi\text{KXEXX}(p)\text{P}\); negatively charged amino acid-dependent SUMOylation motif (NDSM), \(\psi\text{KXEXX}\text{EEEE}\) and others (Flotho and Melchior, 2013).

Some SUMO substrate proteins also contain a SUMO interaction motif (SIM) that helps to recruit the UBC9-SUMO thioester to the target proteins via non-covalent interaction with SUMO. In such SIM-dependent SUMOylation, target proteins are SUMOylated on multiple lysine residues, which are not necessarily part of the SUMO-consensus motifs. The transcriptional corepressor Daxx protein contains two independent SUMO-interacting motifs (Santiago et al., 2009), which help in mediating its interaction with other proteins.
The SUMOylation substrates

SUMOylation targets nuclear proteins, cytoplasmic proteins as well as transmembrane proteins. The majority of SUMOylated proteins are nuclear (Seeler and Dejean, 2003). In the nucleus, SUMOylation is involved in nuclear trafficking, gene expression, genomic stability and chromosomal integrity. The nuclear SUMO-substrate proteins are mostly transcription factors, DNA repair proteins, replication proteins as well as centromere and kinetochore proteins (Jürgen Dohmen, 2004).

The cytoplasmic protein RanGAP1 was the first SUMOylated protein to be identified (Mahajan et al., 1997; Matunis et al., 1996). SUMOylation of RanGAP1 by SUMO E3 ligase (RanBP2) is known to regulate its localization to the nuclear pore complex. Further, SUMOylation regulates the cytoplasmic signal transduction proteins, by altering their activity, stability and subcellular localization (Jürgen Dohmen, 2004).

The glucose transporters GLUT1 and GLUT4 are two examples of SUMOylated transmembrane proteins involved in transporting glucose in the brain (Martin et al., 2007). Thus SUMO modifies proteins irrespective of their localization in the cell.
The biological functions of SUMOylation

A large number of SUMOylated proteins have been identified by biochemical as well as genetic screens accompanied by mass spectrometry. However, the functional implications of SUMOylation and the mechanisms that regulate it are still not completely understood. One widely used experiment to study the function of SUMO conjugation to a target protein is by altering the SUMO acceptor lysine or the entire SUMO consensus sequence. Other experimental approaches involve overexpressing or knocking-down the enzymes in the SUMOylation cascade and observing the functional effects on both wild type and mutant SUMO substrate.

One of the major functional consequences of SUMOylation is altering the transcription regulator activity. Many SUMO substrates are transcription factors or transcription co-factors. In many studies, SUMOylation enhances the repressive activities of transcription factors including the Forkhead transcription factor (FOXL2), Promyelocytic leukemia zinc finger protein (PLZF) and the Erythroid Kruppel-like factor (EKLF) (Kang et al., 2003; Kuo et al., 2009; Marongiu et al., 2010; Siatecka et al., 2007). It has been shown that SUMOylation promotes the interaction of transcription factors with co-repressors (Girdwood et al., 2003; Yang and Sharrocks, 2004) leading to transcriptional repression activity of target genes. In other cases, SUMOylation results in
increased activity of transcriptional activators. For example, the SUMOylation of Pax-6 results in increasing its transcriptional activity (Yan et al., 2010).

SUMOylation also regulates protein-protein interaction. SUMOylation helps stabilize interaction between proteins and also recruits other proteins with SUMO interaction motifs and SUMO consensus motifs to the target protein complex. For example, the Promyelocytic leukemia (PML) protein covalently binds SUMO at three lysine residues, which helps in recruiting other proteins and eventually leads to the formation of nuclear bodies (Zhong et al., 2000).

Understanding the functional effect of SUMOylation on target proteins can be challenging. Even for SUMO substrates, where the putative SUMO acceptor lysine sites have been confirmed in vivo, finding the functional consequences of SUMOylation can be a daunting task. One possible explanation is that, the SUMO sites are redundant. The SUMOylated target could be a part of a protein complex held together by multiple SUMO binding sites and SUMO interaction motifs. Therefore, mutating one SUMO site or even the entire SUMO consensus sequence may not have any measurable consequence.
**SUMOylation of neuronal proteins and its role in synapse formation and synaptic activity**

As a post-translational modification, SUMOylation is being widely studied in neuronal proteins. SUMOylation is involved in many physiological processes including neuronal maturation and differentiation as well as neuropathological disorders. Both *Sumo1* and *Ubc9* mRNA are highly expressed in neuronal stem cells and in the hippocampal dentate gyrus and pyramidal neurons in rat brain (Watanabe et al., 2008). In general, SUMOylation regulates the electrochemical signaling in neurons and modulates their synaptic plasticity (Feligioni et al., 2013). SUMOylation mediates neuronal plasticity through the regulation of synaptic signaling and receptor trafficking (Luo et al., 2013). Disruption of SUMOylation of neuronal layer-specific transcription factors could also disrupt the architecture of neuronal layers in the brain (Gwizdek et al., 2013).

SUMOylation of transcription factors plays a role in synapse formation. The functional consequences of SUMOylation are diverse and substrate specific. SUMOylation of two proteins Calcium/calmodulin-dependent serine protein kinase (CASK) and the Myocyte Enhancer Factor 2A (MEF2A) were shown to play an important role in synapse formation and in the development of spines (Wilkinson et al., 2010). CASK is a synaptic scaffold protein widely implicated in intellectual disability. SUMOylation of CASK by SUMO1 at the K679 residue,
results in reduced spine size and density (Chao et al., 2008). Fusion of SUMO to CASK decreases its interaction with the erythrocyte membrane protein band 4.1 (4.1), resulting in reduced spine size and density (Chao et al., 2008) as it disrupts the localization and function of postsynaptic membrane proteins. On the other hand, SUMOylation of transcription factor MEF2A promotes synapse formation. MEF2A is involved in development of dendritic claws at the presynaptic terminal. Covalent conjugation of SUMO1 with MEF2A at residue K403 has been shown to regulate synapse formation (Shalizi et al., 2006).

SUMOylation-dependent modulation of synaptic activity is thought to involve both postsynaptic and presynaptic proteins. Finally, SUMOylation targets several glutamate receptors as well as kainate receptors at both the presynaptic and the postsynaptic membrane, and plays an important role in the modulation of synaptic activity (Craig and Henley, 2012).

Like other post-translational modifications, SUMOylation can perform diverse functions. Determining the effect of SUMOylation on the physiological functions performed by ZBTB20 in the developing neurons would provide deeper understanding about its role in neurodevelopmental disorders. As the murine Zbtb20 dimerizes and is involved in transcriptional regulation, the role of ZBTB20 SUMOylation on nuclear localization, transcription regulation, dimerization and
stability have been studied. In the future, it would be valuable to determine how the SUMOylation of ZBTB20 is regulated in neuronal cells.
References


CHAPTER 2

FUNCTIONAL CHARACTERIZATION OF HUMAN ZBTB20 AND
IDENTIFICATION OF ZBTB20 INTERACTING PROTEINS

Introduction

Autism spectrum disorder (ASD) and intellectual disability (ID) are frequently reported neurodevelopmental disorders with a combined prevalence of approximately 3% in the general population (Kim et al., 2011). ASD and ID often overlap; 50-70% of patients with ASD also have ID while approximately 10% of children with ID show autistic symptoms (Oeseburg et al., 2011). Both ASD and ID have a well-established genetic component (Abrahams and Geschwind, 2008; Devlin and Scherer, 2012; Ropers, 2010). However the cause is still unknown in a majority of ASD and ID cases.

To understand the genetic cause of global developmental delay and autistic features in a female child, Srivastava and coworkers (Koul et al., 2013; Rimsky et al., manuscript in preparation) characterized an apparently balanced de novo translocation t(3;12)(q13.2; p11.2). Two transcription factor genes, BTB (broad complex, tramtrack, bric-a-brac)-zinc finger gene, ZBTB20 at 3q13.2 and the
SRY (sex determining region Y)-box 5 gene, SOX5, at 12p11.2 were disrupted by the translocation breakpoints. This study is focused on the role of ZBTB20 gene however the role of SOX5 gene was also studied in our laboratory (Rimsky et al., manuscript in preparation).

Moreover, several microdeletions encompassing the ZBTB20 gene were identified in several patients with ASD and or ASD/ID (Molin et al., 2012; Vuillaume et al., 2013; Wisniowiecka-Kowalnik et al., 2013), Two missense heterozygous ZBTB20 variations, P46R and G419V were found in patients with ASD and or ASD/ID in our laboratory and absent in known databases and population-matched controls. These variants were studied in cultured rat cortical neurons and observed to affect spine morphology and dendritic arborizations (Koul et al., 2013; Rimsky et al., manuscript in preparation).

The ZBTB20 gene is located at chromosomal position 3q13.2. ZBTB20 consists of 10 exons, spanning approximately 880 kb of genomic DNA as shown in chapter 1 (Figure 1.1). The ZBTB20 gene (also known as DPZF, HOF, Zfp288) undergoes alternative splicing and encodes two isoforms of 668- and 741-amino acids, both containing a BTB domain at the N-terminal and five C2H2-type zinc fingers at the C-terminal. ZBTB20 gene is highly expressed in human fetal brain (Koul et al., 2013; Rimsky et al., manuscript in preparation) and murine forebrain, cortex and hippocampus (Mitchelmore et al., 2002; Nielsen et al., 2007; Nielsen
et al., 2010). **ZBTB20** knockout mice show a reduction in the size of the hippocampus (Xie et al., 2010) and transgenic mice with ectopic expression of **ZBTB20** show behavioral abnormalities (Nielsen et al., 2007). These findings suggest a potential role of ZBTB20 in the human brain, especially in the development of hippocampal neurons.

The ZBTB20 protein is member of the BTB family (Kelly and Daniel, 2006) of transcription factors. The BTB domain family belongs to a class of nuclear DNA binding transcription factors, involved in biological processes including development, differentiation, tumorigenesis and chromatin remodeling (Kelly and Daniel, 2006). The murine Zbtb20 protein directly binds and represses the alpha-fetoprotein gene (**Afp**) promoter and regulates the expression of the Afp protein postnatally in the liver (Xie et al., 2008). The Zbtb20 protein also directly represses the fructose-1,6-bisphosphatase 1 (**Fbp1**) gene, involved in glucose stimulated insulin secretion in the pancreatic β-cells. Thus the Zbtb20 protein plays a role in regulating glucose homeostasis in mice (Sutherland et al., 2009; Zhang et al., 2012). The mouse Zbtb20 has also been shown to repress many neocortex layer-specific transcription factor genes in the murine hippocampus (Nielsen et al., 2013). Therefore, the BTB and zinc finger protein Zbtb20 acts as a transcriptional repressor, involved in regulating gene expression in various cell types.
The BTB domain containing proteins have been implicated in gene repression through conformational changes in the chromatin structure (Costoya, 2007). Several BTB domain containing proteins like promyelocytic leukemia zinc-finger (PLZF) and B cell lymphomas 6 (Bcl-6) directly interact with co-repressors, silencing mediator of retinoid and thyroid receptor (SMRT), the nuclear receptor co-repressor (N-CoR), histone deacetylase 1 (HDAC-1) and SIN3 transcription regulator family member A (Sin3A) (Privé et al., 2005). These repressors are components of the histone deacetylase complex (HDAC), involved in transcription repression by removing acetyl groups from the histones. Several BTB proteins are also involved in gene repression by binding to components of the basal transcriptional machinery like the *Drosophila melanogaster* BTB proteins, bric-a-brac 1 (Bab1) and bric-a-brac 2 (Bab2) involved in the development of *Drosophila* ovaries, legs, antenna and abdomen (Pointud et al., 2001). Therefore, BTB domain containing proteins are frequently involved in transcriptional repression. An important biological property of the BTB and zinc finger proteins is protein-protein interaction. The BTB domains mediate both homotypic and heterotypic interactions, including BTB and zinc finger proteins PLZF and Bcl-6 (Stogios et al., 2005). The BTB and zinc finger proteins frequently function as substrate specific adaptors for Cullin E3 ubiquitin ligases, targeting substrates to the ubiquitin proteasome pathway (Xu et al., 2003). Thus, the BTB and zinc finger family of transcription factors are involved in many biological processes in the cell.
In this study, I present the functional characterization of human ZBTB20 protein in mammalian cells. ZBTB20 has a nuclear localization signal at the N-terminus and localizes primarily to the nucleus in mammalian cells. ZBTB20 long and short isoforms form homodimers and heterodimers and the N-terminus region containing the BTB domain is critical for dimerization. To identify brain expressed ZBTB20 interacting proteins, a yeast-two hybrid screen was performed using the human fetal brain library and eight novel protein interactions were identified. Three of them, NELL2 (neural epidermal growth factor-like 2), LTBP4 (Latent transforming growth factor beta binding protein 4), and Granulin precursor, are all secreted glycoproteins, which have common epidermal growth factor (EGF)-like repeats. Thus the identification of novel ZBTB20 interacting proteins would elucidate the physiological functions of the ZBTB20 protein as well as the ZBTB20 associated pathways and protein complexes in the human brain.

**Materials and Methods**

**Mammalian expression vectors**

ZBTB20 open reading frames corresponding to the ZBTB20 short and long isoforms were amplified from the Human Fetal Brain MATCHMAKER cDNA library (Clonetech) previously in the laboratory (Rimsky et al., Manuscript in preparation). The amplified cDNA constructs were subcloned into the
pcDNA3.1/V5-His-TOPO vector, which was tagged with V5 (long and short isoform ZBTB20) or GFP (long isoform) using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). Plasmids were sequenced to confirm and verify the insert with vector specific and insert specific primers as shown in Tables 2.1, 2.2 and 2.3. The constructs were further verified using the TNT T7 Quick Coupled Transcription/Translation System (Promega) and the \textit{in vitro} translated proteins were analyzed by SDS-PAGE. The V5-tagged ZBTB20 long and short isoform mammalian expression constructs were also verified \textit{in vivo} by transfecting in HEK293H cells. The \textit{ZBTB20} mutant plasmid \textit{ZBTB20-(BTB)}-V5 lacking the N-terminal BTB domain (1-198 amino acids) of ZBTB20 was generated previously in the Srivastava laboratory using Quick Change Site-Directed Mutagenesis Kit (Stratagene) and sequenced to confirm and verify the deletion using vector specific and insert specific primers as shown in Tables 2.1, 2.2 and 2.3.
Table 2.1: Primers used for amplifying ZBTB20 long, ZBTB20 short and ZBTB20-(BTB) constructs

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<th>Product Size (bp)</th>
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<tr>
<td>ZBTB20 Short isoform</td>
<td>F-ATGACCGAGCGCATCAGACATC R-TCCGTCAGACACATGCATCCTCAT</td>
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<td>2004</td>
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<tr>
<td>ZBTB20-(BTB)</td>
<td>F-GATGTGTTCGCCGGAAT R-TCCGTCAGACATGCATCCTCAT</td>
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<td>1629</td>
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Table 2.2: ZBTB20 sequencing primers for long and short isoforms

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</tr>
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<td>R-GGTGAAAGTCTTTGCGAG</td>
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<tr>
<td>ZBTB20 long isoform</td>
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<tr>
<td>ZBTB20 Short isoform</td>
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Table 2.3: Vector primer list

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<td>BD-R</td>
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<td>Reamp-AD5’</td>
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<tr>
<td></td>
<td>Reamp-AD3’</td>
<td>R-CCGCGGAATTAGCTATGCAAG</td>
<td></td>
</tr>
<tr>
<td>pcDNA3.1D/V5-HisTOPO</td>
<td>PC</td>
<td>F-GGAGACCGCAAGCTGGCTAG</td>
<td>60</td>
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<tr>
<td></td>
<td>BGH</td>
<td>R-TAGAAGGCACATGAG</td>
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<tr>
<td>pcDNA3.1/N-T-GFP-TOPO</td>
<td>NT-GFP</td>
<td>F-CCAAATCTGCGCTTTCGAA</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>BGH</td>
<td>R-TAGAAGGCACATGAG</td>
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</table>
Yeast two-hybrid screening

A yeast two-hybrid screen was performed using the Matchmaker Gold Yeast Two-Hybrid System protocol (Clontech) and the Mate and Plate Human Fetal Brain cDNA library. The cDNAs in the Mate and Plate Human Fetal Brain library were cloned in the pGADT7-Rec prey vector and pre-transformed in the Y187 yeast cells (Clontech). Using a TOPO TA cloning kit (Invitrogen), the bait plasmid, pGBKTK7-ZBTB20 long was constructed by subcloning ZBTB20 long isoform cDNA (1-741 amino acids) in the pGBKTK7 vector bait vector. The vector was sequenced and verified using vector specific primers as shown in Table 2.3.

The pGBKTK7-ZBTB20 long plasmid was then transformed into AH109 yeast (MATa strain) using the Frozen-EZ yeast Transformation II kit (Zymo Research). The autoactivation and the toxicity analysis were performed by mating the pGBKTK7-ZBTB20 long bait plasmid with the empty pGADT7-Rec prey plasmid plating on single dropout nutritional selection media SD-Trp, double dropout nutritional selection media SD-Leu-Trp, triple dropout nutritional selection media SD-His-Leu-Trp-X-α-Gal as well as quadruple dropout nutritional selection media SD-Ade-His-Leu-Trp-X-α-Gal. The pGBKTK7-ZBTB20 long bait plasmid tested negative for both autoactivation as well as toxicity analysis.
The ZBTB20 bait plasmid in AH109 was mated to the pre-transformed human fetal brain cDNA library in the Y187 yeast strain (MATα strain) by incubating overnight at 30°C in enriched YPDA (Yeast Peptone Dextrose Adenine) media. Mated yeast was selected by plating on SD-His-Leu-Trp-X-α-Gal as well as SD-Ade-His-Leu-Trp-X-α-Gal media and the positive clones were picked.

Plasmid isolation from the positive clones was performed, using the Zymoprep Yeast plasmid Miniprep II kit. The ZBTB20 interacting cDNAs in the pGADT7-Rec plasmid were transformed in the One Shot Top10 (Invitrogen) competent bacteria. The cDNA insert in the prey vector was sequenced using the pGADT7-Rec specific primers (sequence described above) and submitted to a BLAST search. The cDNA's encoding for an in-frame protein sequence was characterized to identify the protein. On-to-one mating between the characterized pGADT7-Rec prey vector in Y187 cells and the pGBKT7-ZBTB20 long bait vector in AH109 yeast cells was performed to verify the interaction. The mated yeast cells were plated on triple dropout nutritional selection media SD-His-Leu-Trp-X-α-Gal as well as quadruple dropout nutritional selection media SD-Ade-His-Leu-Trp-X-alpha-Gal to confirm the interaction.

The control plasmids used for mating were: positive controls, pGBK7-53 and pGADT7-T and negative controls, pGBK7-Lam and pGADT7-T, provided with the MATCHMAKER Gold Yeast Two-hybrid kit (Clontech).
Cell culture and transfection

HEK293H cells, a variant of human embryonic kidney cell line 293, were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma) at 37 °C and 5% CO₂. Approximately 1 million HEK293 cells were plated on Poly-L-Lysine pre-coated 60 mm dishes and transfected with 3 µg of appropriate plasmids (described under co-immunoprecipitation and Western blot analysis in serum- and antibiotic-free medium using Lipofectamine 2000 (Invitrogen) according to the conditions specified by the manufacturer.

The PC12 cell line, a pheochromocytoma of the rat adrenal medulla, was maintained in Ham's F-12K medium (Kaighn's Mod. Corning) supplemented with 15% horse serum, 3% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma) at 37 °C and 5% CO₂. The PC12 cells were grown in 24 well dishes pre-coated with 1 µg/ml Poly-L-Lysine and 10 µg/ml laminin. Approximately 150,000 PC12 cells were transiently transfected with 1 µg plasmid in serum- and antibiotic-free medium using NeuroMag (OZ Biosciences) according to the conditions specified by the manufacturer. The PC12 cells were grown in Neural growth factor (NGF) at 0.1 µg/µl concentration, for 36-48 hours for differentiation.
**Immunofluorescence**

Cells seeded on coverslips were fixed and permeabilized using 4% paraformaldehyde (Sigma) and 0.1% Triton X-100 (ICN Biomedicals) in PBS. Cells were then blocked in blocking buffer (0.4% BSA and 2% horse serum in PBS) for 30 minutes at room temperature. Blocked cells were then incubated with primary antibodies diluted in blocking buffer, at appropriate concentrations for 1 hour. The primary antibody, mouse anti-V5 (Invitrogen), was used at a 1:500 dilution to determine the intracellular localization of the ZBTB20 long isoform. Nuclear staining was carried out using 4',6-diamidino-2-phenylindole (DAPI; Molecular probes) for 5 minutes. Secondary antibodies used were Rhodamine-conjugated anti-mouse IgG and Alexa Fluor 488 Phalloidin, incubated for an hour at room temperature. The slips were washed, mounted and observed under the fluorescence microscope (Carl Zeiss). The emission filters were 594 nm and 488 nm for Rhodamine and Phalloidin-conjugated fluorescence, respectively.

**Co-immunoprecipitation and Western blot analysis**

Approximately 1 million HEK293H cells were grown to approximately 80% confluence in 60mm dishes and transfected in serum- and antibiotic-free medium using Lipofectamine 2000 (Invitrogen). For the co-immunoprecipitation
analysis of the ZBTB20 long isoform with the light chain of MAP1B, 3 µg each of GFP-ZBTB20 long/GFP-empty and MAP1BLC1-FLAG were co-transfected in the HEK293H cells. For ZBTB20 homo- and hetero-dimerization analysis, GFP-ZBTB20 long was co-transfected with ZBTB20 long-V5/ ZBTB20 short-V5/ ZBTB20 [-BTB]-V5.

48 hours after transfection cells, cells were washed with cold PBS and lysed using 1%NP-40 lysis buffer (1% NP-40; 150 mM NaCl; 50 mM Tris [pH 8.0] and protease inhibitor cocktail (Sigma) for 10 minutes on ice. Cell lysate was sonicated and centrifuged at 12,000 X g at 4 °C to get a clear supernatant. Protein concentration was measured using coomassie (Bradford) protein assay reagent (Thermo Scientific). 300 µg of protein lysate was pre-cleared and then incubated with anti-rabbit magnetic beads (Thermo scientific) pre-coated with either 1 µg rabbit anti-V5 antibody (Sigma) or 1 µg rabbit anti-Flag antibody (Sigma) in 1% BSA, 1% NP-40 lysis buffer and protease inhibitor cocktail for 4 hours at 4 °C. The immunoprecipitate-conjugated beads were washed 6 times for 10 minutes each at 4 °C in 1% NP-40 lysis buffer and protease inhibitor cocktail (Sigma). Protein was eluted by incubating at 95 °C for 3 minutes in 30 µl 1X Sample Buffer and probed with mouse anti-V5 (Invitrogen) antibody at 1:5000 dilution, mouse anti-GFP (Santa Cruz) at 1:500 dilution and mouse anti-FLAG (Sigma) at 1:4000 dilution separately. SuperSignal West Dura (Thermo Scientific) was used to detect immunoreactive bands.
Results

Intracellular localization of ZBTB20

To determine the subcellular localization of ZBTB20 in mammalian cells, the ZBTB20 long isoform was overexpressed in PC12 cells (Figure 2.1). The V5-tagged ZBTB20 long isoform was transiently transfected in PC12 cells and immunofluorescence analysis was performed. Fifty PC12 cells transfected with ZBTB20 long-V5 plasmid were analyzed in triplicate. Cells showing only nuclear localization of ZBTB20 (Figure 2.1A) were counted and compared with cells showing both cytoplasmic and nuclear localization of ZBTB20 (Figure 2.1B) and expressed as percentage subcellular distribution. Approximately 80% PC12 cells transiently transfected with ZBTB20 long isoform showed nuclear localization (Figure 2.1.C). Thus, a majority of PC12 cells showed nuclear localization of ZBTB20.

Since ZBTB20 was mostly localized to the nucleus of mammalian cells, we looked for putative nuclear localization signals in ZBTB20 using PSORT analysis (http://psort.hgc.jp/form.html). The analysis revealed that the ZBTB20 long isoform has a conserved nuclear localization signal 4-KKPK-7, at the N-terminus (Figure 2.2). However, no nuclear localization signal was predicted in the ZBTB20 short isoform.
Figure 2.1: Intracellular localization of ZBTB20 long isoform in PC12 cells
Figure 2.1: Intracellular localization of ZBTB20 long isoform in PC12 cells

PC12 cells overexpressing ZBTB20 long-V5, immunostained with a, DAPI (blue, nucleus); b, Phalloidin (green, cytoplasm); c, V5 antibody (red, ZBTB20 long-V5); d, merged image. A. ZBTB20 long-V5 (red) localizes in the nucleus B. cytoplasm. Scale bar = 10 µm. The white arrow shows the location of the ZBTB20 long-V5 in the nucleus of PC12 cells. The yellow arrow shows the location of the ZBTB20 long-V5 in the cytoplasm of PC12 cells. C. Subcellular distribution of ZBTB20 long-V5 in PC12 cells analyzed using immunofluorescence staining. Data is represented as percentage of PC12 cells showing nuclear and cytoplasmic localization of ZBTB20 long-V5. Error bars represent standard deviation from two independent replicates.
Figure 2.2: Predicted and conserved nuclear localization signal (NLS) in ZBTB20 long isoform

A. The position of the putative NLS motif and its amino acid sequence is highlighted in pink, along with the BTB domain and five zinc fingers in the schematic of the ZBTB20 long isoform. B. ZBTB20 long isoform sequence (1-51 amino acid only) is aligned using HomoloGene. The 4 residue NLS motif (KKPK, in pink) is conserved across various species.
Homotypic and heterotypic dimerization of ZBTB20

The BTB domain is a well-studied protein-protein interaction domain (Stogios et al., 2005). It is has been shown to be responsible for dimerization in various BTB domain containing proteins. We co-expressed GFP-tagged ZBTB20 long isoform with V5-tagged ZBTB20 short or long isoform in HEK293H cells, to determine if ZBTB20 forms homo- or heterodimers. Rabbit anti-V5 antibody was used to co-immunoprecipitate the transfected HEK cell protein lysate. Both the lysate (L) and immunoprecipitate (IP) were separated on a SDS PAGE gel and subjected to Western blot analysis using both ant-V5 and anti-GFP antibody. The V5-tagged ZBT20 short isoform as well as the V5-tagged ZBTB20 long isoform bind the GFP-tagged ZBT20 long isoform as shown in Figure 2.3. Interaction of ZBTB20 long isoform with itself (ZBTB20 long isoform) as well as with the ZBTB20 short isoform indicates that the ZBTB20 long isoform forms both homodimers and heterodimers in mammalian cells.

To determine whether the N-terminal region containing the BTB domain is responsible for ZBTB20 dimerization, we co-expressed GFP-tagged ZBTB20 long isoform with the N-terminal domain deleted from the ZBTB20, ZBTB20 [-BTB]-V5 in HEK293H cells. We observed that the interaction between GFP-ZBTB20 long and ZBTB20 [-BTB]-V5 was reduced as compared to the interaction between GFP-ZBTB20 long and ZBTB20 long-V5 expressed.
constructs. As shown in the IP lane in Figure 2.3, the signal detected by Western blot analysis using anti-GFP antibody for ZBTB20 [-BTB]-V5 and GFP-ZBTB20 long co-immunoprecipitation reaction indicates that the ZBTB20 N-terminus region containing the BTB domain is critical for homo-and heterotypic interaction.

**Identification of ZBTB20-interacting proteins in human fetal brain**

We screened for ZBTB20-interacting proteins in the human fetal brain library using the yeast two-hybrid screen (Figure 2.4). ZBTB20 long isoform cDNA expressed in frame with the Gal4-DNA binding domain of pGBKT7 was used as a bait vector for screening. ZBTB20-interacting colonies, which grew on triple dropout SD-His/-Leu/-Trp X-α-gal media as well as higher stringency quadruple dropout SD-Ade/-His/-Leu/-Trp X-α-gal media were identified. The bait and the prey plasmids were isolated from the yeast, transformed in *E. coli* and sequenced to determine the insert in pGADT7-Rec plasmid. Using translated BLAST, blastx and tblastn, cDNA coding for in-frame proteins were determined.
Figure 2.3: Homotypic and heterotypic interaction of ZBTB20 long and short isoforms

Western blot analysis of the co-immunoprecipitation reaction of GFP-ZBTB20 long (red arrow) co-transfected with ZBTB20 long-V5 (blue arrow) or ZBTB20 short-V5 (orange arrow) or mutant ZBTB20[-BTB]-V5 (green arrow) in HEK293H cells, showing lysate (lane L, input) and co-immunoprecipitates (lane IP, immunoprecipitate). As seen in the IP lanes (lower pane), GFP-ZBTB20 long is detected in ZBTB20 long-V5 and ZBTB20 short-V5 IP lanes but weak GFP-ZBTB20 signal is detected in the ZBTB20[-BTB]-V5 IP lane. Anti-V5 antibody (upper panel) and anti-GFP (lower panel) is used for the Western blot analysis. Rabbit-anti-V5 antibody is used for pull down.
Five clones of Microtubule-associated protein1B Light chain, MAP1BLC1 were identified in the ZBTB20 yeast two-hybrid screen. Interaction between ZBTB20 and MAP1BLC1 in yeast was verified by individually transforming pGBK7-ZBTB20 long and pGADT7-MAP1BLC1 plasmid into AH109 and Y197 yeast strains respectively followed by one-to-one mating. The mated yeast strains were plated on SD-His/Leu/Trp X-α-gal and higher stringency SD-Ade/His/Leu/Trp X-α-gal media (Figure 2.5B). The growth of mated yeast-harboring ZBTB20 and Map1BLC1 plasmids on high stringency nutritional dropout media confirmed the interaction between ZBTB20 and MAP1BLC1 in yeast. The interaction of ZBTB20 with each of the eight interacting proteins was also similarly confirmed by one-to-one mating in yeast (Figure 2.5). The one-to-one yeast mating interaction between ZBTB20 and UBC9 (Figure 2.5A) was also confirmed by co-immunoprecipitation analysis in HEK293H cells (discussed in Chapter 3).
Figure 2.4: Schematic diagram of yeast two-hybrid screen using Mate and Plate Human fetal brain library

ZBTB20 long isoform is expressed in the bait plasmid (pGBK7) having selection for tryptophan (Trp) transformed in yeast strain AH109. The human fetal brain library expresses cDNA in the prey plasmid (pGADT7-Rec) having selection for leucine (Leu) transformed in Y187 yeast strain. Both the cultures are mated to create diploids, which have both pGBK7-ZBTB20 long and human fetal brain cDNA in pGADT7-Rec plasmids. HIS3, ADE2 and MEL1 reporter genes are activated in response to protein interaction and selected by plating on quadruple dropout media with X-α-Gal (SD-Ade/-His/-Leu/-Trp X-α-gal).
Figure 2.5: UBC9, MAP1BLC1, NELL2, LTBP4, PGRN, CHD3, ZYM5 and ZYM2, identified as ZBTB20-interacting proteins using yeast two-hybrid screening

One-to-one yeast mating of pGBK7-ZBTB20 long in AH109 and (A) pGADT7-UBC9 in Y187; (B) pGADT7-MAP1BLC1 in Y187; (C) pGADT7-NELL2 in Y187; (D) pGADT7-LTBP4 in Y187; (E) pGADT7-PGRN in Y187; (F) pGADT7-CHD3 in Y187; (G) pGADT7-ZYM5 in Y187; (H) pGADT7-ZYM2 in Y187; (I) pGBK7-53 in AH109 was mated with pGADT7-T in Y187 (Positive control); (J) pGBK7-Lam in AH109 was mated with pGADT7-T in Y187 (Negative control). Mated yeast cells were grown on SD-Ade/-His/-Leu/-Trp X-α gal plates. Only clones with interacting proteins grow on quadruple dropout media with X-α Gal (SD-Ade/-His/-Leu/-Trp X-α gal) and turn blue.
Table 2.4 shows the eight unique ZBTB20 interacting proteins identified in the yeast two-hybrid screen. DAVID (Database for Annotation, Visualization and Integrated Discovery; Huang et al. 2007) analysis was performed on all the eight ZBTB20 interacting proteins to determine the common Gene Ontology terms. The DAVID analysis shows (Table 2.5) that three of the ZBTB20-interacting proteins are secreted glycoproteins involved in cell growth regulation. Proteins NELL2 (neural epidermal growth factor-like 2) and LTBP4 (latent transforming growth factor beta binding protein 4) contain epidermal growth factor-like repeats and the protein PGRN (granulin precursor) contains repeats of the granulin/epithelin motif.

Table 2.4: List of ZBTB20 interacting proteins identified by the yeast two-hybrid screen

<table>
<thead>
<tr>
<th># of ZBTB20 Interacting Clones</th>
<th>Protein</th>
<th>Description</th>
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<tbody>
<tr>
<td>3</td>
<td>UBC9</td>
<td>SUMO-conjugating enzyme</td>
</tr>
<tr>
<td>5</td>
<td>MAP1BLC1</td>
<td>Microtubule-associated protein 1B Light chain 1</td>
</tr>
<tr>
<td>3</td>
<td>NELL2</td>
<td>Neural epidermal growth factor – like 2</td>
</tr>
<tr>
<td>1</td>
<td>LTBP4</td>
<td>Latent-transforming growth factor beta-binding protein 4</td>
</tr>
<tr>
<td>2</td>
<td>PGRN</td>
<td>Progranulin</td>
</tr>
<tr>
<td>1</td>
<td>CHD3</td>
<td>Chromodomain helicase DNA binding protein 3</td>
</tr>
<tr>
<td>2</td>
<td>ZMYM5</td>
<td>Zinc finger, MYM-type 5</td>
</tr>
<tr>
<td>1</td>
<td>ZMYM2</td>
<td>Zinc finger, MYM-type 2</td>
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Table 2.5: ZBTB20 interacting proteins grouped using DAVID analysis

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<tr>
<th>Regulation of Growth</th>
<th>Phospho-Protein</th>
<th>EGF like Repeats</th>
<th>Secreted</th>
<th>Glycoprotein</th>
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<tr>
<td>NELL2</td>
<td>CHD3</td>
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<td>NELL2</td>
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<tr>
<td>LTBP4</td>
<td>ZMYM2</td>
<td>PGRN</td>
<td>PGRN</td>
<td>PGRN</td>
</tr>
<tr>
<td>MAP1B LC</td>
<td>MAP1B LC</td>
<td>LTBP4</td>
<td>LTBP4</td>
<td>LTBP4</td>
</tr>
</tbody>
</table>

**Discussion**

In this study, ZBTB20 long isoform was characterized in mammalian cells. Novel brain expressed ZBTB20-interacting proteins were identified using the yeast two-hybrid screen. Using immunofluorescence analysis of the V5-tagged ZBTB20 long isoform transfected in PC12 cells, ZBTB20 was found to localize primarily in the nucleus. Further, using co-immunoprecipitation analysis in transiently transfected HEK293H cells, ZBTB20 long isoform interacted with itself as well as with the ZBTB20 short isoform, forming both homodimers and heterodimers. Finally the N-terminal region of ZBTB20 containing the BTB domain was identified to be important for ZBTB20 dimerization in mammalian cells.

The V5-tagged ZBTB20 long isoform was expressed in PC12 cells and immunostained with anti-V5 antibody to determine the localization of ZBTB20 long isoform. Approximately, 80% of the V5-tagged ZBTB20 transfected PC12
cells showed localization to the nucleus. Additional immunofluorescence experiments could be performed using primary antibody to the endogenous ZBTB20 in various human cell lines. Cell fractionation experiments, followed by Western blot analysis against the tagged ZBTB20 or endogenous ZBTB20 could further support the localization of ZBTB20 to the nucleus.

Moreover, using PSORT, the ZBTB20 long isoform was predicted to contain a conserved nuclear localization signal (NLS) at the N-terminus (4-KKPK-7). Previously, Rimsky and coworkers (Rimsky et al., manuscript in preparation) demonstrated, nuclear localization of the tagged ZBTB20 short isoform to the nucleus of PC12 cells. However a conserved nuclear localization signal in the ZBTB20 short isoform could not be identified in this study. This is suggestive of the fact that the ZBTB20 short isoform could be transported to the nucleus with the help of ZBTB20 long isoform or bound to other proteins, which have nuclear localization signals. It is also possible that the ZBTB20 short isoform has a nuclear localization signal that could not be identified by the PSORT analysis. However, both the ZBTB20 long and the short isoform localize primarily to the nucleus of mammalian cells.

Both the long and short isoforms of ZBTB20 have a BTB domain, which is approximately 100 amino acids in length. The BTB domain has been shown to be involved in protein–protein interaction and mediating transcription regulation
(Privé et al., 2005). It is also critical in the homotypic as well as the heterotypic interactions between BTB and non-BTB proteins (Bardwell and Treisman, 1994). This study shows that the BTB domain is critical in mediating the homo- and hetero dimerization of the human ZBTB20 protein. Previous studies by Mitchelmore and coworkers (2002) in mice showed that both the long and short isoforms of murine Zbtb20 form homo- and heterodimers. They performed in vitro GST pull down experiments using both GST tagged as well as $^{35}$S-labelled short and long isoform mouse Zbtb20 proteins. Using yeast two-hybrid analysis they localized the dimerization interface to the BTB domain at the N-terminus of murine Zbtb20 long and short isoform proteins. Thus the dimerization of human ZBTB20 in the mammalian cells is consistent with the dimerization property of the murine Zbtb20 protein.

The interaction between the ZBTB20 long isoform and Microtubule-Associated Protein 1B light chain1 (MAP1BLC1) was identified for the first time. Using one-to-one yeast two-hybrid mating, the interaction between ZBTB20 and MAP1BLC1 was confirmed in yeast cells. MAP1B is a cytoskeletal protein, highly expressed in the developing nervous system. Among other roles, it is involved during the elongation phase of axon formation (Gonzalez-Billault et al., 2004). Moreover knockout studies of the MAP1B gene also suggest an important role in the development and function of the nervous system (Takei et al., 1997). The MAP1B protein encodes a precursor polypeptide of 2464 amino acids, which
undergoes proteolytic processing to generate the heavy and light chain, MAP1B-HC and MAP1B-LC1 respectively (Riederer, 2007). MAP1BLC1 cDNA corresponding to amino acids 2137-2445 with respect to the full length MAP1B was identified in the yeast two-hybrid screen. Thus ZBTB20 interacts with MAP1BLC1 and this interaction further suggests a role of human ZBTB20 in the developing brain.

ZBTB20 long isoform interacted with NELL2, PGRN and LTBP4 proteins. Each of the three proteins are secreted glycoproteins with Epidermal Growth Factor (EGF)-like repeat. EGF-like repeats are an evolutionarily conserved protein domain consisting of 30-40 amino acids. The EGF domain includes an arrangement of six cysteine residues found mostly in extracellular domain of membrane bound proteins or proteins that are secreted. PGRN or Granulin Precursor or Granulins functions both as a full-length protein or individual granulins. It belongs to a family of secreted, glycosylated peptides that are cleaved from a single precursor protein with repeats of granulin/epithelin motif. PGRN can act both extracellularly modulating signal transduction pathways, as well as intracellularly regulating transcription (Hoque et al., 2010).

The interaction of ZBTB20 with neural epidermal growth factor-like 2 (NELL2) was identified in the yeast two-hybrid screen. NELL2 is a glycoprotein containing several von Willebrand factor C domains and epidermal growth factor (EGF)-like
domains (Kuroda et al., 1999). NELL2 is highly expressed in the mice hippocampus and cortex. Studies in mouse suggest that it plays a role in neural cell growth and differentiation (Oyasu et al., 2000). It is also involved in neuronal signal transduction where it acts as ligand for cell surface receptors.

Further, a TGF β-binding protein 4, LTBP4 was identified in the yeast two-hybrid screen. LTBP4 belongs to the latent TGF-β-binding protein (LTP) and fibrillin family. It has epidermal growth factor-like repeats (EGF) and eight-cysteine repeats. It is involved in controlling and directing the activity of TGFβ1 (transforming growth factor beta family of cytokines). LTBP4 is deposited in the extra cellular matrix (ECM) and forms a complex with TGFβ-like proteins (Saharinen et al., 1998).

CHD3, a chromodomain, helicase/ATPase, and DNA-binding domain (CHD) family protein was also identified as a ZBTB20 interacting protein. CHD3 is a part of the chromatin remodeling and the histone deacetylase complex. It is involved in transcription regulation by deacetylating histones (Woodage et al., 1997).

Both Zinc finger proteins, MYM-type 5 (ZYM5) and Zinc finger, MYM-type 2 (ZYM2) were identified in the yeast two-hybrid screen. ZYM5 and ZYM2 are part of the transcriptional corepressor complex, which associates with histone deacetylases (HDACs) and other corepressor proteins like CoREST (Gocke and
Yu, 2008). ZYM proteins have been shown to interact non-covalently with SUMO and form protein complexes important for formation of PML nuclear bodies (Kunapuli et al., 2006). As ZBTB20 functions as a transcriptional repressor and is shown to be SUMOylated (Chapter 3), it could potentially form protein complexes with ZYM5 and ZYM2 proteins in nuclear bodies.

The identified ZBTB20 interacting proteins could be involved in signaling pathways with ZBTB20 or form protein complexes with ZBTB20, possibly involved in transcription regulation. The interaction of ZBTB20 with eight novel proteins expressed in the human fetal brain cDNA library was confirmed by one-to-one mating in yeast. The interaction between ZBTB20 and SUMO-conjugating enzyme UBC9 was also studied in mammalian cells (Chapter 3). Though the interaction of ZBTB20 with MAP1B-LC1, NELL2, LTBP4, CHD3, PGRN, ZYM5 and ZYM2 was identified in yeast, additional work needs to be done to validate these interactions in mammalian cells. Thus, identifying brain-expressed ZBTB20 interacting proteins is potentially useful for understanding the physiological roles of ZBTB20, elucidating its association with neurodevelopmental disorders and determining new autism spectrum disorder and intellectual disability associated gene
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CHAPTER 3

ZBTB20 INTERACTS WITH THE SUMO-CONJUGATING ENZYME UBC9 AND IS A NOVEL TARGET FOR SUMOYLATION

Introduction

SUMOylation is a posttranslational modification which involves covalent attachment of Small Ubiquitin-like Modifier (SUMO) to the target protein (Hay et al. 2005). There are four SUMO homologs in mammals: SUMO1, SUMO2, SUMO3 and SUMO4 (Hay, 2005). SUMO proteins are approximately 100 amino acids in length and share ~18% sequence identity with ubiquitin. However, unlike ubiquitination, SUMOylation does not target a protein for degradation. SUMOylation has been shown to regulate transcription, protein stability, protein-protein interaction, protein activity and transport of proteins between the nucleus and the cytoplasm (Garcia-Dominguez and Reyes, 2009; Ouyang et al., 2009). Like ubiquitination, the SUMOylation process is an enzymatic cascade, which involves the E1 activating enzyme, E2 conjugating enzyme and E3 ligases. The only E2 enzyme identified to date is ubiquitin conjugating enzyme E2I (UBC9), which plays a major role in determining SUMO substrate specificity and catalyzes covalent attachment of SUMO to
target proteins (Geiss-Friedlander and Melchior, 2007). It was observed that SUMO target proteins that are recognized by UBC9 usually carry a SUMO consensus motif ψKXE/D, where Ψ represents a large hydrophobic residue (leucine, isoleucine or valine), K, E and D are lysine, glutamic acid and aspartic acid respectively and X represents any amino acid (Hecker et al., 2006; Minty et al., 2000; Rodriguez et al., 2001). Additional SUMO consensus sequences (Flotho and Melchior, 2013; Matic et al., 2010) including the inverted consensus motif (E/DXKΨ), hydrophobic consensus motif (ΨΨΨKXE), phosphorylation-dependent SUMOylation motif (ΨKXEXX(pS)P) were also identified. These consensus sequences can be SUMOylated in vitro under high UBC9 concentration in the absence of the SUMO E3 ligases. Thus the SUMO conjugating enzyme UBC9 plays an important role in SUMO consensus site-directed SUMOylation (Flotho and Melchior, 2013).

ZBTB20 belongs to the BTB (Broad-Complex, Tramtrack, and Bric-a-brac) zinc finger family of transcription factors. The ZBTB20 gene has two alternative translation initiation sites encoding the ZBTB20 long and short isoforms, 741- and 668 amino acids respectively. Both the ZBTB20 isoforms are highly expressed in the developing human fetal brain, especially in the fetal hippocampus (Rimsky et al. manuscript in preparation; Nielsen et al. 2013). Murine Zbtb20 is also highly expressed in the hippocampus and is involved in hippocampal neurogenesis, specifically in the determination of hippocampal CA1
field identity (Mitchelmore et al., 2002; Nielsen et al., 2007; Nielsen et al., 2013). Zbtb20 null mice show a reduction in size of the hippocampus as compared to wild type mice (Xie et al., 2010). Mice with ectopic expression of Zbtb20 in non-hippocampal fields show hippocampus-like corticoneurogenesis and behavioral abnormalities (Nielsen et al., 2007). Moreover, murine Zbtb20 functions as a transcriptional repressor. Using the targeted deletion of Zbtb20 in different cell types, Zbtb20 acts as a repressor of target genes (Nielsen et al., 2013; Sutherland et al., 2009; Xie et al., 2008; Zhang et al., 2012).

To elucidate the physiological roles of ZBTB20 in the human brain and identify brain expressed ZBTB20 interacting proteins, a yeast-two-hybrid screen was performed using ZBTB20 long isoform as bait (Chapter 2). This study describes the interaction between ZBTB20 and SUMO conjugating enzyme UBC9 in mammalian cells. Consistent with its interaction with UBC9, two putative conserved SUMO consensus sites at lysine 330 (K330) and lysine 371 (K330) were identified in ZBTB20. Further, the interaction of ZBTB20 with SUMO1 was observed in HEK293H cells and the ZBTB20 K330 residue was found to be critical for SUMO1 binding. Altering ZBTB20 K330 or the entire SUMO consensus motif abolished ZBTB20 SUMOylation. The biological consequences of ZBTB20 SUMOylation were also elucidated. Both wild type ZBTB20 and ZBTB20 SUMOylation mutants showed the same intracellular localization, transcription repression activity and stability. Surprisingly, the ZBTB20 long
isoform SUMOylation mutant K330R, failed to dimerize with the wild type ZBTB20. Since dimerization is critical for the functioning of BTB proteins (Stogios et al., 2005), SUMOylation could potentially play a significant role in regulating the physiological functions of ZBTB20 in the brain.

Material and Methods

Plasmids

The ZBTB20 open reading frame corresponding to the short (668 aa) and long (741 aa) isoforms was amplified from Human Fetal Brain MATCHMAKER cDNA library (Clonetech). The amplified cDNA was subcloned into pcDNA3.1/V5-His-TOPO vector, with V5 tag (long and short isoform) or GFP tag (long isoform) using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). Site-directed mutagenesis using the QuikChange II Site-Directed mutagenesis kit (Stratagene) was performed to generate the following ZBTB20 SUMO mutants constructs: ZBTB20 K330R, Δ330, Δ327-333, K371R, Δ371 and Δ368-374 as shown in Table 3.1. All constructs were sequenced to confirm and verify the alteration. Constructs were further verified using the TNT T7 Quick Coupled Transcription/Translation System (Promega) and analyzed by SDS-PAGE. UBC9 cDNA was also amplified from the Matchmaker Human Fetal Brain Library (Clontech) and subcloned into pcDNA3.1/N-terminal-GFP-TOPO vector.
using NT-GFP Fusion TOPO TA Expression Kit (Invitrogen). The GFP-UBC9 construct was sequenced using vector specific primers.

**Table 3.1: Primers used to generate ZBTB20 SUMOylation mutant constructs in pcDNA3.1/V5-His-TOPO vector**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZBTB20 K330R</td>
<td>F-AGTGGGCAACATCCACATCAGGCAGGATGGR-CCATCTCTGTGATGTGGATGTTGCCACT</td>
<td>50</td>
</tr>
<tr>
<td>ZBTB20 Δ330</td>
<td>F-GGCAACATCCACATCAGGAGATGGAGGACR-GTCTCCATCTCTGGATGATGTTGCC</td>
<td>50</td>
</tr>
<tr>
<td>ZBTB20 Δ327-333</td>
<td>F-GACCCTAGTGGAACGAGGACTGAGGACTCTGAGGATGTTGCC</td>
<td>50</td>
</tr>
<tr>
<td>ZBTB20 K371R</td>
<td>F-CACCGAGAGTGAGGCCAGGAGGACTGAGGACTCTGAGGATGTTGCC</td>
<td>50</td>
</tr>
<tr>
<td>ZBTB20 Δ371</td>
<td>F-CCGAGAGTGAGGCCAGGAGGACTGAGGACTCTGAGGATGTTGCC</td>
<td>50</td>
</tr>
<tr>
<td>ZBTB20 Δ368-374</td>
<td>F-CCGAGAGTGAGGCCAGGAGGACTGAGGACTCTGAGGATGTTGCC</td>
<td>50</td>
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**Yeast two-hybrid screening**

A yeast-two-hybrid screen was performed using Matchmaker Gold Yeast Two-Hybrid System protocol (Clontech) and the pre-transformed Mate and Plate Human Fetal Brain library (Clontech). The ZBTB20 long isoform cDNA was subcloned into the pGBKT7 vector using a TOPO TA cloning kit (Invitrogen). pGBKT7-ZBTB20 was used to transform the AH109 yeast strain using the Frozen-EZ yeast Transformation II kit (Zymo Research) which was then mated.
to the human fetal brain cDNA library pre-transformed in the Y187 yeast strain. Mated yeast were selected for the ZBTB20-interacting colonies by plating on triple dropout synthetic media, SD-His-Leu-Trp-X-α-Gal as well as quadruple dropout synthetic media SD-Ade-His-Leu-Trp-X-α-Gal. Blue colonies that grew on these triple and quadruple dropout nutritional selection media were identified and the interacting cDNA’s were isolated and sequenced. Plasmid isolation from the selected colonies was performed using the Zymoprep II- Yeast plasmid Miniprep kit. The isolated prey plasmid was transformed using One Shot Top10 (Invitrogen) competent cells. By sequencing using prey vector-specific primers, full length UBC9 cDNAs were identified from the yeast two-hybrid screen. pGADT7-Rec UBC9 plasmid was used to transform the Y187 yeast strain which was mated individually with pGBK7-ZBTB20 long isoform in AH109 yeast strain and grown on nutritional dropout media to confirm interaction. The control plasmids used for mating were: positive controls, pGBK7-53 and pGADT7-T and negative controls, pGBK7-Lam and pGADT7-T provided with the MATCHMAKER Gold Yeast Two-hybrid kit (Clontech).

**Cell culture and transfection**

HEK293H cells, a variant of human embryonic kidney cell line 293, were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1%
penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma) at 37°C and 5% CO₂. Approximately, one million HEK293H cells were plated on Poly-L-lysine coated 60 mm dishes and transfected with 3 µg plasmid in serum-and antibiotic free DMEM media using Lipofectamine 2000 (Invitrogen) according to the conditions specified by the manufacturer.

Rat primary neuronal cultures were isolated from cortical hemispheres dissected from one-day-old Sprague Dawley rats, maintained at Lander University. Cells from the rat cortices were suspended in media reconstituted by mixing Neurobasal media (Invitrogen), 2% B27 (Invitrogen), 1% penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma). Primary neuronal cells were maintained in the media supplemented with growth factors including epidermal growth factor (EGF, Millipore) and fibroblast growth factor (FGF, Millipore) added to the media at 20 ng/ml each. The cells were then plated on coverslips for immunofluorescence analysis (described below) and transfected with 1 µg plasmid DNA using Lipofectamine LTX with Plus reagent (Invitrogen) in DME/F12 media (Millipore) according to the conditions specified by the manufacturer.
Immunofluorescence analysis

Rat primary neuronal cells were seeded on Poly-L-lysine pre-coated coverslips in neural basal media with 5% FBS (Sigma). Approximately 2-3 days post-plating, cells were transfected with following plasmids: V5-tagged wild type ZBTB20 long isoform or ZBTB20 SUMO double mutant plasmids (ZBTB20 K330R, K371R and ZBTB20 Δ327-333, Δ368-374) in pcDNA3.1/V5-His-TOPO vector, were transfected for localization studies. GFP-UBC9 and V5-ZBTB20 long isoform plasmids were cotransfected for colocalization analysis. The primary neuronal cell transfections were performed in DME/F-12 (Life Technologies) and Neural Basal media (Millipore) with Lipofectamine LTX and plus reagent (Invitrogen), according to the conditions specified by the manufacturer. Four days post-transfection, immunofluorescence analysis was performed. The transfected cells seeded on coverslips were fixed using 4% paraformaldehyde for 30 minutes at room temperature followed by permeabilization using 0.1% Triton X-100 (ICN Biomedicals) for 5 minutes. Cells were incubated in blocking buffer (0.4% BSA and 2% horse serum in PBS) for 30 minutes at room temperature. Following primary antibodies were mixed together at the indicated dilutions for double staining the cells: mouse anti-V5 (Invitrogen) at 1:500 dilution and rabbit anti-GFP (Roche) at 1:400 dilution; mouse anti-V5 (Invitrogen) at 1:500 dilution and rabbit anti-Neuronal β-tubulin (Covance) used at 1:3000 dilution. Secondary antibodies used were
Rhodamine-conjugated, Alexa Fluor chicken anti-mouse 594 at 1:2000 dilution and FITC-conjugated Alexa Fluor chicken anti-rabbit 488 at 1:800 dilution.

Nuclear staining was performed by incubating the cells in 4’,6- diamidino-2-phenylindole (DAPI; Molecular probes) for 5 minutes. The slips were washed in PBS, mounted on a glass slide using ProLong Gold antifade reagent (Invitrogen). The slides were observed under a fluorescence microscope.

**Co-immunoprecipitation and Western blot analysis**

HEK293H cells were grown in Poly-L-Lysine coated 60mm dishes and transiently transfected with 3 µg of the appropriate V5-tagged and GFP-tagged plasmids in serum- and antibiotic-free medium using Lipofectamine 2000 (Invitrogen). Approximately 48 hours post-transfection, cells were washed with cold PBS and lysed using 1%NP-40 lysis buffer [1% NP-40; 150 mM NaCl; 50 mM Tris (pH8.0)] and protease inhibitor cocktail (Sigma) for approximately 15 minutes on ice. Cell lysate was sonicated and centrifuged at 12,000 X g at 4 °C to get a clear supernatant. Protein concentration was measured using coomassie (Bradford) protein assay reagent (Thermo Scientific). Approximately 300 µg of protein lysate was pre-cleared and then incubated with anti-rabbit magnetic beads (Thermo scientific) pre-coated with 1 µg anti-V5 antibody (anti-rabbit, Sigma) in 1% BSA and 1% NP-40 lysis buffer and protease inhibitor cocktail for 4 hours at 4°C. The immunoprecipitate-conjugated beads were
washed 6 times for 10 minutes each at 4 °C in 1% NP-40 lysis buffer and protease inhibitor cocktail (Sigma). Protein was eluted by incubating at 95°C for 3 mins in 30µl 1X sodium dodecyl sulphate (SDS) sample buffer. Both lysate and co-immunoprecipitate were separated on SDS-PAGE gel and probed with mouse anti-V5 (Invitrogen) antibody at 1:5000 dilution and mouse ant-GFP (Santa Cruz) at 1:500 dilution separately at 4°C. SuperSignal West Dura (Thermo Scientific) was used to detect immunoreactive bands.

**Preparation of protein extracts for SUMOylation**

Approximately 1 million HEK293H cells were plated on Poly-L-lysine coated 60 mm dishes in DMEM media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. After 48 hours post-transfection, cells were harvested for 10min on ice by scrapping in 1% NP-40 lysis buffer, 1X protease inhibitor cocktail and 40mM N-Ethylmaleimide (NEM; Sigma). Cell lysate was sonicated, centrifuged and boiled in SDS sample buffer. The following antibodies were then used for Western blot analysis: mouse anti-V5 (Invitrogen) at 1:5000 dilution, rabbit anti-SUMO1 (Enzo) at 1:1000 dilution and mouse anti-alpha tubulin (Santa Cruz) at 1:1000 dilution.
Gal4-based transcriptional assay

Approximately 2.3\times10^5 HEK293H cells were plated on a Poly-L-lysine coated 12-well dish in DMEM media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Reporter plasmids, pG5-SV40 Luc (firefly luciferase, 200 ng) and pRL-TK (*Renilla* luciferase control reporter, 5 ng), were cotransfected along with 100 ng of Gal4-driven ZBTB20 long isoform wild type (Gal4-ZBTB20 pCMV-FA), ZBTB20 SUMO mutant plasmid (Gal4-ZBTB20 K330R pCMV-FA, Gal4-ZBTB20 K371R pCMV-FA, Gal4-ZBTB20 K330R, K371R pCMV-FA) or control plasmid (Gal4-pCMV-FA) using Lipofectamine 2000 as per conditions specified by the manufacturer.

Approximately 24 hours post-transfection cells were washed in PBS and lysed in Passive Lysis buffer (Promega) by freeze-thaw protocol. Cells were scrapped and then centrifuged to remove cell debris. Using a GloMax Microplate luminometer with dual injectors (Promega) loaded with Luciferase Assay Reagent (LARII, Promega) and Stop and Glow reagent (Promega), both firefly and *Renilla* luciferase readings were obtained from the cell lysate. Readings were measured in triplicate at the same condition. Further, the firefly luciferase activity was normalized with the *Renilla* luciferase activity and expressed as relative fold luciferase activity.
Cycloheximide stability assay

For the cycloheximide (CHX) stability assay, approximately one million HEK293H cells were plated on Poly-L-lysine coated 60 mm dishes. Cells were transfected with 3 µg of V5-tagged ZBTB20 wild type or SUMO acceptor lysine mutant plasmids, ZBTB20 K330R and ZBTB20 K371R. About 12 hours post-transfection, HEK293H cells in the 60mm dishes were counted and split into a 12 well plate such that each well had approximately 2 X10⁶ cells. After 18-20 hours post-plating, cells were treated with 100 µg/ml of Cycloheximide (CHX), reconstituted in dimethyl sulfoxide (DMSO), or in DMSO alone (control wells) for 36 hours. The cells were harvested at the time of treatment (time 0), and 36 hours post-treatment (time 36) by incubating the cells in 1% NP-40 lysis buffer and protease inhibitor cocktail. The protein concentration in the lysate was measured using coomassie (Bradford) protein assay reagent (Thermo Scientific). An equal amount of protein from all the wells were separated on a SDS-PAGE gel and subjected to Western blot analysis using anti-V5 antibody to detect ZBTB20 wild type and ZBTB20 SUMO mutants. The membrane was reprobed with anti-GAPDH antibody to show equal protein loading.
Results

Identification of UBC9 as a ZBTB20-interacting protein by yeast two-hybrid

To increase our understanding of the physiological function of ZBTB20, a yeast two-hybrid screen was performed to search for brain expressed ZBTB20-interacting proteins. The full-length human ZBTB20 long isoform was fused to the Gal4 DNA binding domain to serve as a bait (Figure 3.1A), to screen the Mate and Plate Human Fetal Brain cDNA library in pGADT7-rec vector. Blue colonies, which grew on nutritional triple dropout SD-His/-Leu/-Trp X-α-gal media, as well as higher stringency quadruple dropout SD-Ade/-His/-Leu/-Trp X-α-gal media, were selected. Plasmids from the selected colonies were isolated by transforming in E. coli and sequenced to identify the ZBTB20-interacting cDNAs. Eight unique ZBTB20-interacting proteins were identified in the yeast two-hybrid screen (Chapter 2), though the focus of this study is the interaction of ZBTB20 with SUMO conjugating enzyme UBC9.

Three full-length UBC9 cDNA clones (Figure 3.1B) were identified in the ZBTB20 yeast two-hybrid screen. Interaction between ZBTB20 and UBC9 in yeast was verified by one-to-one mating, by individually transforming pGBK7-ZBTB20 long isoform and pGADT7-UBC9 plasmids into AH109 and Y197 yeast
strains respectively followed by mating the two yeast strains and further plating on SD-Ade/-His/-Leu/-Trp X-α-gal media (Figure 3.1C). The strength of ZBTB20 and UBC9 interaction was determined by measuring beta-galactosidase activity using the yeast liquid β-galactosidase assay (Figure 3.1D). The growth of mated yeast harboring ZBTB20 and UBC9 plasmids on high stringency nutritional dropout media and its beta-galactosidase expression levels confirmed the potential interaction between ZBTB20 and UBC9 in yeast.
Figure 3.1: ZBTB20 interacts with UBC9 in a yeast two-hybrid assay
Figure 3.1: ZBTB20 interacts with UBC9 in a yeast two-hybrid assay

(A) Schematic representation of the BTB domain (BTB) and five zinc finger domains (Znf) in the long isoform of ZBTB20. Orange box at N-terminus indicates region (1-73 aa) specific to the long isoform (B) Full length UBC9 (158 aa) identified in yeast two-hybrid screen. (C) Yeast mating of pGBK7-ZBTB20 in AH109 and pGAD77-UBC9 in Y187, pGBK7-53 in AH109 and pGAD77-T in Y187 (Positive control) and pGBK7-Lam in AH109 and pGAD77-T in Y187 (Negative control). Mated yeast cells were grown on SD-Ade/-His/-Leu/-Trp X-α gal plates. (D) Liquid beta-galactosidase assay to measure the strength of interaction between pGBK7-ZBTB20 and pGAD77-UBC9. Error bars represent standard deviation from three independent replicates.
ZBTB20 interacts with UBC9 in mammalian cells

To further confirm the interaction between ZBTB20 and UBC9 in mammalian cells, a co-immunoprecipitation analysis was carried out. HEK293H cells were transiently transfected with V5-tagged ZBTB20 long isoform and GFP-tagged UBC9 expression vectors. The HEK293H cell lysates were prepared and immunoprecipitated using rabbit anti-V5 antibody followed by Western blot analysis using anti-V5 antibody (Figure 3.2, upper panel) and anti-GFP antibody (Figure 3.2, lower panel). As shown in Figure 3.2, ZBTB20 long isoform-V5 interacted with GFP-UBC9, validating the interaction between wild type ZBTB20 long isoform and SUMO conjugating enzyme UBC9.

To determine the UBC9 binding region of ZBTB20, ZBTB20 lacking the N-terminal region including the BTB domain, ZBTB20(-BTB)-V5 was coexpressed with GFP-UBC9 in HEK293H cells, followed by Western blot analysis of the lysates and immunoprecipitates. GFP-UBC9 was not detected in the immunoprecipitate (Figure 3.2), indicating that the N-terminus region of ZBTB20 is critical for UBC9 binding. The BTB domain was shown to mediate protein-protein interactions in members of the BTB family of transcription factors (Stogios et al., 2005).
Figure 3.2: ZBTB20 interacts with UBC9 in mammalian cells and the N-terminal of ZBTB20 is critical for its interaction with UBC9

Western blot analysis of the co-immunoprecipitation reaction of ZBTB20 long-V5 (red arrow) or ZBTB20-(BTB)-V5 (purple arrow) with GFP-UBC9 (blue arrow), showing lysate (lane L, input) and co-immunoprecipitates (lane IP, immunoprecipitate). Anti-V5 antibody (upper panel) and anti-GFP (lower panel) is used for the Western blot analysis. GFP empty vector (green arrow) is used as negative control.
To investigate the co-localization of ZBTB20 and UBC9 in mammalian cells, immunofluorescence analysis was performed in rat primary neuronal cells (Figure 3.3). GFP-tagged UBC9 and V5-tagged ZBTB20 long isoform were coexpressed in the rat primary neuronal cells followed by immunofluorescence analysis, using antibodies against the GFP and V5 tags. Co-localization of ZBTB20 long isoform-V5 and GFP-UBC9 was indicated by yellow/orange signal detected in the nucleus, cytoplasm and neurite processes of primary neuronal cells (Figure 3.3). However, coexpression of V5-tagged LacZ construct (negative control, not shown) with GFP-tagged UBC9 failed to co-localize in the rat primary neuronal cells. The interaction between ZBTB20 long isoform and UBC9 in HEK293H cells was also specific (GFP-empty vector failed to interact with ZBTB20-V5), indicated by co-immunoprecipitation analysis. Thus from the co-immunoprecipitation and co-localization experiments in mammalian cells, we concluded that ZBTB20 and UBC9 interact \textit{in vivo}. 
Figure 3.3: ZBTB20 long isoform co-localizes with SUMO conjugating enzyme UBC9

ZBTB20 long-V5 and GFP-UBC9 co-expressed in rat primary neuronal cells. (I) Blue (DAPI; nucleus); (II) green (GFP-UBC9); (III) red (ZBTB20 long-V5); (IV) co-localization indicated by yellow/orange signal. Scale bar = 10µm.
ZBTB20 is SUMOylated and co-localizes with SUMO1

We hypothesized that ZBTB20 is SUMOylated as it interacts with UBC9, an enzyme involved in the conjugation of SUMO to target proteins. A majority of SUMOylated proteins have a consensus SUMOylation motif, ΨKXE. Using SUMOylation site prediction software seeSUMO (Teng et al., 2012) and SUMOsp 2.0 (Ren et al., 2009), two putative consensus SUMOylation motifs, IK$^{330}$QE and PK$^{371}$GE (Figure 3.4A) in the ZBTB20 long isoform at conserved lysine residues, K330 and K371, were predicted. Furthermore, the SUMOylation site prediction software seeSUMO predicted that ZBTB20 long isoform K330 residue had a higher SUMO binding score than the K371 residue, as shown by a high classifier output and confidence level values in Figure 3.4B. Both of these putative SUMOylation motifs are well conserved in ZBTB20 orthologues as shown in Figure 3.4C, suggesting that SUMO modification of these motifs could be functionally significant.
Figure 3.4: Predicted human ZBTB20 long isoform SUMOylation sites
Figure 3.4: Predicted human ZBTB20 long isoform SUMOylation sites

(A) Schematic of ZBTB20 showing two SUMO consensus motifs. SUMO consensus sequences at K330 and K371, shown in red, between BTB domain and five zinc finger domains (Znf). (B) SUMOylation residues, K330 and K371 scored on the basis of classifier output and confidence level using seeSUMO. (C) Region surrounding the two putative SUMOylation motifs, IK$^{330}$QE and PK$^{371}$GE (red box) aligned in ZBTB20 orthologues using Homologene.
V5-tagged ZBTB20 long isoform and HA-tagged SUMO1 were transiently transfected in HEK293H cells to determine ZBTB20 SUMOylation \textit{in vivo}. Cells were lysed in NP-40 lysis buffer and N-ethylmaleimide (NEM), a SUMO protease inhibitor. NEM is a sulfhydryl reagent that ablates the enzyme function of cysteine proteases, preventing deSUMOylation. Western blot analysis of the protein lysate using anti-SUMO1 or anti-V5 antibody was performed. Expression of wild type ZBTB20 with SUMO1 yielded two additional higher molecular weight bands when probed with either anti-V5 or anti-SUMO1 antibody (Figure 3.5, lane 1), suggesting that ZBTB20 is modified by SUMO1.

The ZBTB20 lysine 330 and lysine 371 residues were mutated (K330R or K371R) or individually deleted (Δ330K or Δ371K), or deleted for the 7 amino acid conserved sequence surrounding both the lysine residues (Δ327-333 or Δ368-374), to investigate the SUMO acceptor lysine residue involved in ZBTB20 SUMOylation. HEK293H cells were co-transfected with ZBTB20 SUMO mutant constructs and SUMO1 and protein lysates were prepared as described above. Lysine 330 mutations, K330R, Δ330K, and Δ327-333 showed the absence of the higher molecular weight bands (Figure 3.5, lane 2, lane 3 and lane 4) detected both in ZBTB20 wild type as well as in mutations involving the lysine 371 residue: ZBTB20 K371R, Δ371K or Δ368-374 (Figure 3.5, lane 5, lane 6 and lane 7) as shown by Western blot analysis using anti-SUMO1 or anti-V5 antibody. Mutations involving the ZBTB20 K330 residue failed to show
the higher molecular weight bands in the presence of SUMO1, it is thus likely that ZBTB20 K330 is the site of SUMO1 binding.

Figure 3.5: Lysine 330 is likely the site of SUMO1-conjugation in ZBTB20

Western blot analysis of HEK293H lysates overexpressing HA-SUMO1 and ZBTB20 long-V5 or ZBTB20 SUMOylation mutants probed with anti-V5 (upper panel), anti-SUMO1 (middle panel) or anti α-Tubulin (lower panel). Two slower migrating bands (indicated by arrows) are observed in lane 1 (ZBTB20 long-V5 wild type) but not in lane 2 (Δ327-333), lane 3(Δ330) and lane 4 (K330R). Bands similar to wild type but lighter in intensity were observed in lane 5 (Δ368-374), lane 6 (Δ371) and lane 7(K371R). * Nonspecific band. Anti-α-Tubulin is used as loading control.
To investigate whether ZBTB20 and SUMO1 co-localize in mammalian cells, both V5-tagged ZBTB20 long isoform and HA-tagged SUMO1 constructs were transfected in HEK293H cells. Immunofluorescence analysis was performed using anti-SUMO1 and anti-V5 antibodies and co-localization was detected by the presence of yellow/orange signal in the nucleus of HEK293H cells as shown in Figure 3.6.

**ZBTB20 SUMO mutants do not affect the nuclear localization of ZBTB20**

It is known that SUMOylation of SUMO target proteins can regulate their subcellular localization, which in turn regulates protein function as well as protein-protein interaction in the cell (Lomelí and Vázquez, 2011; Marongiu et al., 2010).

The ZBTB20 long isoform localizes primarily in the nucleus as described in Chapter 2. Since SUMOylation is involved in targeting SUMO substrates to the nucleus, we hypothesized that SUMOylation regulates the nuclear localization of ZBTB20. To test this hypothesis, V5-tagged ZBTB20 long isoform (Figure 3.7A), SUMO acceptor lysine double mutant ZBTB20 long K330R, K371R (Figure 3.7B) and ZBTB20 SUMO consensus double mutant ZBTB20 long Δ327-333, Δ368-374 (Figure 3.7C) were transiently overexpressed in rat primary neuronal cells and in HEK293H cells (not shown). In both the
mammalian cells lines, wild type ZBTB20 as well as the ZBTB20 SUMO mutants was primarily localized in the nucleus. Figure 3.7 shows the nuclear localization of wild type and SUMOylation mutant ZBTB20 in rat primary neuronal cells. Thus, contrary to our hypothesis, SUMOylation did not appear to be necessary for the nuclear location of ZBTB20 in mammalian cells.

**Figure 3.6: ZBTB20 long isoform co-localizes with SUMO1**

HEK293H cells overexpressing HA-SUMO1 and ZBTB20 long-V5 constructs. (a) Blue (DAPI, nucleus); (b) green (SUMO1); (c) red (V5-epitope, ZBTB20); (d) merged image. Co-localization is indicated by yellow/orange signal in nucleus. Scale bar = 10µm.
A. ZBTB20 long-V5

Figure 3.7: Both ZBTB20 wild type and ZBTB20 SUMO acceptor site mutants localize in the nucleus of rat primary neuronal cells
B. ZBTB20 long K330R, K371R-V5

![Images of DAPI and Neuron specific tubulin with arrows pointing to localization in the nucleus of rat primary neuronal cells.](image)

Figure 3.7: Both ZBTB20 wild type and ZBTB20 SUMO acceptor site mutants localize in the nucleus of rat primary neuronal cells.
C. ZBTB20 long Δ327-333, Δ368-374 -V5

Figure 3.7: Both ZBTB20 wild type and ZBTB20 SUMO acceptor site mutants localize in the nucleus of rat primary neuronal cells
Figure 3.7: Both ZBTB20 wild type and ZBTB20 SUMO acceptor site mutants localize in the nucleus of rat primary neuronal cells

(A) Wild type ZBTB20 long-V5, (B) ZBTB20 SUMOylation double mutant (K330R in SUMO consensus motif 1 and K371R in SUMO consensus motif 2) and (C) ZBTB20 SUMOylation double mutant deleted for entire SUMO consensus motif 1 (Δ327-333) and SUMO consensus motif 2 (Δ368-374). The indicated constructs were separately over-expressed in rat primary neuronal cells and immunostained with a, DAPI (blue, nucleus); b, neuron specific tubulin (green, cytoskeleton); c, V5 antibody (red, ZBTB20 wild type or ZBTB20 SUMO double mutants); d, merged image. The white arrow indicates the location of the nucleus of the primary neuronal cell being analyzed.
ZBTB20 SUMO mutants do not affect repression activity of ZBTB20

Murine Zbtb20 acts as a transcriptional repressor (Xie et al., 2008, Zhang et al., 2012, Liu et al., 2013). There is approximately 98% sequence similarity between mouse Zbtb20 and human ZBTB20, determined by the ClustalW sequence alignment tool. Since human and mouse proteins are very similar, it was hypothesized that human ZBTB20 acts as a transcriptional repressor.

Using the Gal4-based transcriptional assay, the transcriptional repression activity of ZBTB20 was determined. The Gal4-ZBTB20 fusion vector was cotransfected in HEK293H cells with the luciferase reporter construct having five Gal4-DNA binding sites (pG5-SV40-Luc) and the Renilla luciferase control reporter for normalization. Consistent with the repression activity of mouse Zbtb20, wild type human ZBTB20 also acts as transcriptional repressor. As shown in Figure 3.8, Gal4-ZBTB20 wild type showed approximately 50% reduced relative fold activity as compared to Gal4-empty vector used as control.

Since SUMO modification of transcription repressors facilitates repression (Gill, 2005; Girdwood et al., 2004), the repression activity of ZBTB20 SUMOylation mutants was determined. The Gal4-driven ZBTB20 SUMOylation mutants, Gal4-ZBTB20 K330R, Gal4-ZBTB20 K371R or Gal4-ZBTB20 K330R, K371R were cotransfected with luciferase and Renilla reporters in HEK293H cells, as
described above. All three ZBTB20 SUMO mutants showed repression activity, similar to the wild type ZBTB20, shown in Figure 3.8. Thus using the Gal4-based transcriptional assay, ZBTB20 SUMOylation mutants did not appear to affect the repression activity of human ZBTB20 in HEK293H cells.

![Graph showing transcriptional repression activity of Gal4-driven ZBTB20 wild type and SUMO acceptor site mutants](image)

**Figure 3.8: Transcriptional repression activity of Gal4-driven ZBTB20 wild type and SUMO acceptor site mutants**

HEK293H cells were cotransfected with Gal4-ZBTB20 long wild type or ZBTB20 SUMOylation mutants (Gal4-ZBTB20 K330R, Gal4-ZBTB20 K371R or Gal4-ZBTB20 K330R, K371R) along with pG5-SV40 luciferase reporter and pRL-TK (*Renilla* luciferase) as internal control. Results are expressed as relative fold luciferase activity with respect to the Gal4-empty vector (control) normalized to 1. Error bars represent standard deviation from three samples at the same condition.
ZBTB20-SUMO1 binding does not affect its stability

SUMOylation has been shown to play an important role in regulating the stability of target proteins. To determine the effect of SUMOylation on the stability of ZBTB20, a cycloheximide time-course experiment was performed. Cycloheximide is an inhibitor of protein biosynthesis in eukaryotes and is used to determine the stability of proteins in cell lines. V5-tagged ZBTB20 wild type or ZBTB20 SUMOylation mutants (ZBTB20 K330R or ZBTB20 K371R) were transfected in HEK293H cells, followed by treatment with and without cycloheximide. Protein was isolated both at the start of cycloheximide treatment as well as 36 hours post-treatment, with and without cycloheximide. The cycloheximide treated V5-tagged ZBTB20 wild type protein did not show any significant change in protein expression as compared to untreated cells (Figure 3.9, lane 4,5). Moreover, there was no significant change in protein expression with and without cycloheximide treatment in ZBTB20 SUMOylation mutants K330R (Figure 3.9, lane 6,7) and K371R (Figure 3.9, lane 8,9). Thus both ZBTB20 wild type and SUMOylation mutants appeared to be equally stable. This suggests that SUMOylation likely does not play a role in the stability of ZBTB20.
Figure 3.9: Protein stability of wild type and SUMO mutant ZBTB20

Cycloheximide (CHX) stability assay of HEK293H cells transfected with wild type ZBTB20 long isoform (WT) or ZBTB20 SUMOylation mutants (K330R and K371R). HEK293H cells were treated with 100 µg/ml cycloheximide (+) or DMSO alone (-) for 36 hours. Time 0 hour represents protein lysate obtained at the time of initiation of CHX treatment. Western blot analysis was performed using anti-V5 antibody (upper panel) and with anti-GAPDH (lower panel) as a loading control.
ZBTB20 SUMO acceptor lysine mutant, K330R interferes in the dimerization of ZBTB20

The BTB/POZ (broad complex, tramtrack, bric-a-brac/pox-virus and zinc finger) domain represents a major protein-protein interaction interface for BTB/POZ zinc finger transcription factors (Bardwell and Treisman, 1994). Using co-immunoprecipitation analysis in HEK293H cell lysates (Chapter 2), the homo- and heterotypic interaction between human ZBTB20 long and short isoforms was determined (Figure 3.10, lanes 1, 2, 3 and 4). The N-terminal region containing the BTB domain was found to be critical for the ZBTB20 dimerization (Figure 3.10, lower panel, lane 6). To determine whether the ZBTB20 SUMOylation mutant K330R interferes in ZBTB20 dimerization, V5-tagged ZBTB20 long K330R construct was cotransfected with GFP-tagged ZBTB20 long isoform construct in HEK293H cells. Using V5-antibody for co-immunoprecipitation followed by Western blot analysis using anti-GFP antibody, ZBTB20 K330R-V5 failed to interact with GFP-tagged ZBTB20 long isoform (Figure 3.10, lower panel, lane 8), similar to the result of the immunoprecipitation reaction between ZBTB20-[BTB]-V5 and GFP-ZBTB20 long (Figure 3.10, lower panel, lane 6). Thus the co-immunoprecipitation analysis in HEK293H cells indicates that the lysine 330 SUMO acceptor residue possibly interferes in ZBTB20 dimerization.
Figure 3.10: SUMO acceptor site mutant, ZBTB20 K330R prevents homodimerization with ZBTB20 long isoform

Western blot analysis showing HEK293H cell lysates (lane L, input) and co-immunoprecipitates (Lane IP, immunoprecipitate) co-transfected with the indicated GFP-tagged and V5-tagged plasmids and immunoprecipitated using anti-V5 antibody.

GFP-ZBTB20 long isoform was cotransfected with ZBTB20 long-V5, ZBTB20 short-V5, ZBTB20-[BTB]-V5 or ZBTB20 K330R-V5 in HEK293H cells. Western blot analysis using anti-GFP antibody (lower panel) detected GFP-ZBTB20 long isoform (black arrow) when pulled down using ZBTB20 long-V5 and ZBTB20 short-V5 plasmids but its signal was significantly reduced when pulled down using ZBTB20-[BTB]-V5 and ZBTB20 K330R-V5 plasmids. Ant-V5 antibody (upper pane) confirmed the presence of the V5-tagged plasmids in all the lanes.
Discussion

In this chapter, the interaction of ZBTB20 with E2 SUMO conjugating enzyme UBC9 and SUMO1 has been described. The results described in this study provide evidence suggesting SUMOylation of ZBTB20 long isoform at the lysine 330 residue. Moreover, the effect of ZBTB20 SUMOylation on the various functional roles played by ZBTB20 was explored.

Using ZBTB20 long isoform as bait, SUMO-conjugating enzyme UBC9 was identified in the human fetal brain-yeast two-hybrid screen performed to determine brain expressed ZBTB20-interacting proteins. This interaction was verified by co-expressing both ZBTB20 and UBC9 mammalian expression constructs in HEK293H cells, followed by co-immunoprecipitation analysis. Our co-immunoprecipitation results demonstrated that, ZBTB20 lacking the N-terminus that includes the BTB domain, failed to interact with the SUMO conjugating enzyme UBC9. Thus, the N-terminal region containing the BTB domain is critical for the interaction between ZBTB20 and UBC9, consistent with its role in protein-protein interactions (Stogios et al., 2005).

Since ZBTB20 long isoform interacts with UBC9 the ZBTB20 protein sequence was analyzed for putative SUMO acceptor lysine residues. Using SUMO prediction tools seeSUMO (Teng et al., 2012) and SUMOsp (Ren et al., 2009),
two well-conserved putative SUMO consensus motifs at the lysine 330 and lysine 371 residues in human ZBTB20 protein and its orthologues were identified. Proteomic analyses by others have shown that at least half of all SUMO target proteins contain consensus SUMOylation sites (Matic et al., 2010). The interaction of SUMO1 with ZBTB20 in HEK293H protein extracts, suggests posttranslational modification of ZBTB20 by SUMO1 in vivo. This result has been recently supported by the identification of Sumo1 conjugated murine Zbtb20 in mouse brain by large-scale proteomic analysis (Tirard et al., 2012). Co-immunoprecipitation reaction in HEK293H cells overexpressing HA-tagged SUMO1 and V5-tagged ZBTB20 lysine 330 and lysine 371 mutants, indicate that lysine 330 is likely the site of SUMO1-conjugation in ZBTB20.

Covalent modification of target proteins by SUMOylation regulates various cellular processes (Geiss-Friedlander and Melchior, 2007). SUMOylation facilitates nucleocytoplasmic transport and has been shown to alter intracellular localization of substrate proteins (Mahajan et al., 1997; Matunis et al., 1996). To determine the effect of SUMOylation on nuclear localization of ZBTB20, wild type and ZBTB20 SUMOylation mutants were overexpressed in HEK293H cells and rat primary neuronal cells. However mutating the ZBTB20 SUMO acceptor lysines as well as the SUMO consensus motifs did not alter the intracellular localization of ZBTB20. Thus, the nuclear targeting of ZBTB20 does not appear to depend on an intact SUMOylation site, indicating that SUMOylation is
dispensable for the nuclear localization of ZBTB20. In fact, nuclear localization of many proteins were unaffected by SUMOylation (Snow et al., 2010; Wilson and Rosas-Acosta, 2005).

In this study, ZBTB20 SUMOylation mutants did not show any effect on the stability of ZBTB20 in HEK293H cells. Transiently overexpressing ZBTB20 followed by cycloheximide treatment and Western blot analysis showed that the ZBTB20 wild type and the ZBTB20 SUMOylation mutants have long half-lives. It is however possible that these SUMOylation mutants could differentially affect the stability of ZBTB20 in other cell types.

SUMOylation could have a range of effects on the activity of transcription factors. In most cases, SUMOylation enhanced the repressive activity of transcription factors (Kang et al., 2003; Siatecka et al., 2007), while in other cases SUMOylation increased transcriptional activation (Yan et al., 2010). Consistent with the role of murine Zbtb20 as a transcriptional repressor, human ZBTB20 also functions as a transcriptional repressor. However, the ZBTB20 SUMOylation mutants showed no measurable change in the transcriptional repression activity of ZBTB20. Thus SUMOylation does not appear to play a role in the repression function of ZBTB20 using the Gal4-based transcriptional assay. Since this assay is independent of the DNA binding capability of human ZBTB20, it is still possible that the ZBTB20 SUMOylation mutants facilitate the transcriptional repression
function of ZBTB20. These SUMOylation mutants could impair the interaction of ZBTB20 with other proteins that facilitate repression, interfering in the repression function of ZBTB20. More work needs to be done, to elucidate the role of SUMOylation on the repression function of human ZBTB20.

It was determined that ZBTB20 SUMO mutant K330R impairs dimerization of wild type ZBTB20 long isoform. Inability to dimerize could prevent formation of homodimers and heterodimers as well as disrupt the interaction of ZBTB20 with other proteins. Interestingly murine Zbtb20 shows high expression in the hippocampal pyramidal neurons, where both Ubc9 and Sumo1 are also highly expressed (Watanabe et al., 2008). Moreover the identification of Sumo1 conjugated Zbtb20 in Sumo1 knock-in mice by Tirad and coworkers (2013) shows that Zbtb20 is post-translationally modified by Sumo1 in mouse brain. Thus the inability of ZBTB20 to form dimers in the brain could possibly disrupt its function in hippocampal neurogenesis, besides other functions performed by ZBTB20. Additional studies need to be performed to elucidate the role of SUMOylation on the functions performed by ZBTB20.
References


CHAPTER 4

ZBTB20-DEPENDENT TRANSCRIPTION REGULATION OF NEURONAL GENES AND ITS ROLE IN AUTISM AND INTELLECTUAL DISABILITY

Introduction

Autism spectrum disorders (ASD) and intellectual disability (ID) are neurodevelopmental disorders, often co-morbid, with a combined prevalence estimated at more than 3% of the general population (Kim et al., 2011). Autism spectrum disorders are characterized by impaired language, communication and social skills. Intellectual disability is a limitation in intellectual functioning and adaptive behavior with an intelligence quotient (IQ) below 70. ID is found in approximately 70% of patients with ASD and conversely at least 10% of patients with ID have ASD (Oeseburg et al., 2011).

Both ASD and ID are genetically heterogeneous disorders. It is estimated that hundreds of loci distributed throughout the genome likely contribute to the susceptibility of ASD (State and Sestan, 2012). There is a growing consensus that the transcriptional regulation of many neuronal genes rather than a single
gene contributes to manifestation of ASD and ID (Ebert and Greenberg, 2013; Tsai et al., 2012; West and Greenberg, 2011).

Transcriptome analysis of both autistic and normal brain suggested the role of transcriptional and splicing dysfunction as an underlying cause of neuronal dysfunction in some patients (Voineagu et al., 2011). Recently several human exome studies suggest a prominent role of the genes involved in transcription regulation during brain development in patients with ASD (Iossifov et al., 2012; Neale et al., 2012; O’Roak et al., 2012; Sanders et al., 2012). Moreover transcription factors specific to a particular layer and neuronal subtypes in the neocortex were associated with neurodevelopmental disorders (Kwan et al., 2012). Transcription factors were shown to play an important role in neuronal layer identity, connectivity and axonal projections of pyramidal neurons (Molyneaux et al., 2007). In a mouse study, neocortical layer-specific transcription factors Tbr1, Sox5, Fezf2, Mef2c and Satb2 were found to play a pivotal role in determining the molecular mechanisms underlying neuronal layer identities and connectivities of neocortical projection neurons. Thus, the dysregulation of transcription factors in the brain could lead to the development of ASD and ID.

Murine Zbtb20 was shown to be involved in direct repression of neuronal transcription factor genes implicated in development of neocortical projection
neurons (Nielsen et al. 2013). Using chromatin immunoprecipitation combined with high throughput sequencing (ChIP-Seq), it was shown that the Zbtb20 protein directly binds the transcription factor genes that regulate projection neuron development in the neocortex. Nielsen and coworkers (2013) identified 42 potential transcription factor-encoding genes that were repressed in the CA1-transformed hippocampal subiculum and retrosplenial cortex of the Zbtb20 transgenic mice. It was further observed that most of these Zbtb20 regulated transcription factor genes were upregulated in the hippocampus of the Zbtb20 knockout mice (Nielsen et al., 2010; Nielsen et al., 2013; Xie et al., 2010). Several of these transcription factor genes were implicated in corticofugal projection neuron specification (Fezf2, Tbr1, Sox5), callosal projection neuron identity (Satb2) and upper-layer projection neuron development (Cux1, Cux2, Mef2c). Thus, substantial evidence exists to support the role of Zbtb20 as a transcriptional repressor of the transcription factor-encoding genes involved in the neocortical development.

In this study, by using the human brain integrated microarray dataset (Wang et al., 2010), the human ZBTB20 gene was found to be coexpressed with neuronal transcription factor genes involved in the development of the neocortical layer-specific projection neurons. Consistent with their role in neurogenesis, these genes were found to be associated with the enriched Gene Ontology terms, including synapse formation, synaptic transmission and neurological processes.
Moreover, we observed that the overexpression of the wild type ZBTB20 in human embryonic kidney cells (HEK293H), represses neuronal transcription factor genes *MEF2C, TBR1* and *FEZF2* previously shown to be associated with ASD and ID. The results suggest a possible contribution of ZBTB20-dependent transcription regulation mechanism in the development of ASD and ID.

**Materials and Methods**

**ZBTB20 co-expression analysis**

The genes co-expressed with *ZBTB20* were identified to determine its physiological functions. The procedure to obtain the list of *ZBTB20* co-expressed genes is briefly outlined here. A set of 616 brain-derived microarray expression profiles from 32 different brain regions (Wang et al., 2010) were used for the co-expression analysis. Pearson’s correlation coefficient (r) between the probe sets was measured and probe sets with |r| ≥0.3 were selected as co-expressed genes.

We made a list of the top *ZBTB20* co-expressed genes, both positively and negatively correlated with the *ZBTB20* gene. Both the positively and negatively correlated gene sets were subjected to Gene Ontology functional analysis using the DAVID (Database for Annotation, Visualization and Integrated Discovery)
software tool (Huang et al. 2007). The DAVID software provides a list of functional annotation tools to understand the biological functions of large gene sets. Using functional annotation terms, including cellular process (CC), molecular function (MF) and biological process (BP), the ZBTB20-coexpressed gene clusters were generated by DAVID analysis.

**Expression constructs**

The ZBTB20 open reading frames corresponding to both the short and long isoform ZBTB20 proteins were amplified from the Human Fetal Brain MATCHMAKER (Clonetech) cDNA library (Rimsky et al., manuscript in preparation). Using a pcDNA 3.1 directional TOPO Expression Kit (Invitrogen) the ZBTB20 cDNAs were subcloned into a pcDNA3.1D/V5-His-TOPO vector which was tagged with V5 epitope. The QuickChange Site-directed Mutagenesis Kit (Stratagene) was used to generate the ZBTB20 mutant constructs, P46R in the ZBTB20 long isoform and G346V in the ZBTB20 short isoform pcDNA3.1D/V5-His-TOPO vector (Rimsky et al., manuscript in preparation). The constructs were sequence verified and their expression further confirmed using the TNT T7 Quick Coupled Transcription/Translation system (Promega) and analyzed by SDS-PAGE.
Quantitative reverse transcription PCR (qRT-PCR) and Western blot

Approximately 3 million HEK293H cells were plated in duplicate on 100 mm Poly-L-lysine coated plates. HEK293H cells were transfected with 13 µg ZBTB20 expression plasmids using Lipofectamine 2000 Reagent (Invitrogen) according to the conditions specified by the manufacturer. Approximately 48 hours post-transfection, cells were trypsinized, pelleted and washed with cold Phosphate-buffered saline (PBS, Sigma). Half of the cells were used for RNA extraction and the other half used for protein extraction. RNA extraction was performed using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). RNA was cleaned using Turbo DNA-free kit (Ambion Life Technologies-Invitrogen) followed by lithium chloride precipitation solution (Ambion Life Technologies-Invitrogen). Quantitative qRT-PCR was performed using iScriptTM One-Step RT-PCR kit with SYBR Green and primers as shown in Table 4.1. Primers used for ZBTB20 qRT-PCR analysis were 5'-ATGTTCGTACACACAGGTGAGAAG-3' (forward primer) and 5'-GCTTGTTGCAGATACTACACTGT-3' (reverse primer) to generate a product of 136 base pairs. To determine the normalized expression, ΔΔC(t) was calculated using POLR2A as reference gene in regression mode and analyzed using the Bio-Rad CFX Manager software. The experiments were repeated three times and the p value calculated using the Student’s t-test in Excel.
Table 4.1: Quantitative RT-PCR primers to measure the endogenous expression of neuronal transcription factor genes in HEK293H cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX5</td>
<td>TTCCCATCAAGCACCTGTT</td>
<td>CTTCAGCCAGAGTTAGCACA</td>
<td>121</td>
</tr>
<tr>
<td>TBR1</td>
<td>CGTGTCAATAATTATCCCAAACC</td>
<td>CAGACGTACTCTTCCCCCTGAG</td>
<td>123</td>
</tr>
<tr>
<td>TLE4</td>
<td>TCCTCATTGGAACGCTCAACC</td>
<td>ACAGAAACTCCGGCAGACTAC</td>
<td>130</td>
</tr>
<tr>
<td>ZFPM2</td>
<td>ATGGCCCTCGAAGTTGACAC</td>
<td>TGTTTGCTGGATGACTTG</td>
<td>101</td>
</tr>
<tr>
<td>CUX1</td>
<td>TCACCCCTCAGTAGCTCAGCT</td>
<td>CAGCCAGATCTCAGCTTG</td>
<td>101</td>
</tr>
<tr>
<td>CUX2</td>
<td>GTCCAGACCCAGATGCTTTC</td>
<td>GACACGTACTCCCATACCAAA</td>
<td>92</td>
</tr>
<tr>
<td>FEZF2</td>
<td>GTCACTTGGTGTTCTTTGAGT</td>
<td>ACGCTCAACAGGCATCC</td>
<td>107</td>
</tr>
<tr>
<td>SATB2</td>
<td>Hs00392652.m1</td>
<td>(Probe based assay)</td>
<td></td>
</tr>
<tr>
<td>FOXP2</td>
<td>TGCCATGTGCTTTTCACTGT</td>
<td>AGCACTTCTGTCCATCG</td>
<td>104</td>
</tr>
<tr>
<td>MEF2C</td>
<td>TGGTGTGCTTGTGAAGATGA</td>
<td>AGATTACGGAGTTATGGATGAACG</td>
<td>138</td>
</tr>
<tr>
<td>RORB</td>
<td>TGCAAACCTCCACCACGTAT</td>
<td>GCAGACCCACACCTATGAAG</td>
<td>115</td>
</tr>
</tbody>
</table>

*Annealing temperature used for all primers was 60 ºC.

**Protein extraction and Western blot**

Protein lysis was performed in the RIPA lysis buffer [150 mM NaCl; 1% NP-40; 0.5% Sodium Deoxycholate; 0.1% SDS and 50 mM Tris (pH 8.0)] and the protease inhibitor cocktail (Sigma). The cells were vortexed, incubated in ice for 30 minutes, sonicated and finally centrifuged to obtain the protein supernatant. The protein was quantified using the Pierce BSA protein assay (Thermo Scientific) according to the conditions specified by the manufacturer.
The quantified protein lysate were run on a SDS-PAGE gel. Western blot analysis was performed using the following antibodies to detect endogenous ZBTB20 and the overexpressed ZBTB20 constructs in HEK293H cell lysates: rabbit anti-ZBTB20 (Aviva Systems Biology) at 1:1000 dilution in 5% milk and 2% Bovine serum albumin (BSA; Sigma) in Tris Buffered Saline with Tween 20 (TBST) overnight at 4°C and mouse anti-V5 (Invitrogen) at 1:5000 dilution in 5% milk and 2% BSA in TBST.

Results

**ZBTB20 co-expresses with neuronal transcription factor (TF) genes and genes implicated in neurodevelopment**

The expression of the human *ZBTB20* gene isoforms was examined in a cDNA panel of human fetal tissues (Clonetech). Both the *ZBTB20* gene isoforms show increased expression in the human fetal brain and kidney (Rimsky et al., manuscript in preparation). Expression of human *ZBTB20* was identified specifically in the human fetal hippocampus (Nielsen et al., 2013). Not much is known about the mechanisms underlying the human ZBTB20 expression and the genes that it potentially regulates. On the other hand, expression of murine Zbtb20 in the brain was extensively studied. The mouse *Zbtb20* gene was expressed in neurons of the hippocampus, granule cells in the cerebellum as well
as in differentiated glial cells. Both Zbtb20 isoforms showed overlapping expression in the brain (Mitchelmore et al., 2002; Nielsen et al., 2007). Recently, the Zbtb20-regulated target genes were identified in the murine hippocampus (Nielsen et al., 2013) using ChIP-Seq experiments.

To determine the physiological role of ZBTB20, the genes coexpressed with the human ZBTB20 gene in the brain microarray expression dataset (Wang et al., 2010) were identified. To determine the genes repressed by human ZBTB20 in brain, the list of genes negatively correlated with ZBTB20 was analyzed using the DAVID software tool (Huang da et al., 2007). The genes encoding transcription factors were largely negatively correlated with ZBTB20 in the coexpressed gene list. Further functional analysis revealed that the top negatively correlated genes were enriched with the Gene Ontology terms, including synapse, synapse part, postsynaptic membrane, cell junction, and synaptic transmission functions (Table 4.2). These results are consistent with a recent study (Nielsen et al., 2013), which used chromatin immunoprecipitation (ChIP) combined with high throughput sequencing to show that Zbtb20 directly binds and represses 42 transcription factor genes involved in neurogenesis, axonogenesis, dendritogenesis and neuronal circuit formation. The list of murine Zbtb20 repressed transcription factor genes were analyzed. As expected majority of these transcription factor genes, showed negative correlation in expression with the ZBTB20 gene. As shown in Table 4.3, MEF2C, CUTL2, SATB2, TBR1, FOXP2 and RORB showed
the highest negative correlation with the ZBTB20 gene expression. Previous studies have shown that several of these transcription factor genes are repressed in the hippocampus of Zbtb20 (short isoform) overexpressing transgenic mice (Nielsen et al., 2007; Nielsen et al., 2013) while derepressed in the hippocampus of Zbtb20 null mice (Xie et al., 2010). Thus, ZBTB20 represses the transcription factor genes involved in neurodevelopment.

**ZBTB20-mediated repression of transcription factor genes associated with ASD and ID**

To evaluate ZBTB20-mediated repression of human transcription factor genes, both the long and short isoforms of ZBTB20 protein were individually overexpressed in HEK293H cells (Figure 4.1). The ZBTB20 protein was overexpressed by transiently transfecting V5-tagged ZBTB20 short or long isoform mammalian expression constructs in HEK293H cells. The expression of both ZBTB20 RNA (Figure 4.1A) as well as ZBTB20 protein (Figure 4.1B) was observed to be approximately 1000 fold higher in both ZBTB20 long and short isoform overexpressed HEK293H cells as compared to the untransfected HEK293H cells.
Table 4.2: Functional annotation clustering of top negatively correlated *ZBTB20* co-expressed genes (complete list not shown).

<table>
<thead>
<tr>
<th>Annotation Cluster 1</th>
<th>Enrichment Score: 11.79</th>
<th>Gene count</th>
<th>P_Value</th>
<th>Benjamini</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOTERM_CC_FAT</td>
<td>Synapse</td>
<td>80</td>
<td>2.40E-15</td>
<td>6.30E-13</td>
</tr>
<tr>
<td>GOTERM_CC_FAT</td>
<td>synapse part</td>
<td>61</td>
<td>8.90E-14</td>
<td>1.50E-11</td>
</tr>
<tr>
<td>GOTERM_CC_FAT</td>
<td>postsynaptic membrane</td>
<td>38</td>
<td>1.50E-10</td>
<td>2.00E-08</td>
</tr>
<tr>
<td>GOTERM_CC_FAT</td>
<td>cell junction</td>
<td>87</td>
<td>2.50E-09</td>
<td>2.60E-07</td>
</tr>
</tbody>
</table>

| Annotation Cluster 2 | Enrichment Score: 7.81 | |
|----------------------|--------------------------|------------|------------|-----------|
| GOTERM_BP_FAT         | synaptic transmission     | 64         | 8.50E-13   | 2.70E-09  |
| GOTERM_BP_FAT         | transmission of nerve impulse | 67         | 5.30E-11   | 8.50E-08  |
| GOTERM_BP_FAT         | cell-cell signaling       | 89         | 2.30E-08   | 6.80E-06  |
| GOTERM_BP_FAT         | neurological system process | 114         | 5.50E-02   | 6.20E-01  |
Table 4.3: Transcription factor genes negatively correlated with the ZBTB20 gene expression.

<table>
<thead>
<tr>
<th>Correlation Coefficient</th>
<th>Affymetrix Probe</th>
<th>Gene – Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.51</td>
<td>222357_at</td>
<td>ZBTB20 - zinc finger and BTB domain containing 20</td>
</tr>
<tr>
<td>-0.46</td>
<td>207968_s_at</td>
<td>MEF2C - myocyte enhancer factor 2C</td>
</tr>
<tr>
<td>-0.46</td>
<td>213920_at</td>
<td>CUTL2 - cut-like 2 (Drosophila)</td>
</tr>
<tr>
<td>-0.46</td>
<td>213435_at</td>
<td>SATB2 - SATB homeobox 2</td>
</tr>
<tr>
<td>-0.46</td>
<td>220025_at</td>
<td>TBR1 - T-box, brain, 1</td>
</tr>
<tr>
<td>-0.44</td>
<td>1552902_a_at</td>
<td>FOXP2 - forkhead box P2 (GB: NM_148898,</td>
</tr>
<tr>
<td>-0.41</td>
<td>242385_at</td>
<td>RORB - RAR-related orphan receptor B</td>
</tr>
<tr>
<td>-0.37</td>
<td>217707_x_at</td>
<td>SMARCA2 - SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2</td>
</tr>
<tr>
<td>-0.32</td>
<td>221086_s_at</td>
<td>FEZF2 - FEZ family zinc finger 2</td>
</tr>
<tr>
<td>-0.31</td>
<td>226048_at</td>
<td>MAPK8 - mitogen-activated protein kinase 8</td>
</tr>
<tr>
<td>-0.27</td>
<td>208889_s_at</td>
<td>NCOR2 - nuclear receptor co-repressor 2</td>
</tr>
<tr>
<td>-0.25</td>
<td>215591_at</td>
<td>SATB2 - SATB homeobox 2</td>
</tr>
</tbody>
</table>
Figure 4.1: Overexpression of ZBTB20 long or short isoform in HEK293H cells

Endogenous and exogenous A. ZBTB20 RNA expression measured using qRT-PCR and B. ZBTB20 protein expression observed by Western blot analysis. HEK293H cells overexpressing V5-tagged ZBTB20 long or short isoform plasmids (as indicated above) and un-transfected HEK293H cells were used for ZBTB20 RNA and protein expression analysis. ZBTB20 expression was normalized to POLR2A and represented as fold change of expression relative to un-transfected cells. Anti-ZBTB20 antibody was used for Western blot analysis.
Next, the endogenous expression levels of transcription factor genes previously found to be regulated by Zbtb20 protein in murine brain were analyzed. Nielsen and coworkers (2013) showed that murine Zbtb20 binds to neuronal transcription factor genes that regulate neuronal subtype specification in the developing neocortex. Moreover, Zbtb20 repressed these neocortical transcription factor genes in the CA1 transformed subicular and retrosplenial cortex brain regions in the Zbtb20 transgenic mice.

HEK293H cells overexpressing either the long or short isoform of ZBTB20 were analyzed for the endogenous expression of human transcription factor genes, TBR1, MEF2C, FEZF2, SOX5, ZFPM2, RORB, TLE4, FOXP2, and CUX1 by quantitative reverse-transcription PCR. The overexpression of the ZBTB20 long isoform resulted in a significant reduction in the expression of MEF2C and TBR1 while overexpression of the ZBTB20 short isoform resulted in reduced expression of the FEZF2 gene (Figure 4.2). No significant change in expression was observed for the endogenous expression of SOX5, ZFPM2, RORB, TLE4, FOXP2, and CUX1 in HEK293H cells overexpressed with either of the two isoforms of ZBTB20 protein as shown in Figure 4.2.
Figure 4.2: Endogenous RNA expression levels of transcription factor genes in HEK293H cells overexpressing human ZBTB20 long or short isoform

Endogenous RNA levels of transcription factor genes previously regulated by murine Zbtb20 and associated with neurodevelopmental disorders were quantified by qRT-PCR in HEK293H cells overexpressing human ZBTB20 long isoform (Long WT) and ZBTB20 short isoform (Short WT) isoforms. Transcript levels were normalized to POLR2A and represented as fold change of expression relative to untransfected HEK293H cells. *P< 0.05; n.s., not significant.
Table 4.4: Potential roles and contribution of ZBTB20 and ZBTB20-regulated neuronal transcription factors to neurodevelopmental disorders

<table>
<thead>
<tr>
<th>Transcription factors</th>
<th>Physiological roles</th>
<th>References</th>
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<tr>
<td>ZBTB20</td>
<td>Deletions and mutations in ASD and ASD/ID; Affects dendritic spine morphology and dendritic arborization; Binds and represses genes involved in neuronal subtype specification in mice developing isocortex.</td>
<td>Molin et al., 2012; Nielsen et al., 2013; Wiśniowiecka-Kowalnik et al., 2013) and Rimsky et al., manuscript in preparation.</td>
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<tr>
<td>SOX5</td>
<td>Deletions and mutations in ID, speech delay, variable behavioral abnormalities</td>
<td>Lamb et al., 2012; Lee et al., 2013; Schanze et al., 2013) and Rimsky et al., manuscript in preparation.</td>
</tr>
<tr>
<td>TBR1</td>
<td>Mutations in patients with autisms; Mutations and deletions in patients with ID, developmental delay, ASD, speech disorder, seizures, brain malformations, hypotonia, and behavioral abnormalities; Regulates expression of AUTS2, a gene implicated in ID and autism; Binds XLID encoding protein CASK1</td>
<td>Bedogni et al., 2010; O’Roak et al., 2012; Traylor et al., 2012; Wang et al., 2004</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Deletions and mutations in patients with ID; Associated with ASD, epilepsy and behavioral abnormalities; Negative regulator of synapse number and function</td>
<td>Barbosa et al., 2008; Bienvenu et al., 2013; Le Meur et al., 2010; Li et al., 2008; Novara et al., 2010; Nowakowska et al., 2010; Paciorkowski et al., 2013; Zweier et al., 2010</td>
</tr>
<tr>
<td>FEZF2</td>
<td>Directly repressed by TBR1, implicated in autism; Regulates pyramidal morphology, dendritic patterning, and spine number; An SNP near 5' proximal region of FEZF2 is associated with ASD;</td>
<td>Chen et al., 2005; Han et al., 2011; Kwan et al., 2008; Leone et al., 2008; Molyneaux et al., 2007; Wang et al., 2009</td>
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<td>CUX1/ CUX2</td>
<td>Regulate dendritic branching, spine morphology, and synapses of the upper layer neurons of the cortex; Cux1 regulates ENGRAILED2 autism spectrum disorder-associated haplotype function</td>
<td>Choi et al., 2012; Cubelos et al., 2010; Li et al., 2010</td>
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<tr>
<td>SATB2</td>
<td>Disrupted and deleted in patients with ID microdeletion syndrome; Disrupted in a patient with ASD, developmental dyspraxia; Role in dendritic arborization in mice cerebral cortex</td>
<td>Marshall et al., 2008; Rosenfeld et al., 2009; Talkowski et al., 2012; Zhang et al., 2012</td>
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<tr>
<td>Transcription factors</td>
<td>Physiological roles</td>
<td>References</td>
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<td>RORB</td>
<td>Deleted in patients with ID, speech delay, epilepsy and characteristic facial features; 9q21.13 microdeletion syndrome; Genetic association with bipolar disorder; Genetic association with verbal intelligence</td>
<td>Boudry-Labis et al., 2013; Ersland et al., 2012; McGrath et al., 2009; Partonen, 2012</td>
</tr>
<tr>
<td>FOXP2</td>
<td>Mutations in patients with speech and language impairment; regulates gene networks implicated in neurite outgrowth in the developing brain</td>
<td>Lai et al., 2001; MacDermot et al., 2005; Vernes et al., 2011</td>
</tr>
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Transcription factor genes *MEF2C*, *TBR1* and *FEZF2* are repressed by ZBTB20 overexpression but unaffected by ASD and ID associated *ZBTB20* variants

In the previous section, overexpression of ZBTB20 long isoform resulted in the reduced expression of transcription factor genes *MEF2C* and *TBR1*, while overexpression of the ZBTB20 short isoform resulted in reduced expression of transcription factor gene *FEZF2* in HEK293H cells.

The effect of overexpression of the ASD associated ZBTB20 long isoform variant P46R and ASD and ID associated ZBTB20 short isoform variant G346V on the negatively correlated TF genes was measured in HEK293H cells. Both the ZBTB20 isoforms and the corresponding mutants were overexpressed in HEK293H cells and their protein expression was observed by Western blot analysis using anti-V5 antibody (Figure 4.3). Overexpression of ZBTB20 long isoform mutant P46R in HEK293H cells failed to repress the expression of transcription factor genes *MEF2C* (Figure 4.4A) and *TBR1* (Figure 4.4B). Also, the ZBTB20 short isoform mutant G346V showed no change in the expression of the transcription factor gene *FEZF2* repressed by the overexpression of wild type ZBTB20 short isoform as shown in Figure 4.4C. Thus the ASD and or ID associated ZBTB20 variants showed expression similar to the wild type, indicating lack of repressor activities.
Figure 4.3: ASD and ID associated ZBTB20 variants in the long and short isoforms of ZBTB20

A. ASD and ID associated ZBTB20 protein variants, P46R in long isoform and G346V in short isoform (G419V in long isoform) are shown. The BTB domain (BTB) and five zinc fingers (ZnFs) are present in both ZBTB20 short and long isoforms. A region specific to the long isoform that extends from amino acid 1 to 73 amino acids is indicated (orange box).

B. ZBTB20 protein expression, in HEK293H cells overexpressing V5-tagged ZBTB20 long WT, ZBTB20 long P46R, ZBTB20 short WT and ZBTB20 short G346V. Anti-V5 antibody was used to detect long (black arrow) and short isoform (blue arrow) wild type and mutant ZBTB20 in Western blot analysis.
Figure 4.4: Effect of overexpression of ZBTB20 wild type and mutants on endogenous RNA levels of the indicated transcription factor genes.
Figure 4.4: Effect of overexpression of ZBTB20 wild type and mutants on endogenous RNA levels of the indicated transcription factor genes

Endogenous RNA levels of A. *MEF2C*; B. *TBR1* were quantified by quantitative reverse-transcription PCR in HEK293H cells overexpressing human ZBTB20 long isoform (ZBTB20 Long WT) and ZBTB20 P46R mutant. Endogenous RNA levels of C. *FEZF2* were also quantified by quantitative reverse-transcription PCR in HEK293H cells overexpressing human ZBTB20 short isoform (ZBTB20 WT) and mutant ZBTB20 G346V. Transcript levels were normalized to *POLR2A* and represented as fold change of expression relative to untransfected HEK293H cells. *P* < 0.05; n.s., not significant.
Figure 4.5: Schematic representation of ZBTB20-dependent transcription repression of neuronal transcription factor genes and their association with neurodevelopmental disorders

ZBTB20 acts as a master-regulator of transcription through repressing several neuronal transcription factors, including TBR1, SOX5, FOXP2, CUX1/CUX2, RORB, ZFPM2, FEZF2, and SATB2 (lines in black; Nielsen et al., 2013; Present study). These transcription factors bind and repress additional transcription factors (dashed lines; See Table 4.4 for details and references) and regulate target genes (lines in grey; References in Table 4.4).
Discussion

In this chapter, the co-expression of human ZBTB20 gene with several neuronal transcription factor genes that are associated with neurodevelopmental disorders including autism spectrum disorders, intellectual disability, and speech delay (Table 4.4, Figure 4.5) was determined. The ZBTB20 gene expression in brain was found to be largely negatively correlated with transcription factor genes enriched in Gene Ontology terms including synapse, postsynaptic membrane and synaptic transmission. In this study, overexpression of human ZBTB20 gene in HEK293H cells resulted in the repression of neuronal transcription factor genes TBR1, MEF2C, and FEZF2, previously found to be associated with neurodevelopmental disorders (Table 4.4).

Various studies in mouse brain have shown that murine Zbtb20 is expressed during early differentiation of the CA1 and CA3 pyramidal neurons of the Ammon’s horn and granule cells of the dentate gyrus in the developing hippocampus (Mitchelmore et al., 2002). Zbtb20 expression was strictly confined to the developing hippocampal neurons in the mouse brain. On the other hand, the neighboring neocortical and transitional areas adjacent to the hippocampus are conspicuous by the absence of Zbtb20 expression. Further, Zbtb20 plays an important role in determining the hippocampal CA1 pyramidal neuron identity (Nielsen et al., 2007; Rosenthal et al., 2012; Xie et al., 2010) in the mouse brain.
Zbtb20 represses neuronal subtype-specific neocortical transcription factor genes in the mice hippocampus (Nielsen et al., 2010; Nielsen et al., 2013). Using chromatin immunoprecipitation (ChIP) combined with multi-parallel sequencing (Chip-Seq) Nielsen and coworkers (2013) have shown that murine Zbtb20 binds neuronal transcription factor genes, including *Cux1*, *Cux2*, *Fezf2*, *Foxp2*, *Mef2c*, *Rorb*, *Satb2*, *Sox5*, *Tbr1*, *Tle4* and *Zfpm2*. These transcription factor genes control neuronal subtype specification in the developing neocortex. They further showed that Zbtb20 represses these genes during CA1 pyramidal neuron development in *Zbtb20* transgenic mice. Thus murine Zbtb20 suppresses neocortical cell fate transitions and ensures commitment to hippocampal CA1 pyramidal neuron fate by repressing neocortical transcription factor genes in the developing projection neurons. These findings suggest the potential role of human ZBTB20-dependent transcription regulation in the developing brain.

Recent research on neocortical development has emphasized the role of transcription factors in neuronal identity, neuronal migration and neuronal wiring (Kwan et al., 2012). There are different subclasses of projection neurons in the neocortical deep layers. Specification of projection neurons in deep layer of neocortex requires a network of transcription factors. These transcription factors repress other transcription factor genes of alternative deep-layer fates. Since autism spectrum disorders develop very early in life and impair the functioning of the neocortex, any sort of abnormal neocortical circuit miswiring has been
hypothesized to result in autism spectrum disorders (Kwan et al., 2012). Tbr1, Sox5, Fezf2 and Satb2 are some of the neocortical layer specific transcription factors that are found to be critical for neuronal identity, connectivity and axonal projections and are implicated in autism spectrum disorders as shown in Table 4.4. Thus the disruption of these neuronal transcription factor genes could result in neurodevelopmental disorders including autism spectrum disorders and intellectual disability.

Using quantitative RT-PCR to measure the endogenous expression of transcription factor genes in HEK293H cells overexpressed with ZBTB20 long isoform, the expression of T-box containing transcription factor gene, TBR1 was found to be repressed. TBR1 is involved in laminar identity and axonal migration in early brain cortical development and has been implicated in autism (O’Roak et al., 2012). It regulates the expression of AUTS2, a gene implicated in ASD (Bedogni et al., 2010). TBR1 has also been observed to bind an X-linked intellectual disability (XLID) gene CASK (Wang et al., 2004). Patients with deletions in the 2q24 region encompassing the TBR1 gene have been found to be associated with severe speech and language difficulties, autism-like behavioral problems and moderate to severe ID (Traylor et al., 2012). Moreover many studies have also identified larger deletions encompassing the TBR1 gene (Krepischi et al., 2010; Magri et al., 2011; Palumbo et al., 2012; Takatsuki et al., 2010;Traylor et al., 2012). The common phenotypic features shared by all these
TBR1 gene deletion cases were absent or delayed speech and language as well as ID. Single nucleotide variations of TBR1 have been reported in sporadic ASD cases with or without ID (O’Roak et al., 2012; O’Roak et al., 2012).

Besides TBR1, the expression of MEF2C was also significantly repressed in HEK293H cells overexpressing ZBTB20 long isoform. Various studies have shown the involvement of MEF2C in neurogenesis, neuronal migration and differentiation and its association with ASD and ID (Table 4.4). Deletions in MEF2C have been reported in patients with ID as well as ASD (Novara et al., 2010; Nowakowska et al., 2010; Zweier et al., 2010). Moreover, MEF2C gene deletion associated with ID, autistic features, epilepsy and abnormal hand movements is being increasingly recognized as a neurodevelopmental syndrome (Paciorkowski et al., 2013). MEF2C has also been shown to be involved in regulation of synapse number and function (Flavell et al., 2006). Expression of Mef2C has also been shown to be upregulated in mice deficient in MECP2 gene, implicated in Rett syndrome (Li et al., 2008).

Another neuronal transcription factor gene FEZF2, implicated in autism spectrum disorder (Sanders et al., 2012; Wang et al., 2009) and neuronal development (Chen et al., 2005) is down-regulated by overexpression of the short isoform ZBTB20 in HEK293H cells. FEZF2 is directly repressed by TBR1 (Chen et al., 2005) and has been shown to regulate pyramidal neuron morphology, dendritic
patterning and dendritic spine number (Han et al., 2011; Kwan et al., 2008; Leone et al., 2008; Molyneaux et al., 2007).

The genetic alterations in the ZBTB20 gene along with the transcription factor genes regulated by the ZBTB20 protein has been shown to play an important role in ASD and ID. Since ZBTB20 regulates the expression of several of these neuronal transcription factor genes, it could potentially play the role of a master regulator as shown in Figure 4.5. It is hypothesized that the coordinated expression of the various neuronal genes is critical for homeostasis and a slight imbalance of any of these well-coordinated transcription factor genes could result in neurodevelopmental disorders (Ramocki and Zoghbi, 2008; Toro et al., 2010).

The expression of the ZBTB20 gene is largely negatively correlated with genes implicated in transcription regulation. Many neuronal transcription factor genes enriched in roles associated with neurodevelopment are negatively correlated with the expression of ZBTB20 gene in the ZBTB20 co-expressed gene list. Two of these neuronal transcription factor genes MEF2C and TBR1 previously shown to be associated with ASD and ID were found to be repressed in HEK293H cells overexpressed with the ZBTB20 long isoform. Moreover, overexpression of the ZBTB20 short isoform in HEK293H cells resulted in reduced FEZF2 expression. It is intriguing that many neuronal transcription factor genes found to repressed in Zbtb20 transgenic mice during ectopic CA1 pyramidal neuron development were
not repressed in the ZBTB20 overexpressed HEK293H cells. One possible reason to explain these findings could be that various transcription factors are expressed in a neuronal subtype-specific as well as neocortical layer-specific manner, thus it might be difficult to reproduce the effect of ZBTB20-mediated repression in mammalian cell lines like HEK293H. In the future, neuronal cell lines could be used to study the effect of ZBTB20-dependent regulation of the neuronal transcription factor genes associated with ASD and ID.
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CHAPTER 5

ZBTB20 siRNA KNOCKDOWN

Introduction

The ZBTB20 gene is located at chromosomal position 3q13.2 (Zhang et al., 2001) and consists of 10 exons spanning 880 kb of genomic DNA. The ZBTB20 protein contains the BTB/POZ (Broad Complex, Tramtrack, Bric a Brac/ Poxvirus and zinc finger) domain at the N-terminal and five C2H2 Kruppel-type-zinc fingers at the C-terminal.

The ZBTB20 gene was identified as a strong candidate gene for autism spectrum disorders (ASD) and intellectual disability (ID). One chromosomal copy of the ZBTB20 gene was found to be physically disrupted by a t(3;12) translocation in a patient with autistic features and developmental delay. Two missense ZBTB20 mutations unique to ASD and or ASD/ID and a de novo intragenic deletion encompassing the ZBTB20 gene have been identified in a patient with borderline intelligence, impulsivity and attention deficit. Recently several cases with microdeletions in the region of 3q13.31 have been identified in microdeletion syndrome patients encompassing the ZBTB20 gene. Variable clinical features such as attention deficit, autistic features, intellectual disability
and developmental delay have been reported and provide further evidence of a likely involvement of ZBTB20 gene in the clinical spectrum of neurodevelopmental disorders such as ASD and ID (Rimsky et al., manuscript in preparation; Molin et al., 2012; Vuillaume et al., 2013; Wisniowiecka-Kowalnik et al., 2013).

Murine Zbtb20 has been well studied using both overexpression and knockout experiments to understand its physiological functions. Transgenic mice with overexpression of the Zbtb20 gene in hippocampal subiculum as well as non-hippocampal areas show hippocampus-like corticoneurogenesis and behavioral abnormalities (Nielsen et al., 2007). Furthermore, Zbtb20 knockout mice provide strong evidence of a role for the Zbtb20 protein in hippocampal development, especially in determination of CA1 field identity of the hippocampus. Absence of Zbtb20 resulted in transformation of the CA1 field to a neocortex-like structure and reduction in size of the hippocampus (Xie et al., 2010). Nielsen and coworkers (2013) demonstrated that the Zbtb20 directly binds and represses several neocortical layer-specific transcription factors genes including Myocyte-specific enhancer factor 2C (Mef2c) and T-box brain 1 (Tbr1). Murine Zbtb20-regulated neuronal transcription factor genes have been shown to be associated with neurodevelopmental disorders, including autism spectrum disorder and intellectual disability (Bienvenu et al., 2013;
Nowakowska et al., 2010; O’Roak et al., 2012; Traylor et al., 2012; Zweier and Rauch, 2012).

Since murine Zbtb20 regulates the expression levels of many neurodevelopmental disorder-associated transcription factor genes, we hypothesized that human ZBTB20 may also function as a regulator of these transcription factor genes. A slight imbalance in the expression of any of the neuronal transcription factor genes associated with neurodevelopmental disorders, including ASD and ID, could result in the clinical presentation of the phenotype. Since little is known about human ZBTB20 transcription factor-regulated target genes, we used small interfering RNA (siRNA) to knockdown the ZBTB20 gene expression in two human cells lines to study the effect on ZBTB20-regulated target genes.

Material and Methods

HEK293H cell culture

HEK293H (Human Embryonic Kidney cell line 293H) were cultured using standard protocols. HEK293H cells were maintained in cell media consisting of Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10%
fetal bovine serum (FBS; Atlanta Biologicals), 1% penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma) and maintained at 37°C and 5% CO₂.

Undifferentiated ReNcell VM, a human neural progenitor cell culture

ReNcell VM (Millipore) is neural progenitor cell line derived from human ventral mesencephalon brain tissue. ReNcell VM cells were plated on 60 mm dishes that were pre-coated with laminin (Sigma) in DME/F12 (Millipore) for at least 4 hours at 37°C. The undifferentiated ReNcell VM cells were maintained in the ReNcell maintenance media (Millipore) supplemented with growth factors including fibroblast growth factor (FGF, Millipore) and epidermal growth factor (EGF Millipore) added to the growth media at a concentration of 20 ng/ml each. Cells were detached from the plate using Accutase (Millipore), subcultured every two days and maintained at 37°C and 5% CO₂.

HEK293H siRNA transfection

Approximately a half million HEK293H cells were plated on laminin pre-coated 60 mm dishes in HEK293H cell media (described above) without the antibiotics penicillin/streptomycin for 18-20 hours at 37°C and 5% CO₂. ZBTB20 siRNA (ON-TARGETplus ZBTB20 siRNA; Thermo Scientific) or control siRNA (ON-TARGETplus Non-targeting Pool; Thermo Scientific) suspended in antibiotic-
free media were mixed with the transfection reagent (DharmaFECT; Thermo Scientific). The siRNA and the transfection reagent suspended in antibiotic-free media were incubated for 20 minutes at room temperature and transfected in the HEK293H cells. The transfected HEK293H cells were harvested at 24 hours for RNA analysis and 48 hours for protein analysis. The siRNA sequences in the ON-TARGETplus Human ZBTB20 siRNA SMARTpool are:
GCAAUUCGUGCUCGAGAC, CGACAAGAGCGUCCUACAA, ACACAGGAGUGAGGGCAUA and GCAUGUGUCUGACGGAUAA.

ReNcell VM siRNA transfection

Approximately a half million ReNcell VM cells were plated on laminin pre-coated 60mm dishes in DME/F12 antibiotic-free media and incubated at 37°C and 5% CO₂ overnight. The old media was then replaced with fresh DME/F12 antibiotic-free media. ZBTB20 siRNA (ON-TARGETplus ZBTB20 siRNA; Thermo Scientific) or control siRNA (ON-TARGETplus Non-targeting Pool; Thermo Scientific) was transfected along with the DharmaFECT (Thermo Scientific) transfection reagent according to the manufacture’s protocol.
RNA extraction and qRT-PCR

The ReNcell VM cells were detached from the plates for RNA extraction using Accutase (Millipore), while HEK293H cells were detached using Trypsin + EDTA (Sigma), 24 hours post-transfection. Cells were suspended in RNA lysis buffer (Sigma) and 2-Mercaptoethanol (BME; Sigma) and subjected to RNA extraction using the GenElute Mammalian Total RNA Miniprep Kit (Sigma) protocol. The RNA was cleaned using Turbo DNA-free DNase (Ambion Life Technologies-Invitrogen) kit followed by precipitation using the lithium chloride precipitation solution (Ambion Life Technologies-Invitrogen).

Quantitative RT-PCR was performed using iScript One-Step RT-PCR kit with SYBR Green (BioRad) and the following ZBTB20 forward and reverse primers were used to generate a 136 base pair product:

5'-ATGTTCGTACACACAGGTGAGAAG-3' and
5'-GCTTGTGTGAGATACACTACTGTG-3'. Normalized expression ΔΔC(t) was determined using POLR2A as a reference gene in regression mode and plotted using Bio-Rad CFX manager software. The experiments were repeated three times and the p value between untransfected and siRNA transfected cells was calculated using the Student’s t-test.
Protein lysates and Western blot analysis

Protein was extracted from HEK293H cells and VM cells using the NP40 lysis method. Cells were washed with ice cold phosphate buffered saline and incubated in 1%NP-40 lysis buffer (1%NP-40; 150 mM NaCl; 50 mM Tris pH 8.0) and protease inhibitor cocktail (Sigma) for approximately 10 minutes on ice to dissociate the cells. The cells were sonicated 3 times at 10% amplitude for 2 seconds each followed by centrifugation. The protein concentration was measured using coomassie (Bradford) protein assay reagent. The protein lysate was boiled for 3-5 minutes in SDS sample buffer. The protein was separated on a SDS-PAGE gel, transferred on the membrane and then probed with rabbit anti-ZBTB20 antibody (Aviva Systems Biology) at 1:1000 dilution in 5% milk and 2% Bovine serum albumin (BSA; Sigma) in Tris Buffered Saline with Tween 20 (TBST) overnight at 4°C and mouse anti-GAPDH (Santa Cruz) in 2% BSA TBST at 1:5000 dilution for 1 hour at room temperature, used as loading control.
Results

ZBTB20 gene knockdown in HEK293H and ReNcell VM cells

To determine the effect of reduced expression of the ZBTB20 gene on other genes in the human cell, we performed ZBTB20 knockdown in two human cell lines: HEK293H, a human embryonic kidney cell line and ReNcell VM, a human neural progenitor cell line. The goal of this study is to look for the effect of reduced expression of the ZBTB20 gene on other ZBTB20-regulated target genes including ASD and ID implicated neuronal transcription factor genes.

The ZBTB20 gene is expressed both in HEK293H cells, as well as in ReNcell VM cells. A pool of 4 human ZBTB20-specific siRNA was used to knockdown the ZBTB20 gene expression. Using a gradient of ZBTB20 siRNA concentrations, transfection reagent as well as cell densities, the level of ZBTB20 knockdown in both HEK293H and ReNcell VM cells was measured.

Using 25 nM of ZBTB20 siRNA in 0.5X 10^6 HEK293H cells, approximately 50% knockdown was achieved at the RNA level as shown in Figure 5.1A. The same concentration of non-targeting siRNA (negative control) did not show ZBTB20 knockdown, similar to untreated cells, indicating specificity of ZBTB20 knockdown. Approximately 50% ZBTB20 knockdown was observed only in
HEK293H cells transfected with the ZBTB20 siRNAs, while ZBTB20 expression remained unchanged in both untransfected cells as well as in cells treated with the non-targeting negative control.

To investigate the effect of ZBTB20 knockdown at the protein level, both treated and untreated HEK293H cell lysates were prepared to detect the level of ZBTB20 protein by Western blot analysis. We used a ZBTB20-specific antibody to detect the protein in total protein lysates in ZBTB20 siRNA-treated, non-targeting control-treated and untreated HEK293H cells. The level of ZBTB20 protein in ZBTB20 siRNA-treated HEK293H cells was reduced approximately 50% as compared to non-targeting control-treated protein lysate or untreated HEK293H cells (Figure 5.1B).
Figure 5.1: ZBTB20 knockdown in HEK293H cells using ZBTB20 siRNA
**Figure 5.1: ZBTB20 knockdown in HEK293H cells using ZBTB20 siRNA**

A. Quantitative RT-PCR analysis of ZBTB20 transcript levels and B. Western blot analysis of ZBTB20 protein in ZBTB20 siRNA pool-treated, non-targeting control- treated and untreated HEK293H cells. ZBTB20 transcript levels were normalized to POLR2A and represented as fold change of expression relative to untreated HEK293H cells. *P* < 0.05. Western blot analysis was performed using anti-ZBTB20 antibody. Black arrow indicates endogenous ZBTB20 and blue arrow indicates GAPDH.
Since \textit{ZBTB20} is highly expressed in the hippocampal primordium of developing mouse brain (Mitchelmore et al., 2002) as well as in the human fetal hippocampus (Nielsen et al., 2013, Rimsky et al., Manuscript in preparation) we chose ReNcell VM, a human neural progenitor cell line for our knockdown experiments. We transfected $0.5 \times 10^6$ ReNcell VM cells plated on a 60 mm dish with 75 nM of \textit{ZBTB20} siRNA pool using DharmaFECT transfection reagent. Approximately 50\% knockdown was achieved in the \textit{ZBTB20} siRNA treated ReNcell VM cells, while both control siRNA-treated and untreated ReNcell VM cells did not show reduced \textit{ZBTB20} RNA expression (Figure 5.2)

![Normalized Fold Expression of ZBTB20 Relative to Untreated](image)

\textbf{Figure 5.2: ZBTB20 knockdown in ReNcell VM cells using ZBTB20 siRNA}
Figure 5.2: ZBTB20 knockdown in ReNcell VM cells using ZBTB20 siRNA

Quantitative RT-PCR analysis of ZBTB20 transcript levels in ZBTB20 siRNA SMARTpool treated, non-targeting control treated and untreated ReNcell VM. Transcript levels were normalized to POLR2A and represented as a fold change of expression relative to untreated ReNcell VM.
Discussion

In this study, we knocked down the expression of the ZBTB20 gene in two human cell lines, HEK293H and undifferentiated ReNcell VM cells. RNA isolated from the ZBTB20 gene knockdown human cell lines will be useful in the identification of genes both upregulated and down-regulated as a result of ZBTB20 gene knockdown.

ZBTB20 siRNA SMARTpool used to knockdown ZBTB20 gene expression consists of four 19-nucleotide double-stranded RNA. The RNAi induced silencing complex (RISC) in the cell guides the siRNAs to the target RNA sequence, in this case the last two coding exons of the ZBTB20 gene. The argonaute protein, which is part of the RISC complex, unwinds the double-stranded siRNA, such that only the antisense strand remains bound to the argonaute protein (Dykxhoorn et al., 2003). Finally, the antisense strand together with the RISC complex directs the degradation of the complementary ZBTB20 mRNA.

The ZBTB20 mRNA expression level was knocked down by approximately 50%, measured by quantitative RT-PCR. Studies done in Zbtb20 knockout mice have shown that the murine Zbtb20 gene is essential for survival and postnatal growth. Zbtb20 knockout mice show postnatal lethality and do not survive for
more than 12 weeks of age (Sutherland et al., 2009). Moreover, ZBTB20 RNA expression is reduced by approximately 50% in patients having a ZBTB20 gene deletion. Thus the siRNA mediated 50% ZBTB20 gene knockdown that has been achieved in this study is similar to the ZBTB20 RNA expression level in ZBTB20 deletion patients.

We will perform a human transcriptome analysis using the HEK293H and ReNcell VM ZBTB20 knockdown RNA samples. We will use the GeneChip® Human Transcriptome Array 2.0 (Affymetrix) to identify the genes differentially expressed in ZBTB20 knockdown samples with respect to untransfected cells. The GeneChip Human Transcriptome Array 2.0 is a commercial microarray, which is designed to provide human transcriptome analysis at high-resolution, even for less abundantly expressed genes. Identifying the ZBTB20-regulated target genes in human cells would help us understand the complex interplay between these genes leading to the onset of neurodevelopmental disorders including ASD and ID. This information would also complement the results from the ZBTB20 overexpression analysis done in human embryonic kidney cells.


CONCLUSIONS

The ZBTB20 gene has been found to be associated with autism spectrum disorders (ASD) and intellectual disability (ID) in our laboratory. It has also been identified as a candidate gene for developmental delay and other clinical features observed in the 3q13.31 microdeletion and microduplication syndrome. The mechanisms underlying the functional role of the BTB (Broad Complex, Tramtrack, Bric-a-Brac) -zinc finger containing transcription factor, ZBTB20 in ASD and ID are still not fully understood. While the role of murine Zbtb20 is better characterized, the functional properties and pathways underlying human ZBTB20 are just beginning to be unraveled. The purpose of this study was to functionally characterize the human ZBTB20 protein to elucidate its contribution to ASD and ID. The work presented here has identified potential molecular links by which the ZBTB20 protein functions in mammalian cells and has also provided insight into molecular mechanisms and functional pathways underlying ASD and ID.

The first chapter provides a detailed review about the ZBTB20 protein, its domains and their known functions. The physiological functions of the murine Zbtb20 transcription factor protein, the murine Zbtb20-regulated target genes and its associated biological processes have been described. Analyzing the functional roles of Zbtb20 in mice has enabled a better understanding about the
possible roles of the human ZBTB20 protein. Further, the post-translational modification SUMOylation and its biological functions have been described. Finally, the possible roles of SUMOylation on neuronal proteins were described, which should help understand the possible effects of SUMOylation on ZBTB20.

The second chapter describes the functional characterization of the human ZBTB20 in mammalian cell lines. In mammalian cells, ZBTB20 localizes primarily in the nucleus and the long- and short- isoform ZBTB20 proteins form homodimers and heterodimers. Moreover, the N-terminal region of ZBTB20 containing the BTB domain is found to be critical for its dimerization. Using a yeast two-hybrid screen with ZBTB20 long isoform as bait, eight novel proteins that interact with ZBTB20 were identified. Further functional analysis of the ZBTB20-interacting proteins in mammalian cells could help define the molecular mechanisms underlying the physiological functions of ZBTB20 in the brain.

The third chapter describes the SUMOylation of ZBTB20 and the functional effects of ZBTB20-SUMO1 conjugation. The interaction of ZBTB20 with E2 SUMO conjugating enzyme UBC9 and SUMO1 in human embryonic kidney cells has been studied. Moreover, the effects of ZBTB20 SUMOylation on its stability, intracellular localization, repression function and dimerization have been examined. The interaction of ZBTB20 with SUMO1 and UBC9 has opened new
areas of research into the role of ZBTB20 SUMOylation on neuron development in brain and other ZBTB20-regulated biological processes.

The fourth chapter describes the ZBTB20-dependent transcription regulation of coexpressed neuronal transcription factor genes \( \text{MEF2C, TBR1 and FEZF2} \), previously found to be associated with ASD and ID. The expression of neuronal transcription factor genes \( \text{MEF2C, TBR1 and FEZF2} \) was significantly reduced in HEK293H cells overexpressing either the long or the short isoform ZBTB20 protein. Future studies of various neuronal transcription factor genes regulated by human ZBTB20 protein would provide new insight into the development of ASD and ID.

Finally, the fifth chapter describes the siRNA-mediated knockdown of \( \text{ZBTB20} \) in two human cell lines, HEK293H cells and the ReNcell VM cells, a human neural progenitor cell line. The \( \text{ZBTB20} \) knockdown RNA sample will be used to analyze the expression of genes regulated by ZBTB20.

The ZBTB20 functional studies presented here have helped to further the understanding of the functional role of human ZBTB20 and SUMO1 modified ZBTB20 in mammalian cells. ZBTB20 K330 residue, which is part of the SUMOylation consensus sequence, was shown to play a role in ZBTB20 homodimerization. It is possible that SUMOylation facilitates protein-protein
interaction of ZBTB20. In fact, loss of ZBTB20 SUMOylation could affect the interaction of ZBTB20 with zinc finger proteins ZMYM5 and ZMYM2, which are part of the transcriptional corepressor complex. Proteins containing the BTB domain frequently interact with components of the transcriptional corepressor complex. These transcriptional corepressor proteins associate with histone deacetylase complex and other corepressor proteins that are involved in chromatin modification. In this work, ZBTB20 coexpression analysis has revealed significant negative correlation with NCOR2 (nuclear receptor co-repressor-2), which is a component of the histone deacetylase complex. ZBTB20 expression is also negatively correlated with SWI/SNF, components of the chromatin-remodeling complex as well as other neuronal transcription factor proteins. Thus ZBTB20 could potentially interact with co-repressors and other proteins to cause repression of gene expression by modifying chromatin, consistent with the role of BTB containing proteins.

ZBTB20 interacting proteins and ZBTB20 regulated target genes will be further investigated using RNA isolated from ZBTB20 gene knockdown in HEK293H and ReNcell VM cells. Identification of ZBTB20-regulated genes would help in delineating the molecular mechanisms and pathways underlying ZBTB20 function. Moreover, studies showing ZBTB20-dependent transcription regulation could provide further evidence supporting the role of ZBTB20 in neurodevelopmental disorders.