

The role of *Pax6* in brain development and its impact on pathogenesis of autism spectrum disorder

Takako Kikkawa^a, Cristine Casingal^a, Seung Hee Chun^a, Hiroshi Shinohara^b, Kotaro Hiraoka^c, and Noriko Osumi^a

^aDepartment of Developmental Neuroscience, United Center for Advanced Research and Translational Medicine (ART), Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

^bDepartment of Histology and Neuroanatomy, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan

^cDivision of Cyclotron Nuclear Medicine, Cyclotron and Radioisotope Center, Tohoku University, 6-3, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan

*Corresponding author.

Noriko Osumi, PhD

Department of Developmental Neuroscience,
United Center for Advanced Research and Translational Medicine (ART),
Tohoku University Graduate School of Medicine,
2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

Tel.: +81 22 717 8201.

Fax: +81 22 717 8205.

E-mail address: osumi@med.tohoku.ac.jp (N. Osumi)

Abstract

Pax6 transcription factor is a key player in several aspects of brain development and function. Autism spectrum disorder (ASD) is a neurodevelopmental disorder in which several loci and/or genes have been suggested as causative candidate factors. Based on data obtained from meta-analyses of the transcriptome and ChIP analyses, we hypothesized that the neurodevelopmental gene *PAX6* regulates and/or binds to a large number of genes (including many ASD-related ones) that modulate the fate of neural stem/progenitor cells and functions of neuronal cells, subsequently affecting animal behavior. Network analyses of *PAX6*/ASD-related molecules revealed significant clusters of molecular interactions involving regulation of cell-cell adhesion, ion transport, and transcriptional regulation. We discuss a novel function of Pax6 as a chromatin modulator that alters the chromatin status of ASD genes, thereby inducing diverse phenotypes of ASD and related neurodevelopmental diseases.

Keywords

Pax6, autism, transcriptional regulation

Abbreviations

ASD, autism spectrum disorder; ChIP, chromatin immunoprecipitation; CP, cortical plate; Di, diencephalon; dTel, dorsal telencephalon; EGL, external granular layer; GO, Gene Ontology; HB, hindbrain; IGL, internal granular layer; MB, midbrain; MGI, Mouse Genome Informatics; MRI, magnetic resonance imaging; Pax, paired box; PCL, Purkinje cell layer; RG cells, radial glial cells; SFARI, Simons Foundation Autism Research Initiative; vTel, ventral telencephalon; VZ, ventricular zone; WAGR, Wilms tumor in the kidney, Aniridia in the eye, Genital ridge defect, and mental Retardation.

Introduction

Pax6 is one of the key transcription factors in brain development and was originally cloned as a member of the paired box (Pax) family based on its homology to the *Drosophila* gene *paired* (Walther and Gruss, 1991). Pax6 contains two DNA binding domains: a *paired box*-domain and a homeodomain (Kessel and Gruss, 1990; Treisman et al., 1991; Walther et al., 1991). The paired domain is subdivided into two DNA binding domains, the PAI and the RED motifs (Cai et al., 1994; Czerny et al., 1993; Jun and Desplan, 1996; Treisman et al., 1991; Xu et al., 1995). The molecular structure of Pax6 is well conserved, and the functions of Pax6 are interchangeable across species; the fly *eyeless*, mouse Pax6 and human PAX6 genes can all induce compound eyes in the imaginal discs of the fly (Halder et al., 1995).

Pax6 has crucial roles not only in eye formation but also in brain development (Fig. 1A). There are many studies on neurogenesis of the cortex, and brain structure has increased in size and complexity throughout mammalian evolution (see review by (Osumi et al., 2008)). These changes may be due to the role of Pax6 in balancing proliferation and differentiation of neural stem/progenitor cells (Estivill-Torrus et al., 2002; Fukuda et al., 2000; Gotz et al., 1998; Heins et al., 2002; Warren et al., 1999). Because Pax6 is a transcription factor, it regulates expression of many genes during corticogenesis. Recent genome-wide transcriptomic analyses have identified a few thousands of Pax6 downstream genes that are involved in cortical development (Kikkawa et al., 2013; Sansom et al., 2009; Sun et al., 2015; Walcher et al., 2013; Xie et al., 2013).

If corticogenesis drastically fails, microcephaly will develop. Similarly, subtle abnormalities occur in neural development, resulting in neurodevelopmental disorders, such as intellectual disability, attention deficit-hyper activity disorder, and autism spectrum disorder (ASD). Many loci and/or genes have been suggested as potential causative factors for ASD. Since PAX6 is one of the responsible genes for ASD (see below, (Davis et al., 2008; Maekawa et al., 2009; Yamamoto et al., 2014)), Pax6 regulatory molecular networks might be involved in pathogenesis of ASD. In this review, we discuss the involvement of PAX6 in the onset of neurodevelopmental diseases based on

meta-analyses of comprehensive datasets of Pax6 downstream and Pax6-binding genes.

Expression patterns and functions of *Pax6* in the brain

Many studies in rodents have identified brain regions in which Pax6 is expressed in the embryonic and/or adult stages (see (Georgala et al., 2011; Hiraoka et al., 2016; Manuel et al., 2015; Osumi et al., 2008) and the references therein). Pax6 expression is first activated around the time of neural plate induction (Inoue et al., 2000). In the neural tube, Pax6 is expressed in neural stem and progenitor cells, i.e., radial glial (RG) cells. Pax6 plays an important early role in establishing specific brain territories, including the forebrain/midbrain border and the border between the dorsal and ventral telencephalon (i.e., the cerebral cortex and basal ganglia, respectively) (Fig. 1B). In the developing neocortex, Pax6 expression turns off in differentiated neurons; thus, the Pax6-positive nuclear layer demarcates the ventricular zone from the cortical plate (Fig. 1C). Loss of Pax6 function causes premature neurogenesis in the neocortex, resulting in a net reduction in the number of neurons (Estivill-Torres et al., 2002; Fukuda et al., 2000). The demarcation of brain territories based on Pax6 expression is also used to track the migration of specific neurons such as “lot cells”, which subsequently guide mitral cells to project from the olfactory bulb to the tubercles (Tomioka et al., 2000). In the hindbrain and spinal cord, Pax6 plays an essential role in establishing progenitor domains for somatic motor neurons (Ericson et al., 1997; Osumi et al., 1997; Takahashi and Osumi, 2002).

In the developing brain, Pax6 is also expressed robustly in specific regions, including the preoptic area, the olfactory neuroepithelium, the ventral thalamus, the internal germinal layer of the dorsal thalamus and epithalamus, the anterior amygdaloid area, the nucleus raphe dorsalis, and the cochlear, vestibular, and hypoglossal nuclei (Duan et al., 2013; Stoykova and Gruss, 1994). In the amygdaloid complex, Pax6 is required for the specification and development of the lateral, basolateral, and basomedial nuclei (Soma et al., 2009; Tole et al., 2005). Pax6 also plays a key role in establishing specific excitatory and inhibitory neuronal subpopulations in the amygdala (Cocas et al., 2011). Pax6 is expressed in the developing diencephalon (Stoykova and Gruss,

1994), and development of the thalamocortical tract requires Pax6 expression (Kawano et al., 1999; Pratt et al., 2000; Pratt et al., 2002; Simpson et al., 2009). In the developing midbrain, Pax6 plays a pathfinding role in dopaminergic projections from the substantia nigra (SN) and ventral tegmental area (VTA) (Vitalis et al., 2000). In the developing cerebellum, Pax6 is expressed in granule cells in the external granular layer (EGL); these cells differentiate and migrate radially across the Purkinje cell layer (PCL) to the internal granular layer (IGL) (Fig. 1D). Pax6 regulates the migration of post-mitotic neurons from the rhombic lip to form the granule cell layers in the cerebellum and precerebellar nuclei, including the pontine nucleus (Benzing et al., 2011; Engelkamp et al., 1999). Pax6 also regulates the polarization of cerebellar granule cells during parallel fiber formation (Yamasaki et al., 2001).

In the adult rodent brain, Pax6 expression persists in the subventricular zone of the lateral ventricle and in the subgranular zone of the dentate gyrus in the hippocampus (Hevner et al., 2006; Kohwi et al., 2005; Maekawa et al., 2005; Nacher et al., 2005). Pax6 expression is detected in neurons within the olfactory bulb, the amygdala, the pallidum, the thalamus, the hypothalamus, the midbrain, and the cerebellum (Haba et al., 2009; Hack et al., 2005). A recent study by Duan et al. (2013) reported that nearly 40% of Pax6-positive cells in the cortex are neurons. Dorsal telencephalon-derived cortical excitatory neurons are negative for Pax6. However, Pax6 is expressed not only in the dorsal telencephalon but also in the caudal ganglionic eminence (CGE), which produces cortical interneurons (Tang et al., 2012). Thus, Pax6-positive neurons in the cortex may be the CGE-derived inhibitory interneurons. Therefore, Pax6 has several important functions, including the specification, differentiation, and migration of neurons; Pax6 also likely plays a role in the maintenance of neurons, as well as in the pathfinding of neuronal circuits via an indirect mechanism. Notably, Pax6 is expressed weakly in astrocytes throughout the entire central nervous system, where it appears to play a role in astrocyte maturation (Sakurai and Osumi, 2008). Importantly, these patterns of Pax6 expression in rodents appear to be conserved within the human brain as well (Terzic and Saraga-Babic, 1999).

Recently, Miller and colleagues performed a comprehensive

transcriptome study combined with ultra-high-resolution magnetic resonance imaging (MRI) in the brains of human fetuses at mid-gestation (Miller et al., 2014). In their study, the authors observed minimal differences in gene expression levels in both humans and rodents between the inner and outer subventricular zones (i.e., the germinal zones), even though the latter zone is substantially larger in humans than in rodents. Moreover, in the developing human brain, PAX6 is expressed in both the inner and outer subventricular zones, the latter of which contains neural progenitor cells and—as mentioned above—is dramatically larger in primates, particularly humans, than rodents (see (Miller et al., 2014) and the references therein). This finding may suggest that PAX6 expression has a strong contribution to neural stem cells and progenitor cells in human neural development.

PAX6 is related to ASD

In recent years, the prevalence of ASD has increased considerably in various countries, up to 1/68 in the United States (see media release from Centers for Disease Control and Prevention; <https://www.cdc.gov/media/releases/2014/p0327-autism-spectrum-disorder.html>). ASD comprises a broad spectrum of neurodevelopmental disorders. The most recent standard established by the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) defines ASD as the presence of both *i*) defects in social interactions, including vocal communication; and *ii*) stereotypic/repetitive behaviors and restricted interest, beginning in early childhood (e.g., by three years of age). However, several additional symptoms can be present, including abnormalities in sensory systems, motor control, sleep disorders, gastrointestinal disturbances, epilepsy, and comorbid psychiatric conditions (Chen et al., 2015; Geschwind, 2009). Indeed, it is commonly noted that from a clinical perspective, no two autistic people are alike. This heterogeneity with respect to clinical presentation reflects the high complexity associated with the mechanisms that underlie the pathogenesis of ASD.

Based on classic genetic analyses of monozygotic twins, the heritability of ASD is extremely high, ranging from 60% to as high as 90% (Deng et al., 2015; Gupta and State, 2007). This finding has prompted researchers to perform

genome-wide genetic analyses, allowing to screen many genes across chromosomes (Pinto et al., 2010; Weiss et al., 2009; Yu et al., 2013). Notably, many *de novo* mutations have been associated with ASD (Chang et al., 2015; Pinto et al., 2014; Sanders et al., 2011; Sebat et al., 2007).

Because the symptoms of ASD generally present in early childhood, the disorder is believed to originate from defects that occur during brain development when the expression of many genes is harmonically coordinated. As noted above, the transcription factor Pax6 is a neurodevelopmental molecule that governs brain development, neurogenesis, and gliogenesis (Manuel et al., 2015; Osumi, 2001; Osumi et al., 2008; Ypsilanti and Rubenstein, 2016). Thus, there has been interest in how human PAX6 is involved in neurodevelopmental diseases such as ASD.

In the human genome, the *PAX6* gene is located on the chromosomal region 11p13, and its deletion causes WAGR (Wilms tumor in the kidney, Aniridia in the eye, Genital ridge defect, and mental Retardation) syndrome (Ton et al., 1991). In the mouse genome, the *Pax6* gene was identified from the genetic analysis of a spontaneous mutant called *Small eye* (Hill et al., 1991), and other mutations have also been identified in the *Small eye* mutant rats (Matsuo et al., 1993; Osumi et al., 1997).

Clinically, patients with WAGR syndrome are often diagnosed as having ASD. Several studies have indeed reported abnormalities in the psychosocial states of patients who have a mutation in the *PAX6* gene; these patients often present aggression, mental retardation, and/or autism (for review, see Davis et al. (2008) and the references therein). A previous genome-wide linkage study found that the chromosomal region 11p12-13 is associated with ASD (Abrahams and Geschwind, 2008; Szatmari et al., 2007). We previously sequenced all of the exons and flanking introns of the *PAX6* gene in 285 Japanese patients with ASD and found 15 single-nucleotide polymorphisms that were present in the autistic patients but absent in 2120 controls (i.e., non-autistic) subjects (Maekawa et al., 2009). A recent case report has further suggested that a 1.6 Mb region containing *PAX6*, *WT1*, and *PRRG4* is responsible for the severe developmental delays and autistic behaviors in WAGR syndrome (Yamamoto et al., 2014).

Behavioral phenotypes have been examined in rodent models that have deficiencies in Pax6 function. Cortex-specific *Pax6* knockout mice (*Emx1-Cre; Pax6^{fl/fl}* mice) also showed deficiencies in sensorimotor information integration and both hippocampus-dependent short-term and neocortex-dependent long-term memory recalls (Tuoc et al., 2009). We examined the behavior of spontaneous *Pax6* mutant heterozygous (*rSey^{2/+}*) rats as a model for a neurodevelopmental disorder and found deficits in vocal communication, social behavior, emotional behavior, sensorimotor gating, and fear-conditioned memory (Umeda et al., 2010). We have confirmed behavioral abnormalities in *Pax6* heterozygous mutant mice (*Sey/+*), but very intriguingly, the phenotypes are different in offspring derived from young or aged *Pax6* mutant fathers, i.e., offspring born to young *Pax6* mutant fathers show vocalization impairment, while offspring born to aged *Pax6* mutant fathers show hyperactivity (Yoshizaki et al., 2016). Importantly, these findings indicated that there is an interaction of a genetic risk factor and a non-genetic factor (e.g., paternal aging), to which basic researchers working on animal models of neurodevelopmental diseases should pay more attention. In addition, current human genetic studies may overlook some risk genes if the genes are related to multiple diseases (e.g., ASD and hyperactivity). In any case, it is possible that *Pax6* may affect various animal behaviors related to neurodevelopmental diseases.

Below, we discuss the molecular possibilities that *PAX6* may be associated with the pathogenesis of ASD. Based on meta-analyses of the *Pax6* downstream transcriptome and chromatin immunoprecipitation (ChIP) studies, we show that *Pax6* may regulate and/or bind to hundreds of ASD-associated genes. Taking these facts into account, we propose a novel scenario to explain the multifactorial mechanism that underlies the complex and heterogeneous symptoms associated with ASD.

The role of PAX6-regulated and PAX6-binding molecules related to ASD

A major function of *PAX6* is transcriptional regulation of various target genes via binding to their regulatory regions. Since *PAX6* is a highly conserved gene among species as mentioned above (Manousaki et al., 2011), we searched

ASD-associated human genes among Pax6-regulated/-binding rodent genes that are expressed in the developing brain (Fig. 2A). Here, we assessed both downregulated and upregulated genes in the developing brain of *Pax6* mutants because ASD-related genes include both loss-of-function and gain-of-function conditions.

We first identified 1,074 genes that are either upregulated or downregulated in the developing brain of *Pax6* mutants relative to wild-type animals (defined as a >1.5-fold change in either direction) based on microarray datasets of ours and other laboratories (Kikkawa et al., 2013; Sansom et al., 2009; Walcher et al., 2013). Next, we used published ChIP-on-Chip data to identify genes that have promotor regions bound by the Pax6 protein. We focused on the 2,696 Pax6-binding genes suggested by ChIP-on-Chip analyses using the mouse cortical primordium; genes significantly positive for Pax6-binding were selected by criteria of Sansom et al. (2009) and Xie et al. (2013).

A comprehensive list of ASD-associated genes is maintained in the Simons Foundation Autism Research Initiative (SFARI) database, a curated dataset of putative ASD candidate genes/loci (Banerjee-Basu and Packer, 2010). We therefore screened this publicly accessible web-based dataset containing 910 SFARI genes (updated in September 2017), among which 886 have homologs in the mouse (Blake et al., 2017). As a result, we found 95 PAX6-regulated/ASD-related genes and 167 PAX6-binding/ASD-related genes, including 16 overlapped ones, listed in the order of SFARI scores based on the number of corresponding reports (Supplementary Table 1).

SHANK3, at the top of our list, is a well-known ASD-associated gene along with its family members *SHANK1* and *SHANK2* (Durand et al., 2007; Leblond et al., 2014; Moessner et al., 2007). *SHANK3* encodes a scaffold protein that localizes to the postsynaptic density structure and interacts with Neuroligin (NLGN) and Neurexin (NRXN) to form the connection between presynaptic and postsynaptic neurons (for review, see (Banerjee et al., 2014)). Because Pax6-regulated genes also include *NLGN1* and *NRXN3*, it is reasonable to hypothesize that synaptic dysfunction can be caused by perturbations in a Pax6-dependent molecular pathway.

PTEN, a tumor suppressor gene, is expressed in the developing central nervous system (Luukko et al., 1999). *Pten*-deficient mice have an enlarged cerebral cortex because of the negative regulation of neural stem/progenitor cells proliferation (Groszer et al., 2001; Kwon et al., 2006; Page et al., 2009). Some patients with ASD have a transient increase in cerebral growth during early childhood (Amaral et al., 2008). Interestingly, *PTEN* deletion corresponding to a mutation in ASD patient with macrocephaly (Marchese et al., 2014) increased proliferation of PAX6-positive neural stem/progenitor cells in organoids induced from human fetal cerebral primordium, resulting in surface folding (Li et al., 2017). However, there is a discrepancy between phenotypes; *Pax6* mutant heterozygous rats show decreases in volume in various brain regions (Hiraoka et al., 2016). Possible explanations for the different phenotypes between *PTEN* and *Pax6* mutants are as follows: i) more than one thousand of *Pax6*-regulated genes may regulate the balance between proliferation and differentiation of neural stem/progenitor cells, and ii) *PTEN* is a PAX6-binding gene but not a PAX6-regulated gene; therefore, the expression level of *PTEN* may not be changed in *Pax6* mutant embryos.

PAX6 protein interacts with *FOXP2*, which is known as a “language gene” in both humans and songbirds (Fisher and Scharff, 2009). Thus, a deficit in *FOXP2* function may be associated with ASD, given that language impairment is a hallmark feature of ASD (Scherer et al., 2003). Notably, *FOXP2* binds directly to intron 1 in *CNTNAP2*, a gene that also plays a major role in language development and in the onset of ASD (Alarcon et al., 2008; Peter et al., 2011; Vernes et al., 2008). In the mouse cerebellum, *Foxp2* and *Pax6* are expressed specifically in Purkinje cells and granule cells, respectively (Fig. 1D; (Fujita and Sugihara, 2012; Yamasaki et al., 2001)). In *Pax6* homozygous mutant rat embryos, migration of granule cells is abnormal (Yamasaki et al., 2001). Given that cerebellar Purkinje cells receive input from granule cells, the development of Purkinje cells might be affected by impaired development of granule cells. In the developing neocortex, *Foxp2* expression appears to be repressed in RG cells (in which *Pax6* is highly expressed), whereas *Foxp2* expression is present in layer VI cortical neurons (in which *Pax6* is not expressed) (Ferland et al., 2003). Deep layer cortical neurons project long distances, and *Foxp2* is important for

extending neurites (Vernes et al., 2011). Therefore, impaired Pax6 function may affect Foxp2 expression, ultimately causing impaired cerebellar and/or neocortical function. A ChIP analysis using zebrafish embryos showed that Pax6 can bind to an enhancer of FoxP2, which is evolutionarily conserved from zebrafish to human (Coutinho et al., 2011). We thus hypothesized that PAX6 may bind to *FOXP2* and regulates its expression in mammals.

Another interesting target for PAX6 is *FMR1*, a gene responsible for fragile X syndrome, which is characterized by mental retardation and autistic behaviors. The *FMR1* gene encodes an RNA-binding protein called FMRP. FMRP transports mRNAs that encode synaptic molecules to synapses over extremely long distances (Dictenberg et al., 2008) and regulates translation of mRNA into protein (Ascano et al., 2012; Darnell et al., 2011). During neocortical development, FMRP is expressed in RG cells at the apical and basal tips of long processes and suppresses differentiation of the RG cells into neurons (Saffary and Xie, 2011). A previous study using *Fmr1* KO mice identified *N-cadherin* (*Cdh2*) mRNA as a target of FMRP and revealed neuronal mispositioning that may affect the balance of excitatory and inhibitory input in the neocortex (La Fata et al., 2014). More recently, FMRP was shown to control transportation and localization of mRNA at the basal tip of RG cells (Pilaz et al., 2016). Thus, it would be interesting to determine the precise target mRNAs that are bound by FMRP and thereby transported within RG cells and/or regulated through translation during cortical development.

GO analyses of PAX6-related ASD genes

To understand the role of the molecular networks associated with Pax6, we performed a functional screen of 246 genes (95 PAX6-regulated/ASD genes and 167 PAX6-binding/ASD genes, in which 16 genes are overlapped, Fig. 2B) using the Gene Ontology (GO) Biological Process with the gene-function annotations from the Mouse Genome Informatics (MGI) (Blake et al., 2017). The resulting GO terms reveal enrichment of genes assigned to terms related to functions and development of the nervous system (Fig. 2C, Supplementary Table 2).

The GO term “behavior” containing 54 genes was identified as the most significant GO. As discussed above, ASD is characterized by difficulties with

social interactions—including communication deficits—and stereotypic/repetitive behaviors. Interestingly, the *SLC1A2* gene categorized in “behavior” encodes a major glutamate transporter (GLT-1), and mice that lack *Slc1a2* expression in the forebrain display pathological repetitive behaviors (Aida et al., 2015). Conditional knockout mice that lack cortex-specific expression of the transcription factor *Mef2c* show social behavior abnormalities due to the imbalance of excitatory and inhibitory synapses in the cortex (Harrington et al., 2016). Thus, these PAX6 downstream molecules can explain—at least to some extent—the range of behavioral phenotypes found among patients with ASD.

Unsurprisingly, the GO categories “central nervous system development” and “neurogenesis” also appeared in our list of Pax6-regulated/-binding/ASD-related genes. Indeed, we previously reported that Pax6 regulates the expression of a gene that encodes a brain-type fatty acid binding protein (*Fabp7*/BLBP) and is required for the maintenance of proliferating neural stem/progenitor cells in the developing cortex (Arai et al., 2005), as well as in the hippocampus (Matsumata et al., 2012). Interestingly, genetic evidence suggests that the *FABP7* gene plays a role in ASD and schizophrenia (Maekawa et al., 2010; Shimamoto et al., 2014).

During corticogenesis, complex transcriptional networks play important roles in neuronal specification. For example, the transcription factors DLX1 and DLX2 are mainly expressed in the ventral telencephalon that produces inhibitory interneurons (Cobos et al., 2005). *Pax6* mutant mice have an expanded ventral region of the telencephalon, which expresses *Dlx* family genes that are involved in the production of interneurons (Stoykova et al., 2000). These findings suggest that Pax6 may regulate interneuron development. Furthermore, Pax6 negatively regulates the transcription factor *Fezf2* that control projection identities via a complex network, including *Tbr1* and *Satb2*, which are required for the differentiation of excitatory neurons (Britanova et al., 2008; Hevner et al., 2001; Srinivasan et al., 2012; Sun et al., 2015). Therefore, these transcription factors regulate the expression of their target genes, gradually producing specific cortical neurons in a time-dependent manner. We therefore hypothesized that ASD is caused by inadequate expression of PAX6-regulated genes in the developing cortex in the embryonic stages.

Interaction of PAX6-related proteins with ASD

To elucidate the interaction of Pax6 with other ASD proteins, we analyzed the Pax6-related protein network using STRING version 10.5 database (Szklarczyk et al., 2017). Notably, the resulting graph shows interesting clusters of molecular interactions (Fig. 2D, Supplementary Table 3).

One of these clusters is “Cell surface receptor signaling pathway involved in cell-cell signaling”, which contains the cadherin family members CDH8 and CDH10 that play an important role in cell adhesion. This is an expected finding, as chimeric analyses have previously shown that Pax6 plays a role in cell adhesion during cortical development (Inoue et al., 2001; Talamillo et al., 2003); abnormal cell aggregation is occasionally observed in the brain cytoarchitecture of patients with neurodevelopmental diseases (Wegiel et al., 2010). These cadherins contain an intracellular domain that binds β -catenin (encoded by the *CTNNB1* gene) and a juxtamembrane domain that binds p120-catenin (Katafiasz et al., 2003; Ozawa et al., 1989). A recent study found that Pax6 mediates β -catenin signaling, driving the self-renewal of RG cells in the developing neocortex (Gan et al., 2014). *CTNND2*, which encodes delta-2-catenin, is also involved in the cadherin–catenin cell adhesion complex (Zhou et al., 1997). A previous study found that *Ctnnd2* is regulated by Pax6 in the developing neocortex and retina in the mouse (Duparc et al., 2006). Notably, the *CTNND2* gene was recently identified in a human genetics study based on female-enriched severe cases of ASD (Turner et al., 2015); loss of delta-catenin may strongly be correlated with mental retardation in a severe form of ASD. Protocadherins, especially those clustered on the 5q31 region, could also be intriguing genes because a combination of these protocadherins was shown to be important for diversification of neurons (Mountoufaris et al., 2017; Toyoda et al., 2014).

The categories “Regulation of calcium ion transport” and “Regulation of ion transport” include genes that encode ion channels. Many studies have suggested that several disease-inducing mutations in ion channel genes play a role in the pathogenesis of ASD (“channelopathies”; e.g., reviewed in (Schmunk and Gargus, 2013)). Because *Pax6* is postnatally expressed at high levels in

neurons within the olfactory bulb, amygdala, thalamus, hypothalamus, and cerebellum (summarized in (Hiraoka et al., 2016)), Pax6 may play a role in the pathogenesis of ASD by modulating multiple channels that respond to changes in membrane potential and driving neuronal action potentials. We also found a cluster with nitric oxide synthase 1 (Nos1) at its center. Nitric oxide displays many properties as a neurotransmitter (Ignarro et al., 1990; Toda and Okamura, 1990). Nos1 is expressed in a distinct subpopulation of interneurons in the cortex, hippocampus, and olfactory bulb (Tricoire and Vitalis, 2012). In the olfactory bulb, the density of Nos1+ cells was reduced in *Pax6* heterozygous *Sey^{DeY}* mice (Curto et al., 2014). Further analyses of the role of Pax6 in the ion channel and Nos1 pathways might yield new insight into ASD pathogenesis.

PAX6 functions as a chromatin remodeling modulator

We found that only 16 genes within the 246 putative Pax6/ASD-related genes were *bona fide* ones that were bound and regulated by Pax6. One possibility is that the change in expression levels could not be detected by microarray analyses using whole cortical primordia because some molecules may be downregulated or upregulated in specific cell types (e.g., neural stem cells, neurons, and glial cells) or specific parts of the cell (e.g., the apical or basal part of RG cells). We also considered that Pax6 may have a function other than a canonical transcription factor. Consistent with this notion, a novel function of Pax6 as a chromatin modulator has been proposed in recent years.

Fig. 2D shows EP300, a transcriptional co-activator, in the center of the cluster “regulation of transcription”; EP300 acts as a histone acetyltransferase that regulates transcription via structural changes in chromatin and appears to have molecular interactions with several Pax6/ASD-associated molecules, including PTEN, TAF1, HOXB1, MED12, STAT1, CAMK4, ESR1, and PAX6 (Fig. 2D). Indeed, Pax6 was previously reported to interact with EP300 for transactivation of a gene encoding glucagon (Hussain and Habener, 1999). A more recent study found that recruitment of EP300 into AUT2 (autism susceptibility candidate 2)/polycomb complex induces gene activation via histone modification (Gao et al., 2014). Thus, Pax6 may play a role in chromatin remodeling together with other chromatin molecules in pathogenesis of ASD.

Pax6 also interacts with several BAF (SWI/SNF-like Brg1/Brm-associated factor) complexes that are involved in chromatin remodeling during neural development both *in vitro* and *in vivo* (Ninkovic et al., 2013; Tuoc et al., 2013a; Tuoc et al., 2013b). A previous study (Tuoc et al., 2013a) showed that BAF170 competes with BAF155 subunit of the BAF complex in the promoter of Pax6 target genes. BAF155 decreases a repressive condition of transcription (i.e., DNA methylation and/or H3K27me3) and conversely increases an activated condition (H3K9Ac). Meanwhile, BAF170 recruits the REST (RE1-silencing transcription factor)-corepressor complex to these promoter regions and represses the expression of Pax6 downstream genes involved in the embryonic cortical neurogenesis. Therefore, it is reasonable to assume that different combinations of Pax6 and BAF155 or BAF170 in the complex may affect the status of the euchromatin structure and regulate the transcription of Pax6 target genes involved in the embryonic cortical neurogenesis.

Another important chromatin remodeling factor that may be related to ASD is chromodomain helicase DNA binding protein 8 (CHD8) (Neale et al., 2012; O'Roak et al., 2012; Sanders, 2015; Talkowski et al., 2012). Heterozygous *Chd8* mutant mice show ASD-like behavior and activation of REST, which suppresses the transcription of neuronal genes, thereby causing a delay in corticogenesis (Katayama et al., 2016). Intriguingly, we did not identify CHD8 but its closely related family members, CHD2 and CHD7, in our Pax6/ASD-associated molecules.

Pax6 is also reported to recruit H3K4-specific methyltransferase Mll1, Mll2, and Set1a in promoters and distal enhancers of developmentally controlled genes in lens, subsequently changing their H3K4 methylation states (Sun et al., 2016). These findings provide new insight into complicated modification of the chromatin state of ASD genes by Pax6 as a chromatin remodeling modulator. This is why we took into account not only downregulated but also upregulated genes in the developing brain of *Pax6* mutants based on microarray analyses. All these lines of evidence suggest a new role of Pax6 as a chromatin regulator, which may be related to the pathogenesis of ASD.

Conclusion

A variety of complex genetic and epigenetic factors play a role in the etiology of ASD. PAX6 dysfunction can lead to abnormal brain development via several pathways. As discussed above, we speculate that complex Pax6 regulatory molecular networks may be involved in the onset of ASD during brain development. Given that distinct missense mutations in the *PAX6* gene cause distinct phenotypes (Walcher et al., 2013), and given that the *PAX6* gene contains many regulatory elements (for a recent review, see (Manuel et al., 2015)), the clinical outcomes can vary widely. Recent studies also identified specific microRNAs that regulate the expression of Pax6 in the developing mouse brain (Cheng et al., 2014; de Chevigny et al., 2012; Kaspi et al., 2013; Needhamsen et al., 2014); interestingly, mutations in these microRNA loci can also indirectly affect the function of Pax6 in a highly complex manner. The emergence of novel sequencing technologies (RNA-seq, HiC-seq, ChIP-seq, and others) enabled us to perform genome-wide analyses to produce more big data. We hope that the combination of such approaches for identifying complicated genomic architectures will help identify new Pax6 regulatory networks in the future.

Acknowledgments

We thank Drs. Takeo Yoshikawa, Takeo Kubota, and Takeshi Sakurai for valuable suggestions and for critically reading our original manuscript. We also thank Dr. Takeshi Obayashi for technical advice on the meta-analyses. The Pax6 and FoxP2 double-immunostaining image in the cerebellum was kindly provided by Dr. Taku Sugiyama. This work was supported by MEXT Grant-in-Aid for Scientific Research on Innovative Areas (#16H06530) to N.O. and JSPS KAKENHI (#16K20902) to T.K. This work was also supported by Financial Support for English Editing of International Research Papers in Tohoku University Center for Gender Equality Promotion.

References

- Abrahams, B.S., Geschwind, D.H., 2008. Advances in autism genetics: on the threshold of a new neurobiology. *Nat Rev Genet.* 9, 341-355.
- Aida, T., et al., 2015. Astroglial glutamate transporter deficiency increases synaptic excitability and leads to pathological repetitive behaviors in mice. *Neuropsychopharmacology.* 40, 1569-1579.
- Alarcon, M., et al., 2008. Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene. *Am J Hum Genet.* 82, 150-159.
- Amaral, D.G., Schumann, C.M., Nordahl, C.W., 2008. Neuroanatomy of autism. *Trends Neurosci.* 31, 137-145.
- Arai, Y., et al., 2005. Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. *J Neurosci.* 25, 9752-9761.
- Ascano, M., Jr., et al., 2012. FMRP targets distinct mRNA sequence elements to regulate protein expression. *Nature.* 492, 382-386.
- Banerjee, S., Riordan, M., Bhat, M.A., 2014. Genetic aspects of autism spectrum disorders: insights from animal models. *Front Cell Neurosci.* 8, 58.
- Banerjee-Basu, S., Packer, A., 2010. SFARI Gene: an evolving database for the autism research community. *Dis Model Mech.* 3, 133-135.
- Benzing, K., et al., 2011. A novel approach to selectively target neuronal subpopulations reveals genetic pathways that regulate tangential migration in the vertebrate hindbrain. *PLoS Genet.* 7, e1002099.
- Blake, J.A., et al., 2017. Mouse Genome Database (MGD)-2017: community knowledge resource for the laboratory mouse. *Nucleic Acids Res.* 45, D723-D729.
- Britanova, O., et al., 2008. Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. *Neuron.* 57, 378-392.
- Cai, J., et al., 1994. Dissection of the Drosophila paired protein: functional requirements for conserved motifs. *Mech Dev.* 47, 139-150.
- Chang, J., et al., 2015. Genotype to phenotype relationships in autism spectrum disorders. *Nat Neurosci.* 18, 191-198.
- Chen, J.A., et al., 2015. The emerging picture of autism spectrum disorder:

- genetics and pathology. *Annu Rev Pathol.* 10, 111-144.
- Cheng, Q., et al., 2014. PAX6, a novel target of miR-335, inhibits cell proliferation and invasion in glioma cells. *Mol Med Rep.* 10, 399-404.
- Cobos, I., et al., 2005. Mice lacking Dlx1 show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nat Neurosci.* 8, 1059-1068.
- Cocas, L.A., et al., 2011. Pax6 is required at the telencephalic pallial-subpallial boundary for the generation of neuronal diversity in the postnatal limbic system. *J Neurosci.* 31, 5313-5324.
- Coutinho, P., et al., 2011. Discovery and assessment of conserved Pax6 target genes and enhancers. *Genome Res.* 21, 1349-1359.
- Curto, G.G., et al., 2014. Pax6 is essential for the maintenance and multi-lineage differentiation of neural stem cells, and for neuronal incorporation into the adult olfactory bulb. *Stem Cells Dev.* 23, 2813-2830.
- Czerny, T., Schaffner, G., Busslinger, M., 1993. DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev.* 7, 2048-2061.
- Darnell, J.C., et al., 2011. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell.* 146, 247-261.
- Davis, L.K., et al., 2008. Pax6 3' deletion results in aniridia, autism and mental retardation. *Hum Genet.* 123, 371-378.
- de Chevigny, A., et al., 2012. miR-7a regulation of Pax6 controls spatial origin of forebrain dopaminergic neurons. *Nat Neurosci.* 15, 1120-1126.
- Deng, W., et al., 2015. The Relationship Among Genetic Heritability, Environmental Effects, and Autism Spectrum Disorders: 37 Pairs of Ascertained Twin Study. *J Child Neurol.* 30, 1794-1799.
- Dictenberg, J.B., et al., 2008. A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome. *Dev Cell.* 14, 926-939.
- Duan, D., et al., 2013. Spatiotemporal expression patterns of Pax6 in the brain of embryonic, newborn, and adult mice. *Brain Struct Funct.* 218, 353-372.
- Duparc, R.H., et al., 2006. Pax6 is required for delta-catenin/neurojugin expression during retinal, cerebellar and cortical development in mice.

- Dev Biol. 300, 647-655.
- Durand, C.M., et al., 2007. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet.* 39, 25-27.
- Engelkamp, D., et al., 1999. Role of Pax6 in development of the cerebellar system. *Development.* 126, 3585-3596.
- Ericson, J., et al., 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell.* 90, 169-180.
- Estivill-Torrus, G., et al., 2002. Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development.* 129, 455-466.
- Ferland, R.J., et al., 2003. Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. *J Comp Neurol.* 460, 266-279.
- Fisher, S.E., Scharff, C., 2009. FOXP2 as a molecular window into speech and language. *Trends Genet.* 25, 166-177.
- Fujita, H., Sugihara, I., 2012. FoxP2 expression in the cerebellum and inferior olive: development of the transverse stripe-shaped expression pattern in the mouse cerebellar cortex. *J Comp Neurol.* 520, 656-677.
- Fukuda, T., et al., 2000. Histogenesis of the cerebral cortex in rat fetuses with a mutation in the Pax-6 gene. *Brain Res Dev Brain Res.* 120, 65-75.
- Gan, Q., et al., 2014. Pax6 mediates β -catenin signaling for self-renewal and neurogenesis by neocortical radial glial stem cells. *Stem Cells.* 32, 45-58.
- Gao, Z., et al., 2014. An AUTS2-Polycomb complex activates gene expression in the CNS. *Nature.* 516, 349-354.
- Georgala, P.A., Carr, C.B., Price, D.J., 2011. The role of Pax6 in forebrain development. *Dev Neurobiol.* 71, 690-709.
- Geschwind, D.H., 2009. Advances in autism. *Annu Rev Med.* 60, 367-380.
- Gotz, M., Stoykova, A., Gruss, P., 1998. Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron.* 21, 1031-1044.
- Groszer, M., et al., 2001. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science.* 294, 2186-2189.
- Gupta, A.R., State, M.W., 2007. Recent advances in the genetics of autism. *Biol*

- Psychiatry. 61, 429-437.
- Haba, H., et al., 2009. Subtype-specific reduction of olfactory bulb interneurons in Pax6 heterozygous mutant mice. *Neurosci Res.* 65, 116-121.
- Hack, M.A., et al., 2005. Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat Neurosci.* 8, 865-872.
- Halder, G., Callaerts, P., Gehring, W.J., 1995. New perspectives on eye evolution. *Curr Opin Genet Dev.* 5, 602-609.
- Harrington, A.J., et al., 2016. MEF2C regulates cortical inhibitory and excitatory synapses and behaviors relevant to neurodevelopmental disorders. *Elife.* 5.
- Heins, N., et al., 2002. Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci.* 5, 308-315.
- Hevner, R.F., et al., 2001. Tbr1 regulates differentiation of the preplate and layer 6. *Neuron.* 29, 353-366.
- Hevner, R.F., et al., 2006. Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci Res.* 55, 223-233.
- Hill, R.E., et al., 1991. Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature.* 354, 522-525.
- Hiraoka, K., et al., 2016. Regional Volume Decreases in the Brain of Pax6 Heterozygous Mutant Rats: MRI Deformation-Based Morphometry. *PLoS One.* 11, e0158153.
- Hussain, M.A., Habener, J.F., 1999. Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. *J Biol Chem.* 274, 28950-28957.
- Ignarro, L.J., et al., 1990. Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biophys Res Commun.* 170, 843-850.
- Inoue, T., Nakamura, S., Osumi, N., 2000. Fate mapping of the mouse prosencephalic neural plate. *Dev Biol.* 219, 373-383.
- Inoue, T., et al., 2001. Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. *Development.* 128, 561-569.

- Jun, S., Desplan, C., 1996. Cooperative interactions between paired domain and homeodomain. *Development*. 122, 2639-2650.
- Kaspi, H., et al., 2013. Brief report: miR-290-295 regulate embryonic stem cell differentiation propensities by repressing Pax6. *Stem Cells*. 31, 2266-2272.
- Katafiasz, B.J., et al., 2003. Characterization of cadherin-24, a novel alternatively spliced type II cadherin. *J Biol Chem*. 278, 27513-27519.
- Katayama, Y., et al., 2016. CHD8 haploinsufficiency results in autistic-like phenotypes in mice. *Nature*. 537, 675-679.
- Kawano, H., et al., 1999. Pax-6 is required for thalamocortical pathway formation in fetal rats. *J Comp Neurol*. 408, 147-160.
- Kessel, M., Gruss, P., 1990. Murine developmental control genes. *Science*. 249, 374-379.
- Kikkawa, T., et al., 2013. Dmrt1 regulates proneural gene expression downstream of Pax6 in the mammalian telencephalon. *Genes Cells*. 18, 636-649.
- Kohwi, M., et al., 2005. Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *J Neurosci*. 25, 6997-7003.
- Kwon, C.H., et al., 2006. Pten regulates neuronal arborization and social interaction in mice. *Neuron*. 50, 377-388.
- La Fata, G., et al., 2014. FMRP regulates multipolar to bipolar transition affecting neuronal migration and cortical circuitry. *Nat Neurosci*. 17, 1693-700.
- Leblond, C.S., et al., 2014. Meta-analysis of SHANK Mutations in Autism Spectrum Disorders: a gradient of severity in cognitive impairments. *PLoS Genet*. 10, e1004580.
- Li, Y., et al., 2017. Induction of Expansion and Folding in Human Cerebral Organoids. *Cell Stem Cell*. 20, 385-396.
- Luukko, K., et al., 1999. Expression of LKB1 and PTEN tumor suppressor genes during mouse embryonic development. *Mech Dev*. 83, 187-190.
- Maekawa, M., et al., 2005. Pax6 is required for production and maintenance of progenitor cells in postnatal hippocampal neurogenesis. *Genes Cells*. 10, 1001-1014.

- Maekawa, M., et al., 2009. A novel missense mutation (Leu46Val) of PAX6 found in an autistic patient. *Neurosci Lett.* 462, 267-271.
- Maekawa, M., et al., 2010. Polymorphism screening of brain-expressed FABP7, 5 and 3 genes and association studies in autism and schizophrenia in Japanese subjects. *J Hum Genet.* 55, 127-130.
- Manousaki, T., et al., 2011. Co-orthology of Pax4 and Pax6 to the fly eyeless gene: molecular phylogenetic, comparative genomic, and embryological analyses. *Evol Dev.* 13, 448-459.
- Manuel, M.N., et al., 2015. Regulation of cerebral cortical neurogenesis by the Pax6 transcription factor. *Front Cell Neurosci.* 9, 70.
- Marchese, M., et al., 2014. Autism-epilepsy phenotype with macrocephaly suggests PTEN, but not GLIALCAM, genetic screening. *BMC Med Genet.* 15, 26.
- Matsumata, M., et al., 2012. The effects of Fabp7 and Fabp5 on postnatal hippocampal neurogenesis in the mouse. *Stem Cells.* 30, 1532-1543.
- Matsuo, T., et al., 1993. A mutation in the Pax-6 gene in rat small eye is associated with impaired migration of midbrain crest cells. *Nat Genet.* 3, 299-304.
- Miller, J.A., et al., 2014. Transcriptional landscape of the prenatal human brain. *Nature.* 508, 199-206.
- Moessner, R., et al., 2007. Contribution of SHANK3 mutations to autism spectrum disorder. *Am J Hum Genet.* 81, 1289-1297.
- Mountoufaris, G., et al., 2017. Multicenter Pcdh diversity is required for mouse olfactory neural circuit assembly. *Science.* 356, 411-414.
- Nacher, J., et al., 2005. Expression of the transcription factor Pax 6 in the adult rat dentate gyrus. *J Neurosci Res.* 81, 753-761.
- Neale, B.M., et al., 2012. Patterns and rates of exonic de novo mutations in autism spectrum disorders. *Nature.* 485, 242-245.
- Needhamsen, M., et al., 2014. Regulation of Human PAX6 Expression by miR-7. *Evol Bioinform Online.* 10, 107-113.
- Ninkovic, J., et al., 2013. The BAF complex interacts with Pax6 in adult neural progenitors to establish a neurogenic cross-regulatory transcriptional network. *Cell Stem Cell.* 13, 403-418.

- O'Roak, B.J., et al., 2012. Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. *Nature*. 485, 246-250.
- Osumi, N., et al., 1997. Pax-6 is involved in the specification of hindbrain motor neuron subtype. *Development*. 124, 2961-2972.
- Osumi, N., 2001. The role of Pax6 in brain patterning. *Tohoku J Exp Med*. 193, 163-174.
- Osumi, N., et al., 2008. Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. *Stem Cells*. 26, 1663-1672.
- Ozawa, M., Baribault, H., Kemler, R., 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J*. 8, 1711-1717.
- Page, D.T., et al., 2009. Haploinsufficiency for Pten and Serotonin transporter cooperatively influences brain size and social behavior. *Proc Natl Acad Sci U S A*. 106, 1989-1994.
- Peter, B., et al., 2011. Replication of CNTNAP2 association with nonword repetition and support for FOXP2 association with timed reading and motor activities in a dyslexia family sample. *J Neurodev Disord*. 3, 39-49.
- Pilaz, L.J., et al., 2016. Dynamic mRNA Transport and Local Translation in Radial Glial Progenitors of the Developing Brain. *Curr Biol*. 26, 3383-3392.
- Pinto, D., et al., 2010. Functional impact of global rare copy number variation in autism spectrum disorders. *Nature*. 466, 368-372.
- Pinto, D., et al., 2014. Convergence of genes and cellular pathways dysregulated in autism spectrum disorders. *Am J Hum Genet*. 94, 677-694.
- Pratt, T., et al., 2000. A role for Pax6 in the normal development of dorsal thalamus and its cortical connections. *Development*. 127, 5167-5178.
- Pratt, T., et al., 2002. Disruption of early events in thalamocortical tract formation in mice lacking the transcription factors Pax6 or Foxg1. *J Neurosci*. 22, 8523-8531.
- Saffary, R., Xie, Z., 2011. FMRP regulates the transition from radial glial cells to

- intermediate progenitor cells during neocortical development. *J Neurosci.* 31, 1427-1439.
- Sakurai, K., Osumi, N., 2008. The neurogenesis-controlling factor, Pax6, inhibits proliferation and promotes maturation in murine astrocytes. *J Neurosci.* 28, 4604-4612.
- Sanders, S.J., et al., 2011. Multiple recurrent de novo CNVs, including duplications of the 7q11.23 Williams syndrome region, are strongly associated with autism. *Neuron.* 70, 863-885.
- Sanders, S.J., 2015. First glimpses of the neurobiology of autism spectrum disorder. *Curr Opin Genet Dev.* 33, 80-92.
- Sansom, S.N., et al., 2009. The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. *PLoS Genet.* 5, e1000511.
- Scherer, S.W., et al., 2003. Human chromosome 7: DNA sequence and biology. *Science.* 300, 767-772.
- Schmunk, G., Gargus, J.J., 2013. Channelopathy pathogenesis in autism spectrum disorders. *Front Genet.* 4, 222.
- Sebat, J., et al., 2007. Strong association of de novo copy number mutations with autism. *Science.* 316, 445-449.
- Shimamoto, C., et al., 2014. Functional characterization of FABP3, 5 and 7 gene variants identified in schizophrenia and autism spectrum disorder and mouse behavioral studies. *Hum Mol Genet.* 23, 6495-6511.
- Simpson, T.I., et al., 2009. Normal ventral telencephalic expression of Pax6 is required for normal development of thalamocortical axons in embryonic mice. *Neural Dev.* 4, 19.
- Soma, M., et al., 2009. Development of the mouse amygdala as revealed by enhanced green fluorescent protein gene transfer by means of in utero electroporation. *J Comp Neurol.* 513, 113-128.
- Srinivasan, K., et al., 2012. A network of genetic repression and derepression specifies projection fates in the developing neocortex. *Proc Natl Acad Sci U S A.* 109, 19071-19078.
- Stoykova, A., Gruss, P., 1994. Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J Neurosci.* 14, 1395-1412.

- Stoykova, A., et al., 2000. Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. *J Neurosci.* 20, 8042-8050.
- Sun, J., et al., 2015. Identification of in vivo DNA-binding mechanisms of Pax6 and reconstruction of Pax6-dependent gene regulatory networks during forebrain and lens development. *Nucleic Acids Res.* 43, 6827-6846.
- Sun, J., et al., 2016. Pax6 associates with H3K4-specific histone methyltransferases Mll1, Mll2, and Set1a and regulates H3K4 methylation at promoters and enhancers. *Epigenetics Chromatin.* 9, 37.
- Szatmari, P., et al., 2007. Mapping autism risk loci using genetic linkage and chromosomal rearrangements. *Nat Genet.* 39, 319-328.
- Szklarczyk, D., et al., 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* 45, D362-D368.
- Takahashi, M., Osumi, N., 2002. Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. *Development.* 129, 1327-1338.
- Talamillo, A., et al., 2003. Pax6 regulates regional development and neuronal migration in the cerebral cortex. *Dev Biol.* 255, 151-163.
- Talkowski, M.E., et al., 2012. Sequencing chromosomal abnormalities reveals neurodevelopmental loci that confer risk across diagnostic boundaries. *Cell.* 149, 525-537.
- Tang, K., et al., 2012. COUP-TFII controls amygdala patterning by regulating neuropilin expression. *Development.* 139, 1630-1639.
- Terzic, J., Saraga-Babic, M., 1999. Expression pattern of PAX3 and PAX6 genes during human embryogenesis. *Int J Dev Biol.* 43, 501-508.
- Toda, N., Okamura, T., 1990. Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebroarterial muscle. *Biochem Biophys Res Commun.* 170, 308-313.
- Tole, S., et al., 2005. Selective requirement of Pax6, but not Emx2, in the specification and development of several nuclei of the amygdaloid complex. *J Neurosci.* 25, 2753-2760.
- Tomioka, N., et al., 2000. Neocortical origin and tangential migration of guidepost neurons in the lateral olfactory tract. *J Neurosci.* 20,

- 5802-5812.
- Ton, C.C., et al., 1991. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell*. 67, 1059-1074.
- Toyoda, S., et al., 2014. Developmental epigenetic modification regulates stochastic expression of clustered protocadherin genes, generating single neuron diversity. *Neuron*. 82, 94-108.
- Treisman, J., Harris, E., Desplan, C., 1991. The paired box encodes a second DNA-binding domain in the paired homeo domain protein. *Genes Dev*. 5, 594-604.
- Tricoire, L., Vitalis, T., 2012. Neuronal nitric oxide synthase expressing neurons: a journey from birth to neuronal circuits. *Front Neural Circuits*. 6, 82.
- Tuoc, T.C., et al., 2009. Selective cortical layering abnormalities and behavioral deficits in cortex-specific Pax6 knock-out mice. *J Neurosci*. 29, 8335-8349.
- Tuoc, T.C., et al., 2013a. Chromatin regulation by BAF170 controls cerebral cortical size and thickness. *Dev Cell*. 25, 256-269.
- Tuoc, T.C., Narayanan, R., Stoykova, A., 2013b. BAF chromatin remodeling complex: cortical size regulation and beyond. *Cell Cycle*. 12, 2953-2959.
- Turner, T.N., et al., 2015. Loss of delta-catenin function in severe autism. *Nature*. 520, 51-56.
- Umeda, T., et al., 2010. Evaluation of Pax6 mutant rat as a model for autism. *PLoS One*. 5, e15500.
- Vernes, S.C., et al., 2008. A functional genetic link between distinct developmental language disorders. *N Engl J Med*. 359, 2337-2345.
- Vernes, S.C., et al., 2011. Foxp2 regulates gene networks implicated in neurite outgrowth in the developing brain. *PLoS Genet*. 7, e1002145.
- Vitalis, T., et al., 2000. Defect of tyrosine hydroxylase-immunoreactive neurons in the brains of mice lacking the transcription factor Pax6. *J Neurosci*. 20, 6501-6516.
- Walcher, T., et al., 2013. Functional dissection of the paired domain of Pax6 reveals molecular mechanisms of coordinating neurogenesis and proliferation. *Development*. 140, 1123-1136.

- Walther, C., Gruss, P., 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development*. 113, 1435-1449.
- Walther, C., et al., 1991. Pax: a murine multigene family of paired box-containing genes. *Genomics*. 11, 424-434.
- Warren, N., et al., 1999. The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. *Cereb Cortex*. 9, 627-635.
- Wegiel, J., et al., 2010. The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes. *Acta Neuropathol*. 119, 755-770.
- Weiss, L.A., et al., 2009. A genome-wide linkage and association scan reveals novel loci for autism. *Nature*. 461, 802-808.
- Xie, Q., et al., 2013. Pax6 interactions with chromatin and identification of its novel direct target genes in lens and forebrain. *PLoS One*. 8, e54507.
- Xu, W., et al., 1995. Crystal structure of a paired domain-DNA complex at 2.5 Å resolution reveals structural basis for Pax developmental mutations. *Cell*. 80, 639-650.
- Yamamoto, T., et al., 2014. Narrowing of the responsible region for severe developmental delay and autistic behaviors in WAGR syndrome down to 1.6 Mb including PAX6, WT1, and PRRG4. *Am J Med Genet A*. 164A, 634-638.
- Yamasaki, T., et al., 2001. Pax6 regulates granule cell polarization during parallel fiber formation in the developing cerebellum. *Development*. 128, 3133-3144.
- Yoshizaki, K., et al., 2016. Paternal Aging Affects Behavior in Pax6 Mutant Mice: A Gene/Environment Interaction in Understanding Neurodevelopmental Disorders. *PLoS One*. 11, e0166665.
- Ypsilanti, A.R., Rubenstein, J.L., 2016. Transcriptional and epigenetic mechanisms of early cortical development: An examination of how Pax6 coordinates cortical development. *J Comp Neurol*. 524, 609-629.
- Yu, T.W., et al., 2013. Using whole-exome sequencing to identify inherited causes of autism. *Neuron*. 77, 259-273.
- Zhou, J., et al., 1997. Presenilin 1 interaction in the brain with a novel member of

the Armadillo family. Neuroreport. 8, 2085-2090.

Figure Legends

Figure 1. Expression patterns and functions of Pax6 in the rodent brain. (A) A summary of Pax6 functions in specific brain areas during embryogenesis. (B) Whole-mount *in situ* hybridization of an E13.5 rat neural tube. (C) Immunostaining of the E14.5 mouse cortex. Pax6 protein (magenta) is restricted to the ventricular zone (VZ), where neural progenitor cells reside, while Pax6 is not expressed in the cortical plate (CP) where neurons are located. (D) Immunostaining of Pax6 (green) and FoxP2 (magenta) in the mouse cerebellum at postnatal day 6. Pax6 is expressed in granule cells, which migrate radially across the FoxP2-positive Purkinje cell layer (PCL) to the internal granular layer (IGL). Abbreviations: CP, cortical plate; Di, diencephalon; dTel, dorsal telencephalon; EGL, external granular layer; HB, hindbrain; IGL, internal granular layer; MB, midbrain; PCL, Purkinje cell layer; SN, substantia nigra; VTA, ventral tegmental area; vTel, ventral telencephalon; VZ, ventricular zone. Scale bar: 100 μm .

Figure 2. Putative functional analyses of the roles of PAX6-regulated and PAX6-binding molecules in ASD. (A, B) PAX6-regulated and PAX6-binding genes related to ASD. SFARI-derived ASD-related genes that overlap with “PAX6-regulated” genes (i.e., >1.5-fold downregulated or upregulated in the developing brain of *Pax6* mutants relative to wild-type animals based on microarray analyses including [1] Kikkawa et al., 2013, [2] Walcher et al., 2013, [3] Sansom et al., 2009). ASD-related genes that overlap with “PAX6-binding” genes based on ChIP-on-Chip analyses ([3] Sansom et al., 2009, [4] Xie et al., 2013). (C) GO analyses of 246 overlapped genes derived from the PAX6-regulated and PAX6-binding genes that overlapped with the SFARI genes. Functional screening was performed by using GO Biological Processes in gene-function annotations from MGI. (D) Significant clusters of PAX6-related proteins with ASD. The molecular interaction network of PAX6-regulated and PAX6-binding proteins related to ASD was analyzed using STRING version 10.5 database. Functional clusters are different colors.

Supplemental Information

Supplemental Table Legends

Supplementary Table 1. List of PAX6-regulated and PAX6-binding genes related to ASD. SFARI-derived ASD-related genes that overlap with “PAX6-regulated” genes and are downregulated or upregulated in the developing brain of *Pax6* mutants relative to wild-type animals based on microarray analyses (>1.5-fold change, [1] Kikkawa et al., 2013, [2] Walcher et al., 2013, [3] Sansom et al., 2009). ASD-related genes that overlap with “Pax6-binding” genes based on ChIP-on-Chip analyses ([3] Sansom et al., 2009, [4] Xie et al., 2013).

Supplementary Table 2. GO analyses of PAX6-regulated and PAX6-binding genes related to ASD. GO analyses of 246 overlapped PAX6-regulated and/or PAX6-binding and SFARI genes by using GO Biological Processes in gene-function annotations from the Mouse Genome Informatics (MGI) (see Fig. 2C).

Supplementary Table 3. Significant clusters of PAX6-related proteins with ASD. Molecular interaction network of PAX6-regulated and PAX6-binding proteins related to ASD analyzed using STRING version 10.5 database (see Fig. 2D).

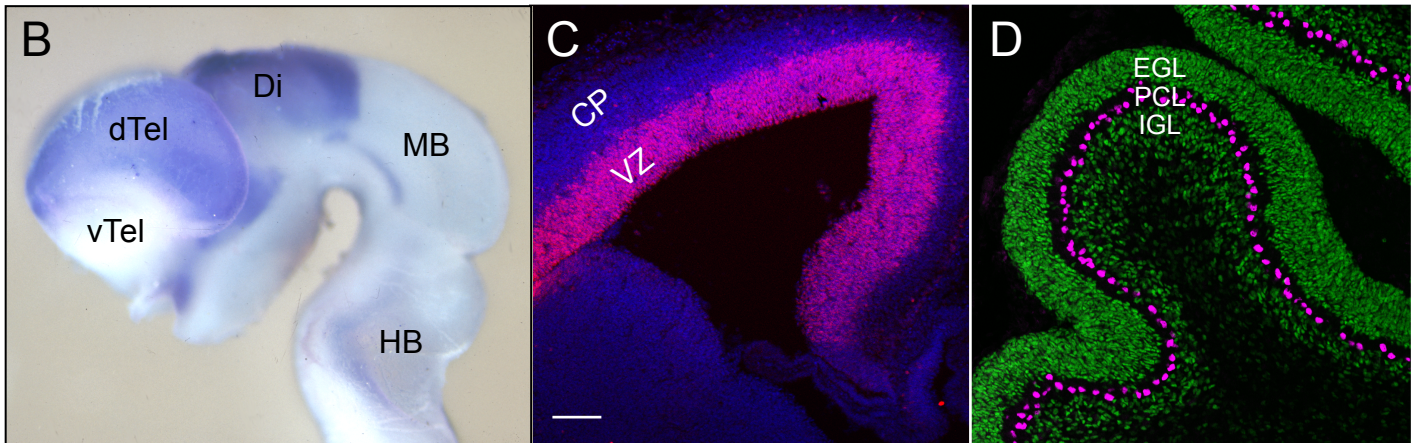
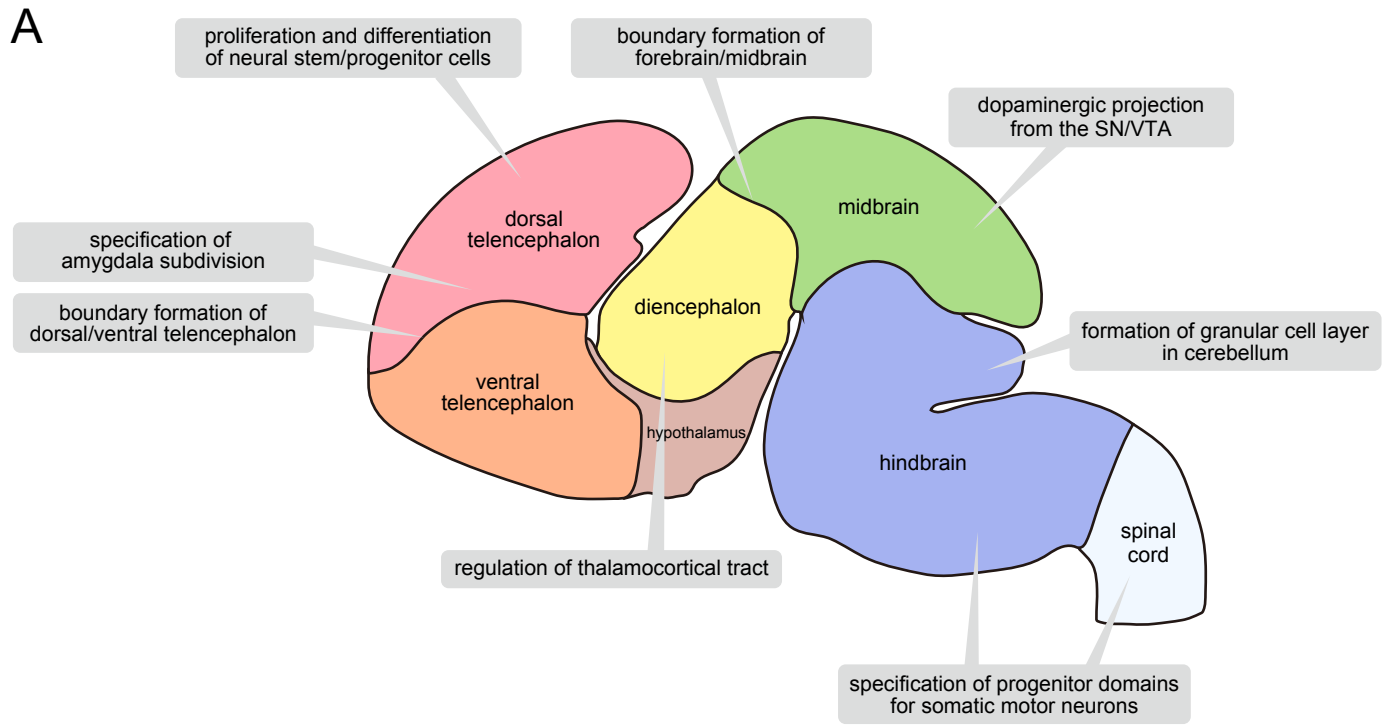


Figure 1

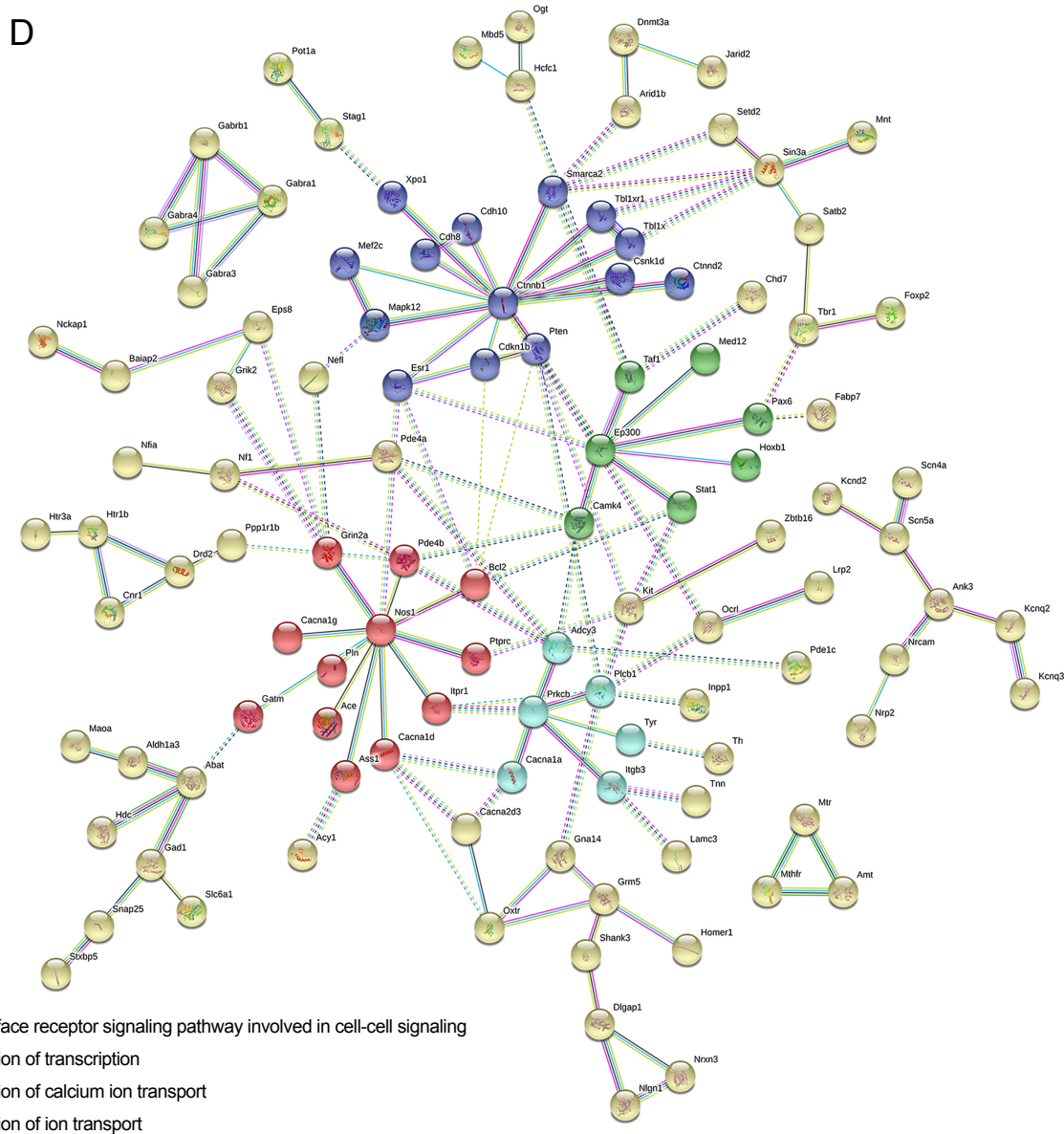
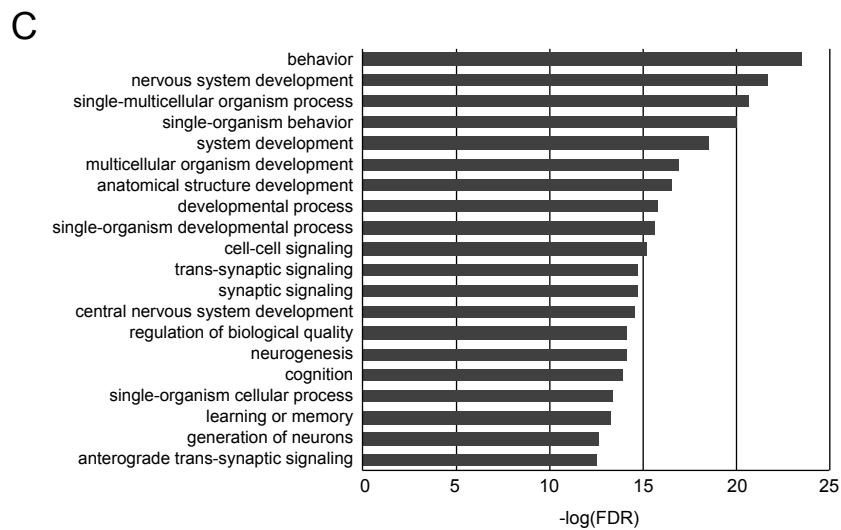
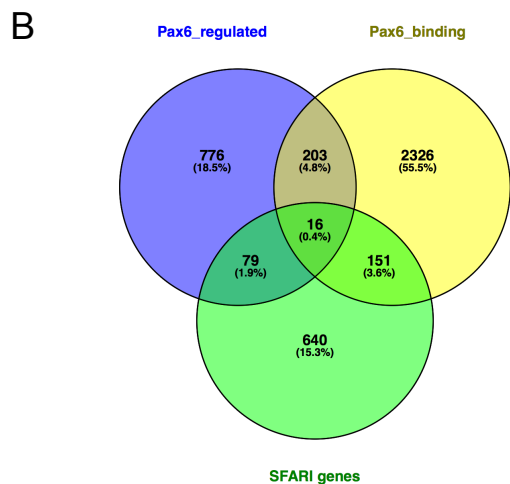
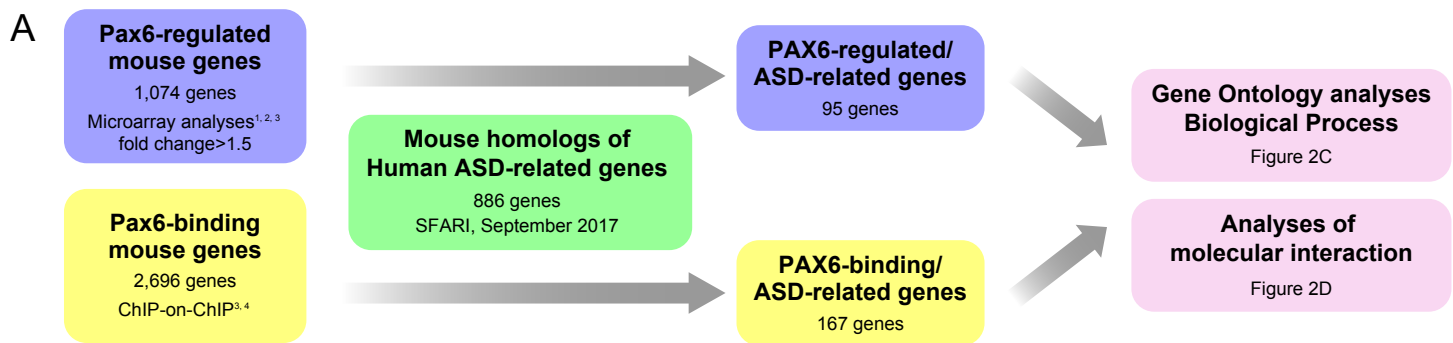


Figure 2