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

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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, Wilmus 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-Torrus 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 genes 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.

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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.

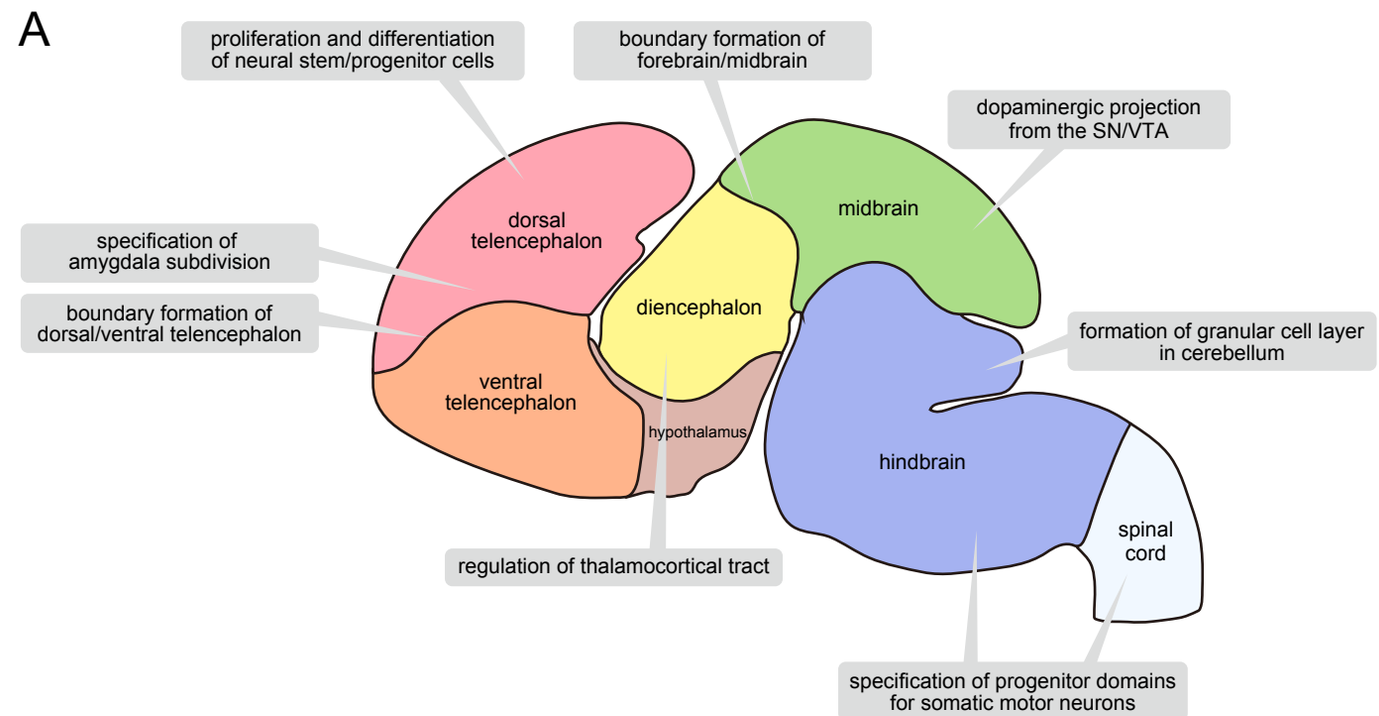
Supplemental Information

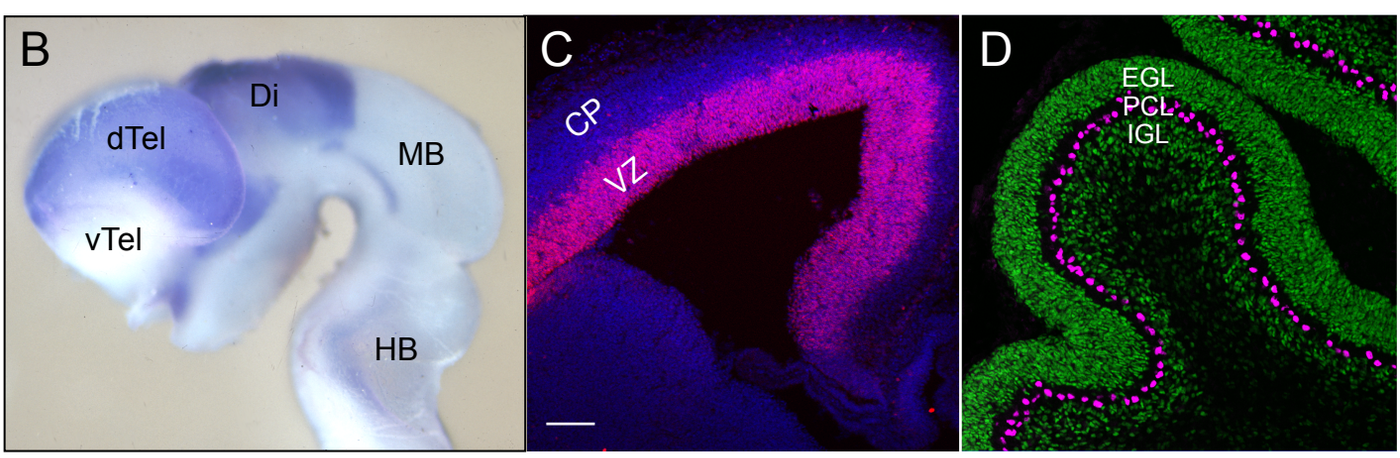
Supplemental Table Legends

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).


A
proliferation and differentiation of neural stem/progenitor cells
boundary formation of forebrain/midbrain
dopaminergic projection from the SN/VTA
specification of amygdala subdivision
boundary formation of dorsal/ventral telencephalon
dorsal telencephalon
midbrain
diencephalon
formation of granular cell layer in cerebellum
ventral telencephalon
hypothalamus
hindbrain
spinal cord
regulation of thalamocortical tract
specification of progenitor domains for somatic motor neurons


B
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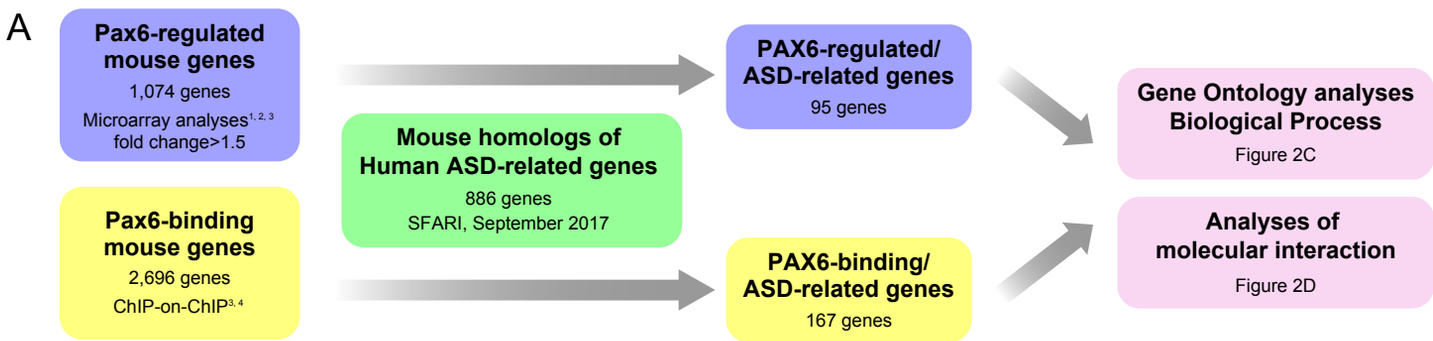
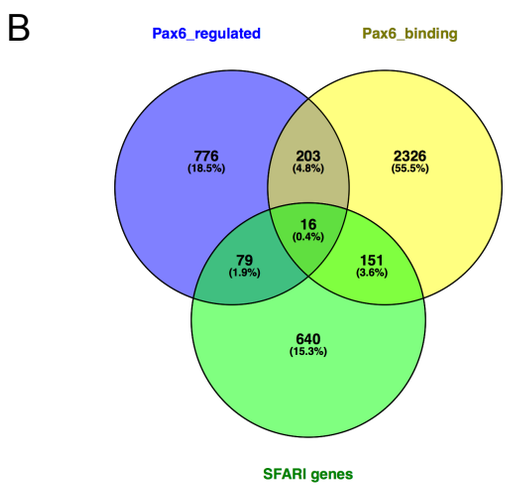
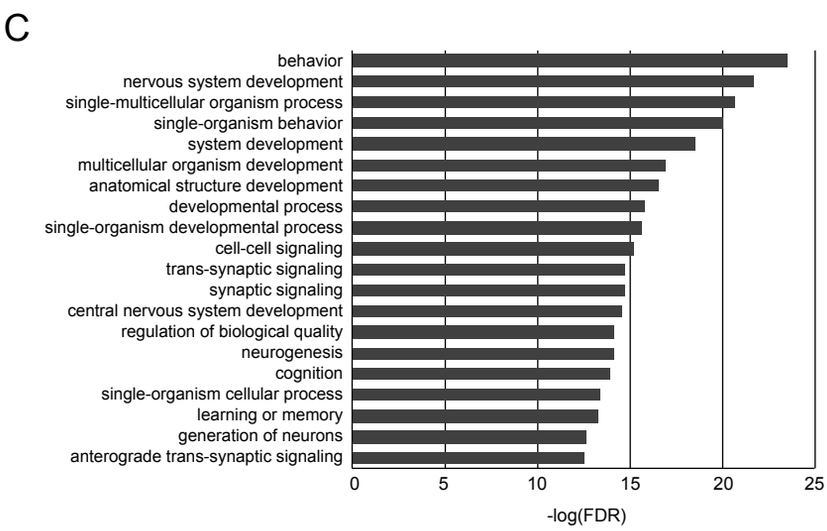
IGL

Figure 1


A
Pax6-regulated mouse genes
1,074 genes
Microarray analyses[1, 2, 3]
fold change>1.5
Pax6-binding mouse genes
2,696 genes
ChIP-on-ChIP[3, 4]
Mouse homologs of Human ASD-related genes
886 genes
SFARI, September 2017
PAX6-regulated/ ASD-related genes
95 genes
PAX6-binding/ ASD-related genes
167 genes
Gene Ontology analyses Biological Process
Figure 2C
Analyses of molecular interaction
Figure 2D


B
Pax6_regulated
Pax6_binding
776 (18.5%)
203 (4.8%)
2326 (55.5%)
16 (0.4%)
79 (1.9%)
151 (3.6%)
640 (15.3%)
SFARI genes


C
behavior
nervous system development
single-multicellular organism process
single-organism behavior
system development
multicellular organism development
anatomical structure development
developmental process
single-organism developmental process
cell-cell signaling
trans-synaptic signaling
synaptic signaling
central nervous system development
regulation of biological quality
neurogenesis
cognition
single-organism cellular process
learning or memory
generation of neurons
anterograde trans-synaptic signaling
0
5
10
15
20
25
-log(FDR)

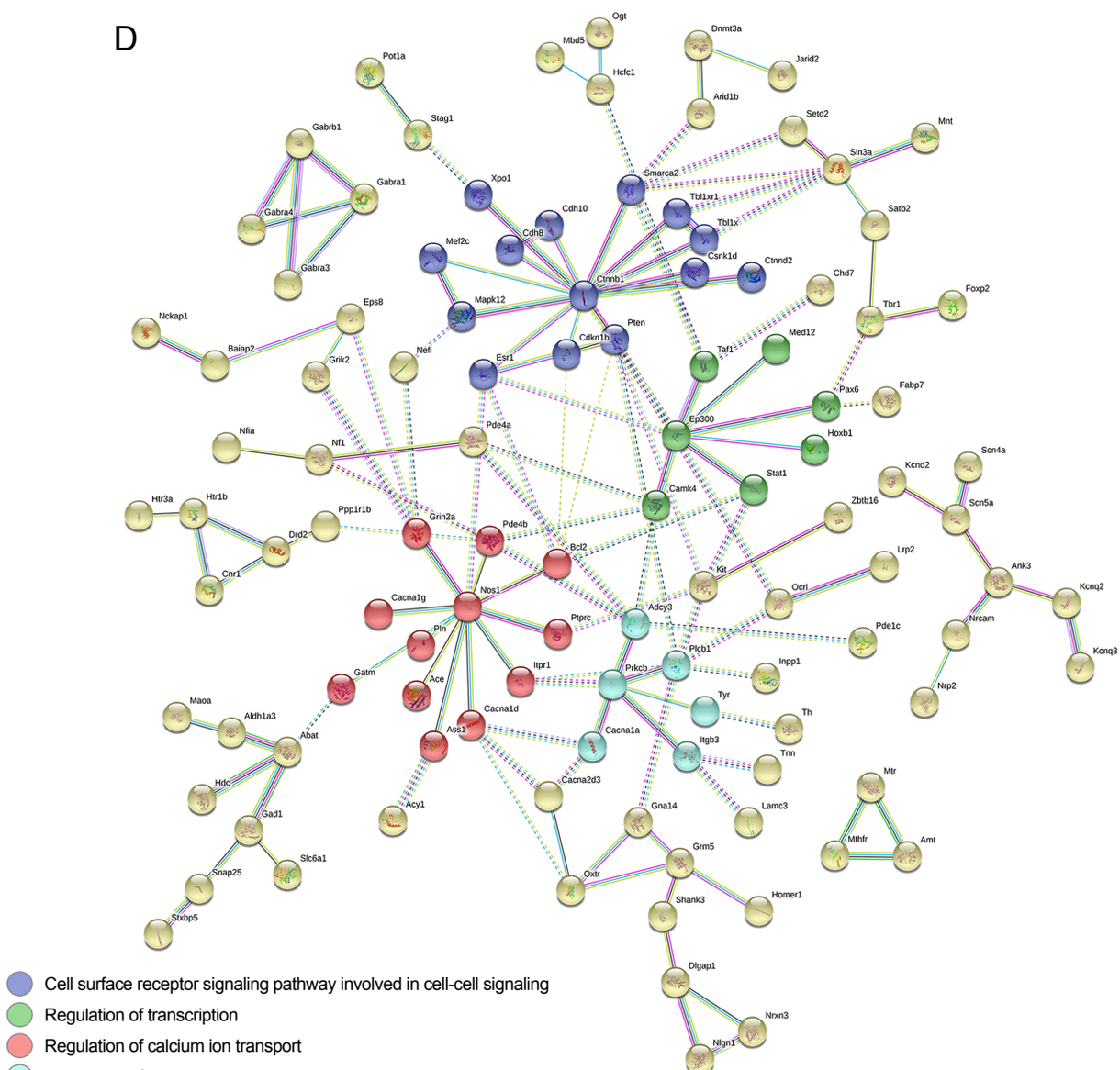

D
Ogt
Mbd5
Dnmt3a
Pot1a
Hcfc1
Jarid2
Arid1b
Stag1
Setd2
Mnt
Gabrb1
Sin3a
Smarca2
Xpo1
Gabra1
Tbl1xr1
Gabra4
Cdh10
Satb2
Tbl1x
Cdh8
Mef2c
Csnk1d
Ctnnd2
Gabra3
Chd7
Ctnnb1
Foxp2
Eps8
Mapk12
Tbr1
Nckap1
Pten
Cdkn1b
Med12
Baiap2
Nefl
Taf1
Grik2
Esr1
Fabp7
Pax6
Ep300
Nfia
Pde4a
Hoxb1
Nf1
Scn4a
Kcnd2
Stat1
Htr3a
Htr1b
Camk4
Zbtb16
Ppp1r1b
Grin2a
Scn5a
Drd2
Pde4b
Lrp2
Bcl2
Cnr1
Kit
Ank3
Kcnq2
Cacna1g
Nos1
Ocrl
Adcy3
Nrcam
Ptprc
Pde1c
Pln
Plcb1
Kcnq3
Gatm
Itpr1
Prkcb
Inpp1
Ace
Nrp2
Tyr
Maoa
Cacna1d
Th
Aldh1a3
Ass1
Cacna1a
Itgb3
Abat
Tnn
Hdc
Cacna2d3
Mtr
Gad1
Acy1
Gna14
Lamc3
Mthfr
Amt
Grm5
Slc6a1
Snap25
Oxtr
Homer1
Shank3
Stxbp5
Dlgap1
Nrxn3
Nlgn1
Cell surface receptor signaling pathway involved in cell-cell signaling
Regulation of transcription
Regulation of calcium ion transport
Regulation of ion transport

Figure 2