SAPIENZA
UNIVERSITÀ DI ROMA

SAPIENZA

University of Rome

PhD course in Genetics and Molecular Biology

PhD cycle: XXXIII

Differential expression of circular RNAs in a mouse model of autism spectrum disorders

PhD Student

Silvia Gasparini

Supervisor

Prof. Carlo Presutti

Coordinator

Prof. Fulvio Cruciani

INDEX

GLOSSARY

ASD	Autism spectrum disorder
ncRNAs	Non-coding RNAs
circRNAs	Circular RNAs
BTBR	BTBR T + tf/J
B6	C57BL/6J
lncRNAs	Long non-coding RNAs
DEC	Differentially expressed circRNA
DEG	Differentially expressed gene
DEmiRNAs	Differentially expressed miRNAs
miRNAs	microRNAs
mRNAs	messenger RNAs
RPBs	RNA Binding Proteins
Cb	Cerebellum
Pfx	Prefrontal cortex
HP	Hippocampus

ABSTRACT

Autism spectrum disorder (ASD) comprises a heterogeneous group of pervasive developmental disabilities characterized by compromised social interactions and communication skills, and by restrictive and repetitive behaviors. Emerging evidence suggests the involvement of non-coding RNAs (ncRNAs) in the pathophysiology of ASD. circRNAs arisen in the last decades as a novel class of ncRNAs and recent reports have shown their implication in the pathogenesis of several human neurological diseases. circRNAs are endogenous stable molecules, characterized by a covalently closed structure resulting from a backpslicing reaction. They are evolutionary conserved, abundant and significantly enriched in the brain. Although the biological function is still unknown, specific circRNAs are regulated by neuronal activity and have been implicated in plasticity mechanisms.

In this study, we present the expression profile of circRNAs in the hippocampus of BTBR T + tf/J (BTBR) mouse model for Autism Spectrum Disorder (ASD), compared to age-matched C57BL/6J (B6) mice. We identified several circRNAs whose expression is consistently altered, and 12 circRNAs and their corresponding linear counterparts were validated by RT-qPCR analysis. The ASD-related circCdh9 and circRmst have been further characterized in terms of molecular structure and expression. To evaluate their functional role in a physiological context, we characterized their expression during mouse development, neuronal differentiation and homeostatic plasticity. Interestingly, our results suggest a possible involvement of circCdh9 and circRmst in brain development and neuronal differentiation. Moreover, to comprehensively investigate the transcriptomic profile of the hippocampus of BTBR mice, we analyzed the gene and miRNA expression patterns. We performed enrichment analysis of BTBR differentially expressed RNA species and found interesting biological and molecular pathways associated to ASD phenotype. Lastly, we compared the circRNAs and gene expression profiles and we identified 6 genes highly modulated as circular and linear isoforms, indicative of a low correlation in the expression of circRNAs and their host genes.

In conclusion, our study has identified and analyzed differentially expressed circRNAs in the BTBR hippocampus, and we deepen characterized two ASD-associated circRNAs candidates. By integrating the circRNAs and gene expression profiles, we found a coregulation in the expression of specific genes ASD related. Moreover, we explored for the first time the miRNA expression profile in the hippocampus of BTBR mice, and we found 18 significantly modulated miRNAs. Functional studies are in progress to shed more light on the physiological function of circCdh9 and circRmst and their putative role in the pathophysiology of ASD.

INTRODUCTION

1. circRNAs Discovery Studies

The first evidence of circular RNAs was reported in 1976 by Sanger et al, who observed by electron microscopy viroids containing RNAs in the circular form [1]. Initially identified in viruses as "covalently closed circular RNA molecules", these circRNAs were found to be produced by endogenous RNAs following non-canonical splicing. The discovery of transcripts containing "scrambled exons" was made at first by Nigro et al and Coquerelle et al, who reported the expression of human circRNA isoforms from the DCC and ETS-1 genes, as stable cytoplasmic molecules [2], [3]. Since that time, the existence of circular RNA was reported in many studies [4]–[7] but for a handful of genes, and the low levels of expression led to consider these "non-linear mRNA" or exon-shuffling products [8], [9], as transcriptional noise.
In the last years, the advent of highly performative sequencing techniques and the application of *de novo* splicing algorithms, allowed to expand the number of circRNAs deriving from hundreds human genes[10]–[14]. After these initial reports, circRNAs were described in zebrafish, C. elegans, fruit flies and in other metazoans, and their identification in evolutionarily divergent species(plants, fungi, protists) led to revalue the circular RNA isoforms as a ubiquitous and conserved feature of the eukaryotic tree of life [15].

2. General Features of circRNAs

2.1 Characteristics and Methods of Identification

circRNAs is a class of long non-coding RNAs originated by a form of alternative splicing called backspling [2]–[4]. When the pre-mRNA splicing machinery joins a downstream 5' splice site(donor) to an upstream 3' splice site (acceptor), a covalently closed circular molecule is generated. The backsplicing junction region, the lack of free ends, polyadenylated tails and capping represent unique features that distinguish circRNAs from other RNA molecules. These characteristics have been exploited to identify circRNAs in different organisms, tissues, and cell types [10]–[12]. High-through RNA-sequencing of polyA depleted library, followed by the alignment of spliced reads spanning the backsplicing junctions to the reference genome, has revealed the presence of thousands of circRNAs [16], [17]. Several computational algorithms have been developed in the last decade [18]–[20], and the increasing number of circRNAs detected led to the production of multiple circRNAs databases [21]–[25]. Validation of circRNAs and their expression is required since the identified transcripts with scramble exons could represent artefacts of reverse transcription or the result of trans-splicing events [26]–[29]. The combination of independent biochemical methods such as the use of divergent primers in RT-PCR experiments, the treatment with RNAse R exonuclease [30], allows confirming the presence of the backsplicing junction and the circular conformation of the transcripts detected by RNA-seq.

2.2 Biogenesis, Degradation and Transport

circRNA biogenesis depends on the splicing machinery and competes with the formation of the linear transcripts [31], [32]. Generally, a backsplicing reaction is less efficient than linear splicing, since the intron looping that brings into proximity the downstream 5' splice site with the upstream 3' splice site result to be sterically unfavourable for the activity of the spliceosome machinery [33]. Moreover, studies demonstrated that circRNA levels increase when core spliceosome factors are inhibited [34]and that they represent the preferred RNA outcome when occurring readthrough transcription events. Abundant circRNAs have long introns with complementary repeats whose base-pairing facilitates circRNA biogenesis [12], [35]. There are two models proposed for circRNA formation (Fig. 1): the first is called "lariat-driven circularization", where canonical splicing leads to the production of a lariat intermediate. Both the internal splicing reaction or the debranching of the lariat can generate exonic circRNAs (ecircRNAs/E-circRNAs), exon-intron circRNAs (ElciRNAs) or intronic circRNAs (ciRNAs) [10], [12], [36], [37]. The "intron-pairing-driven circulation" model addresses to the flanking introns the formation of a circular transcript based on the sequence complementarity. Thus, inverted repeat elements enriched in the upstream and downstream introns, can mediate the base pairing and promote the circularization. In this regard, Alu sequences represent cis-acting elements involved in the backsplicing regulation [38]–[40]. The expression levels of circRNAs can vary on the efficiency of intronic sequences pairing: the secondary structure of the pre-mRNA can differentially regulate the circularization, thus originating different circRNAs from the same genes. Otherwise, a secondary structure whose high stability is mediated by the pairing of introns can inhibit the circRNA biogenesis [10]–[12], [14], [41], [42]. Despite the importance of intronic repeats, circRNAs biogenesis can additionally be influenced by the combinatorial action of trans-acting factors on cis-acting elements. RNA-binding proteins (RBPs) can increase the levels of circRNAs by binding specific recognition motifs in the flanking introns, as for the case of the RBPs Quaking (QKI)[43], the Drosophila muscleblind (Mbl)[31] or FUS [44]. On the other hand, several heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine-arginine proteins (SR) may interfere with the backsplicing reaction and reduce circRNA levels [45]. Also, both the adenosine deaminase (ADAR) enzymes [13], [38]which edit adenosine to inosine in double-strand RNAs, and the ATP-dependent RNA helicase A (DHX9), independently or cooperatively inhibit the formation of many circRNAs, by influencing the strength of interaction or unwinding the base pairing of the intronic inverted repeats [46], [47]. More recently, it was described that the NF90 NF110 proteins, which participate in the host antiviral response, are involved in the production of circRNAs by promoting the stability of the intronic repetitive elements [48].

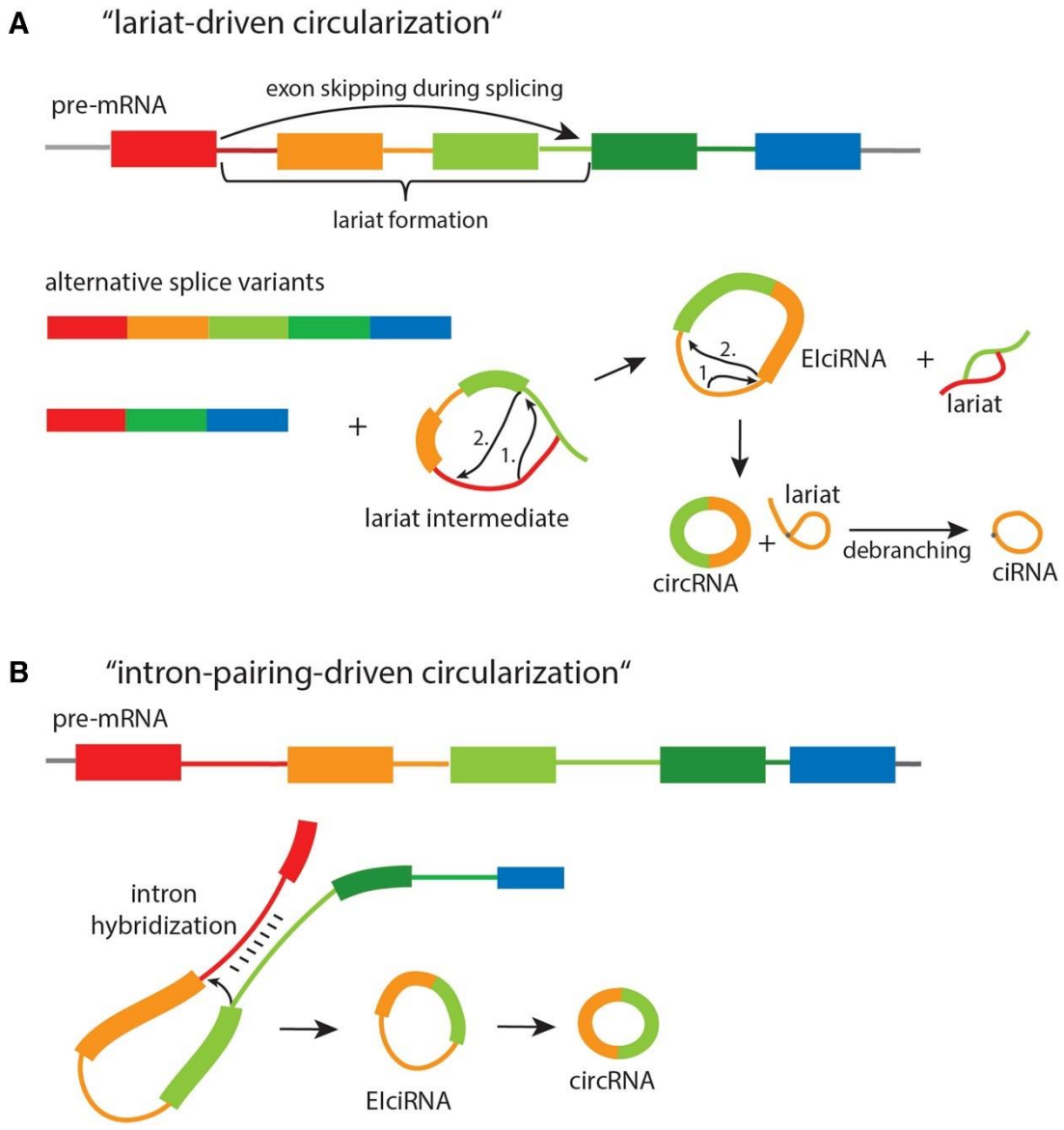


Figure 1. circRNA Formation. Lariat-driven circulation mechanism (A). Intron-pairing-driven circularization mechanism (B) (Santer et al., Mol. Ther. 2019).

In contrast to linear transcripts, the covalently closed structure confers to circRNAs high stability by preventing the action of RNA exonucleases. Thus, the half-lives of circRNAs have been demonstrated to be around 24 hours or longer [49]. It is still unclear how cells can degrade circRNAs. In recent years several mechanisms have been proposed for circRNAs turnover. The CDR1as/ciRS-7 circRNA contains a perfect complementary target site for the miRNA mir-671, and its cleavage and subsequent degradation is mediated by Argonaute-2 (Ago2) protein. This

specific regulatory pathway seems to be unique for CDR1as [50]–[52]. However, other RNA endonucleases appear to be involved in the elimination of circRNAs. The ribonuclease P (RNAse P) can trigger the degradation of circRNAs containing the N6-methyladenosine modification [53] and, following viral infection, circRNA molecules are subjected to the endoribonucleolytic cleavage of RNAse L [54]. circRNAs clearance may be achieved by exocytosis mechanisms. Some studies reported that some circRNAs are packaged into extracellular vesicles, like exosomes [55], [56], and that the active export can reduce the cellular levels of circRNAs, besides be relevant for cell communication.

The vast majority of exonic circRNAs are found in the cytoplasm [57], while a subset of ciRNAs and EIciRNAs is enriched in the nucleus [37], [58]. How exactly circRNAs are exported from the nucleus to the cytoplasm is poorly understood. A recent study, based on the RNAi screening of proteins involved in nuclear export in Drosophila DL1 cells, found that the DExH/D-box helicase Hel25E is necessary for the nuclear export of long circRNA (>881nt). The two human homologs UAP56 (DDX39B) and URH49 (DDX39A) are implicated in the export of long (>1298 nt) and short (< 356 nt) circRNAs, respectively [59] Therefore, the different length of circRNAs seems to regulate the trafficking outside the nucleus by recruiting specific binding proteins that may be considered as size sensors and actively export circRNAs.

3. circRNAs Biological Functions

3.1 circRNA as regulators of gene transcription and splicing

Due to the complementarity to the parental genomic DNA, circular RNAs localized in the nucleus can regulate their host gene expression. ciRNAs and EIcirRNAs have been described to localize near their promoter regions and modulate the host gene transcription efficiency by interacting with the RNA Po II and the U1 small nuclear ribonucleoprotein [37], [58]. Conn et al, observed that the circRNA SEP3 exon6 forms an R loop with the parental genomic DNA, inducing the stalling of RNA Pol II, and in turn promotes the expression of its cognate linear isoform lacking exon 6 through the recruitment of splicing factors [60]. The FECR1 circRNA has been demonstrated to act as a modulator of the epigenetic chromatin status of its host gene FL1 [61]. This nuclear circRNA acting as a modulator of DNA methylation and demethylating enzymes promotes the expression of FL1

3.2 circRNAs sponging activity

circRNAs can function as competing endogenous RNA (ceRNA) molecules by interacting with miRNAs and interfering with their activity (Insert in Fig. 2). The regulatory interaction between CDR1as and miR-7 has been described in detailed in several studies [11], [51], [62]. At first, Hansen et al, observed in CDR1as sequence the presence of 73 evolutionarily conserved miR-7 binding sites and their interaction was confirmed experimentally. The down-regulation of miR-7 targets after knocking-down CDR1as reinforced the hypothesis of a functional sponge activity [62]. However, the depletion of CDR1as locus in mouse led to contrasting results. The KO mouse

expressed a phenotype related to neuropsychiatric disorders, and mRNA targets of miR-7 were found to be up-regulated, while miR-7 expression levels were significantly decreased [51]. Kleaveland et al, established a regulatory network that assigned to CDR1as an entirely new function. According to this model, CDR1as binding prevents the degradation of miR-7 from the long non-coding RNA Cyrano, that harbours a high complementary site that induces miR-7 tailing and trimming, and in turns regulates miR-7 localization at the synapses [52]. There are other examples of circRNAs that bind to and regulate miRNA activity but, unlike CDR1as, most of them contain a reduced number of miRNA binding sites, as further demonstrated by genome-level analysis that found weak circRNAs enrichment for miRNA binding sites [36]. Moreover, the low expression of circRNAs contrasts with their potential affection for miRNA activity. Therefore, the miRNA sponge activity should be seen critically, although it represents the most and well-studied function [11], [12], [62]. Nevertheless, circRNAs binding to miRNAs might potentially imply other functions, besides ceRNA activity, such as regulating miRNA transport, stability or storage.

3.3 circRNAs interaction with proteins

The binding of circRNAs to proteins like RBPs, and the consequent functional interaction has been widely characterized [63]–[71]. circRNAs can function as a decoy, thus interfere with protein activity (Insert in Fig. 2). On the other hand, circRNAs may act as an enhancer of protein function, or as a scaffold by mediating multiple protein complex formation. Due to their high stability, circRNAs binding may exert the function of a molecular reservoir, by recruiting and localizing proteins in specific cellular compartments. Despite the extensively bioinformatic identification of RBPs consensus sequence on circRNAs [72], biochemical validation of these predicted interactions is necessary, since RBP binding sites are weak and parameters developed for linear transcripts should be reviewed for circular molecules.

3.4 Translation of circRNAs

circRNAs have been found to be translated both in vivo and in vitro studies [73]–[80]. However, the vast majority of circRNAs are considered not coding [14]. Since the lack of critical features for cap-dependent translation, such as the capping at 5’ and the poly(A) tail, circRNA can be translated in a cap-independent manner, through the presence of internal ribosome entry site (IRES) (Insert in Fig. 2). Moreover, engineered circRNAs with the N6-methyladenosine (m6A) modification in the 5’untranslated region have been demonstrated to be efficiently translated [81], [82]. Although bioinformatic analysis found thousands of circRNAs harbouring putative open reading frame [83], only a few of examples of endogenous circRNAs have been demonstrated to be translated. However, the identified circRNA-derived peptides are truncated versions of the full-length proteins [78], and they might have their function, or act as endogenous competitors of their linear counterparts. Studies are necessary to assess the functional relevance of most circRNA-derived truncated proteins, and prediction analysis revealed that these peptides are similar to the N-terminal regions of the host gene protein. Since some circRNAs are translated from IRES elements, upon specific cellular stimuli, the circRNA-derived peptide expression could regulate

stress responses or other physiological situations. Moreover, the accumulation of these peptides, due to the long-lived nature of circRNAs in cellular compartments, like neurons, could serve as memory molecules and encode information to be stored.

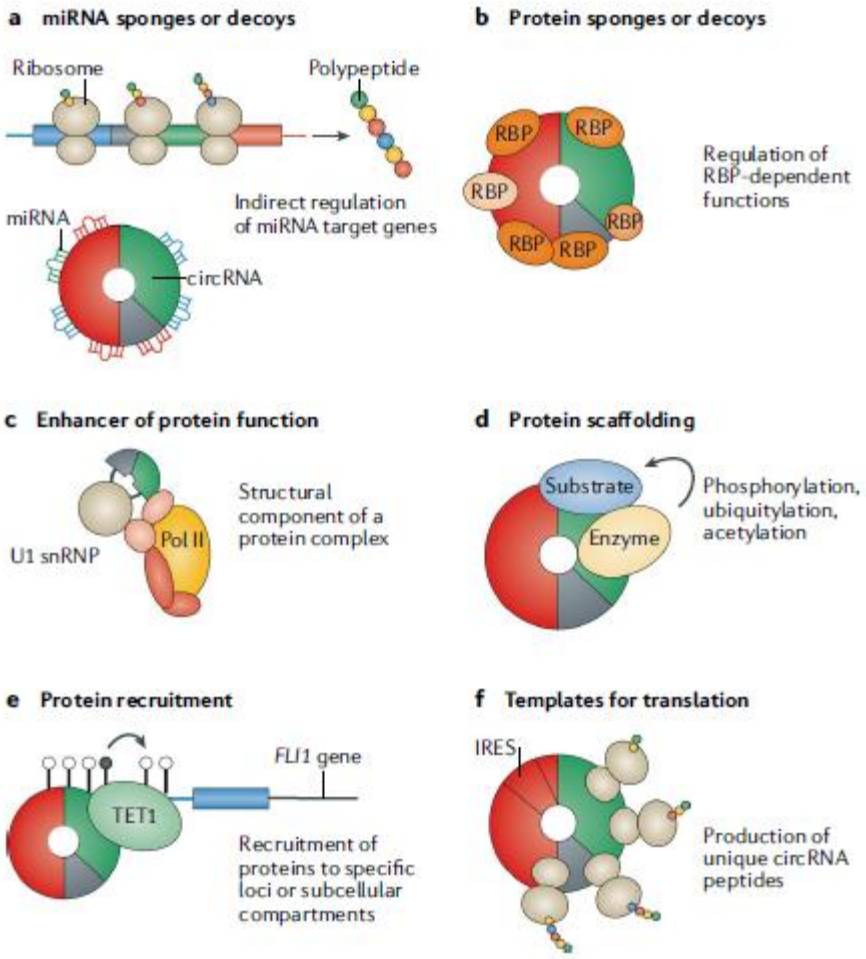


Figure 2. General mechanisms of circRNA functions (Kristensen et al., Nat Rev, Genetics 2019)

4 circRNAs in the brain

4.1 Neuronal circRNAs

Although backsplicing is generally less efficient than canonical splicing, thus leading to a low abundance of circRNA transcripts, some circRNAs show a higher expression than their linear counterparts. Several studies reported that both in mammals and flies many of the host genes producing circRNAs are exclusively expressed in the brain compared to other tissues [10], [11], [49], [84] and codify for proteins with regulatory functions in neurons and during brain development [13], [14]. Neuronal circRNAs are evolutionarily conserved among mammals, predominantly exonic with a higher percentage of exons subjected to alternative splicing, and in

many cases, their expression is independent of that of the linear counterparts [13], [14]. The enrichment of circRNAs in the brain might be addressed to several factors. The alternative splicing (AS) in the brain act as a general mechanism of gene expression regulation that allows responding to complex biological processes such as neuronal development and plasticity. Thus, the extensive use of AS might be indicative of the high amount of circRNAs in the nervous system. Their abundance could be related to the brain-specific expression of RBPs or other splicing factors that regulate circRNA biogenesis. Besides, the high stability of circRNAs and the low cell division rate of neuronal cells might contribute to their accumulation, mainly observed in aged-brain tissues

4.2 circRNA expression during neuronal development and plasticity

The majority of neuronal circRNAs are found in the cytoplasm, but an enrichment of circRNAs has been detected in synapses, as observed in murine synaptoneurosome fractions [14]. Moreover, they have been found in other subcellular compartments, like axons and dendrites, showing specificity of expression [13], [14]. Many studies reported an evolutionarily conserved and dynamically regulated circRNAs expression in the brain, suggesting that neuronal circRNAs might play a significant role in brain development [13], [14], [85], [86]. Differences in circRNA expression levels have been described both in developing and in terminally differentiated neurons. In murine hippocampal neurons, an increased expression of many circRNAs was detected at the time of the synaptogenesis onset, while a minority of circRNAs resulted in being down-regulated [14]. This shift in circRNAs expression might suggest an involvement of circRNAs in processes related to synapses formation and functioning.

Moreover, some circRNAs have shown a changing in their nucleocytoplasmic localization during development [14]. However, it is not clear whether this accumulation is the result of a regulated mechanism of transport or passive diffusion. Besides, circRNA expression and localization was found to be regulated by neuronal activity [14], [87]. By inducing a form of homeostatic plasticity in primary mouse neurons and in the mouse brain, altered expression of many circRNAs was revealed as well as changes in subcellular compartments localization [14] (Fig. 3). This level of regulation indicates that circRNA might specifically respond to and regulate neuronal activity. Similarly, age-specific expression changes have been observed in the brain tissues of several species [84], [88]–[90]. This regulated dynamic expression could imply a possible regulatory role of circRNAs in ageing processes.

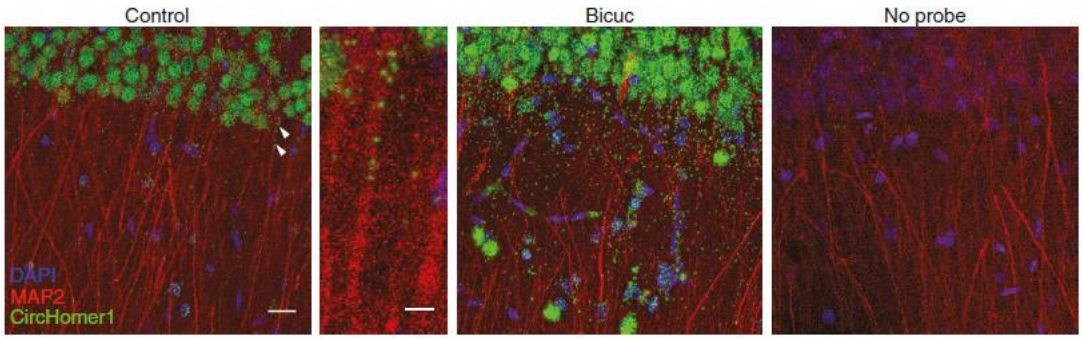


Figure 3. Modulation of circRNAs expression levels following homeostatic plasticity. In situ hybridization showing the significant upregulation of circHomer1 in hippocampal slices following bicuculline treatment (You et al., Nature Neuroscience 2015). Blue:DAPI; Green: circHomer1_a; Red: MAP2

4.3 circRNAs in neuronal diseases

circRNA expression changes have been found in many neurodegenerative and neuropsychiatric disorders. Disease studies in mouse models and human tissues reported a dysregulated expression of circRNAs, potentially due to a deregulation of their biogenesis. Neurological disorders might rely on alterations of cis-elements or trans-splicing factors that influence circRNAs expression. Recently, it has been observed a correlation between SNPs and the expression of circRNAs, suggesting that the alteration of the splicing sites might deregulate circRNAs levels[91] . RBPs known to be involved in the biogenesis of circRNAs have been associated with several neurological diseases, demonstrating that AS defects can influence circRNAs expression. Post-mortem studies on several neurodegenerative and psychiatric disorders such as schizophrenia, amyotrophic lateral sclerosis (ALS), bipolar disorder revealed an overall dysregulation of circRNA expression [92]–[96]. Moreover, circRNAs known to be expressed in the brain and to exert functions like RBPs decoy or miRNA sponging, have found to be altered in the neuronal tissues of patients with neurological diseases, suggesting that circRNAs expression changes might contribute to the pathological phenotype. The case of CDR1as is one of the best characterized, and the dysregulation of CDR1as/miR-7 interaction has been suggested to play a regulatory role in the Alzheimer disease (AD) and Parkinson disease (PD) pathogenesis [97]–[100]. In this context, it has to be mentioned the recent regulatory network axes that involved miRNA-circRNA-mRNA, whose alteration has been indicated in the pathogenesis of neuronal diseases [101]–[103]. Based on the bioinformatic identification of miRNA target sequences on circRNA, most studies correlate the expression levels of circRNA to the predicted miRNA targets mRNA levels. This regulatory effect is likely due to the sponge activity exerted by the circRNAs on their putative miRNA target, whose expression and function should show an opposite regulation. In addition to their putative roles in neurological diseases, circRNAs can be considered excellent biomarker candidates. Their high stability, cell-specific expression pattern, conservation and abundance in blood have already elected circRNAs as suitable biomarkers for several types of cancers. Moreover, many groups detected circRNAs in other human biofluids, such as saliva, plasma as well as in exosomes and extracellular vesicles [104]–[109]. The identification and the potential use of circRNAs as

biomarkers for neuronal diseases would be fundamental since the inaccessibility of brain or spinal cord tissues. In many diseases, such as AD, PD or ALS, the observed alteration of the blood-brain barrier would allow the release of free or exosome- endowed circRNAs in the periphery of the body. Therefore, disease-related circRNA could be released into the circulation, and their altered levels might enable to identify the disease, as well as its progression and response to treatments

5 circRNAs in neurodevelopmental diseases: Autism Spectrum Disorder (ASD)

5.1 General features of ASD

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social communication and restricted and repetitive interests and behaviours [110], [111]. These core symptoms usually coexist with other psychiatric and medical conditions, including intellectual disability, epilepsy, anxiety and gastrointestinal and metabolic problems. The prevalence of ASDs across most countries is roughly 1 per cent of the total population. It is the fastest-growing developmental disability, and research shows that early intervention leads to positive outcomes later in life for people with autism [112]. There are different brain regions involved in the pathogenesis of ASD. Among these, anatomical and functional alterations have been identified in the cerebellum, hippocampus and corpus callosum. Moreover, neuroimaging studies of children with autism have revealed abnormal brain overgrowth in prefrontal and temporal regions [113].

5.2 The molecular genetics of ASD

ASD is a heterogeneous genetic disorder and the common, rare and de novo mutations found to contribute to the risk of the disorder, can account for only 1% of all cases of ASD [114], [115]. Many of the risk genes that have been associated with ASD are crucial components of the activity-dependent signaling networks which regulate synaptic development and plasticity, such as synaptic cell-adhesion molecules, components of the postsynaptic density or receptors. These ASD-risk genes are regulated by neuronal activity and modulate synaptic strengths, turnover and structure since their gene products are involved in chromatin remodeling, transcription, protein expression or cytoskeletal architecture [113], [116], [117]. Consequently, mutations that occur in these genes lead to the disruption of the normal process of experience-dependent synaptic development, resulting in alterations in excitatory or inhibitory neurotransmission. Studies suggested that the high degree of genetic heterogeneity of ASD converge on common molecular pathways implicated in the activity-dependent developmental processes [110]. Since most of ASD features manifest in the first few years of life, corresponding to a critical window for brain development and synapses maturation, ASD has been recently defined as a “synaptic-defect disease” [116], and the dysregulation of the activity-dependent signaling networks that control synapse refinement might play a crucial role in the etiology of ASD.

5.3 Splicing defects and altered levels of non-coding RNAs

Among the molecular mechanisms whose failure have been proposed to cause or contribute to ASD phenotypes, the alternative splicing results particularly relevant. Aberrant RNA processing of synapse-related genes has been observed in autistic patients, suggesting that splicing defects can be considered risk factors [118]. Genetic variants that occur in cis-regulatory splice sites within known risk genes might determine alternative splicing dysfunction, that in turn could influence the inhibitory and excitatory neurotransmission in brain areas related to behavioral autistic phenotype [110], [114], [116]. Similarly, mutations that affect expression levels and activity of trans-splicing factors can produce atypical splicing patterns [119]. Recent studies investigated the relevance of non-coding RNAs in ASD. miRNAs represent regulatory molecules that can interfere with mRNA stability and/or impair mRNA translation. They have been demonstrated to act as key regulators of processes that mediate neurogenesis and synaptic plasticity. Genome-wide transcriptome analysis in brain structures of post-mortem ASD patients, revealed dysregulated expression levels of miRNAs, suggesting a potential contribution of this class of ncRNAs autism pathological condition [119]–[122].Until a few years ago, a direct association between circRNA expression levels has not yet been demonstrated. However, splicing factors involved in the regulation of circRNAs abundance, have been reported to be dysregulated in ASD brain, suggesting that cirRNAs could exert a role in this disorder.
As mentioned before, a high enrichment of circRNAs at synapses and regulated expression during neurogenesis and neuronal activity has been extensively characterized, suggesting that circRNAs might play essential roles in neuronal development, synaptic formation and activity. circRNAs putative involvement in ASD has been recently proposed by two studies, which observed in both human post-mortem brain tissues of ASD patients and in a mouse model for ASD, an altered expression profile of many circRNAs [103], [123]. Moreover, some studies reported that many circRNAs originate from host gene loci associated with atypical neurodevelopmental phenotypes, included ASD, such as RMST and CDH9 [124]–[126]. Besides these findings, the potential role of circRNAs in the etiology of neurodevelopmental disorders is poorly understood.

5.4 Cdh9 and Rmst: gene loci associated with neurodevelopmental functions

Cdh9 belongs to the cadherin superfamily, which has been reported to exert important roles in the core developmental events that are essential for the proper assembly of neural circuitry, such as synaptogenesis, dendrite arborization and dendritic spine regulation [127]–[130]. The recent genetic association of Cdh9 with autism suggests that the protein-coding gene for this type II cadherin might be implicated in the pathogenesis of ASD. The developmentally regulated expression of Cdh9 observed in several ASD-relevant brain regions and its identification as an autism risk gene, strongly support Cdh9 role in neuronal developmental processes [131]–[134]. Although circCdh9 physiological functions are still unknown, in our recent report, we described a deregulated expression profile of this circular isoform in ASD-related brain regions of the BTBR mouse [123].

The long non-coding Rmst represent a crucial regulator of neurogenesis. In the nucleus of neuronal progenitors, the lncRmst has been found to interact with the transcriptional factor SOX2. The recruitment on the promoters of SOX2 target genes is mediated by the binding with lncRmst, that in turns induces the neuronal differentiation [135]. The Rmst gene locus drives the expression of

several circular isoforms [136], one of which has been described to be enriched in synaptic compartments of hippocampal neurons, such as cell bodies and dendritic projections. Moreover, changes in the expression of circRmst have been observed during neuronal differentiation and in the developing mouse brain, suggesting a potential role in brain development [13], [14]. Together with circCdh9, we found altered expression levels of circRmst in the BTBR brain regions relevant for ASD pathophysiology [123].

As mentioned before, non-coding RNAs have been implicated in many neurological diseases, and emerging evidence reinforce circRNAs involvement in neurodevelopmental processes. Thus, the investigation of circCdh9 and circRmst expression during neuronal development and synaptic plasticity would allow understanding their functions in normal and pathological conditions, such as ASD.

5.5 Mouse models of ASD

The diagnosis of ASD relies on the evaluation of behavioural phenotypes defined by the two core symptoms of impaired social interaction and communication, and restricted interests. Moreover, the clinical and molecular heterogeneity of ASD represents a significant challenge in the modelling of the disease in rodents. A valid mouse model ideally should reproduce the molecular defects and show behavioural analogues observed in ASD patients. Many genetically engineered mice models have been designed for those ASD cases of which the causative genes or chromosomal alterations are supported by genetic evidence. Among these, are to be mentioned mutant mice for genetic syndromes, such as Fragile X or Rett syndrome, non-syndromic ASD cases due to pathological mutations in genes such as Neurologin or Shank genes, and ASD cases associated with CNVs [137]–[141]. These genetic models recapitulate the dysfunctional behaviours observed in humans, according to well-established and acknowledged behavioural tests. However, the wide spectrum of behavioural phenotypes observed in ASD is far from being reproduced in a single-gene mouse model.

5.6 The BTBR T+tff J mouse: an ASD non-genetic model

Several non-genetic models that demonstrate ASD-related behaviours have been developed and characterized [142]. The prenatal exposure to inflammatory or teratogen agents in mice has been demonstrated to induce aberrant gene expression, morphological brain changes and altered behavioural phenotypes which are correlated to ASD in humans.
The BTBR T1tf/J inbred mouse strain, initially used for diabetes and phenylketonuria studies, exhibits behavioural phenotypes consistent with the two diagnostic symptoms of ASD. Reduced social interaction, impaired socialization and defects in communications, repetitive behaviours such as the high levels of self-grooming and unusual pattern of vocalization, compared to C57BL/6J (B6) control mice, have been observed [143]–[147]. Moreover, BTBR mice display severe anatomical and neurological features resembling those observed in ASD diagnosed patients. In particular, they show a reduced hippocampal commissure, agenesis of the corpus callosum, alteration in the neurogenesis and developmental migration of hippocampal neurons [148], [149]. However, many of the alterations detected in BTBR mice are found in a small fraction of ASD cases suggesting that BTBR autistic-like phenotype might reproduce only a few ASD characteristics.

Studies in the genetic background of BTBR mice revealed single nucleotide polymorphisms in autism candidate genes, while sequencing analysis of the coding and non-coding transcriptome in several BTBR brain regions, compared to B6 control mice, detected altered expression levels in genes associated with the autism-related phenotype[150], [151] . Thus, providing both behavioural, genetic and molecular autism-relevant characteristics, the BTBR mouse represent a valid model to investigate the pathophysiological mechanisms underlying the behavioural symptoms of ASD.

AIMS OF THE STUDY

Recent studies demonstrate that non-coding RNAs (ncRNAs) participate in the fine-tuning programs of regulation of gene expression occurring in neuronal signaling. Among these, circular RNAs (circRNAs) represent a novel class of RNA molecules characterized by a covalently closed structure that gives them high stability, and an extraordinary enrichment in the mammalian nervous system. Interestingly, some circRNAs are dynamically regulated during neuronal developmental processes and in synaptic plasticity mechanisms. These findings suggest the involvement of circRNA in many brain functions. Moreover, growing experimental evidence indicates their implication in several human neurological diseases.

To investigate the potential contribution of circRNAs in the pathophysiology of ASD, we explored the circRNAs expression profile in the hippocampus of BTBR mice. We identified several circRNAs highly deregulated in the BTBR hippocampus, and we decided to deepen study two ASD-related circRNAs: circCdh9 and circRmst. circCdh9 host gene codifies for a type II cadherin involved in neuronal circuit formation, and it has been recently associated with ASD. The Rmst gene locus drives the expression of the long non coding RNA (lncRNA) lncRmst, which is involved in neuronal differentiation. Due to the implication of their host genes in neuronal functions, related to synaptic connections and development, we performed experiments aimed at clarifying the involvement of the circular transcripts in both processes as well.

With this purpose we performed expression profile analysis of circCdh9 and circRmst, and in parallel of Cdh9 mRNA and lncRmst, during mouse brain development, neuronal differentiation and synaptic plasticity.

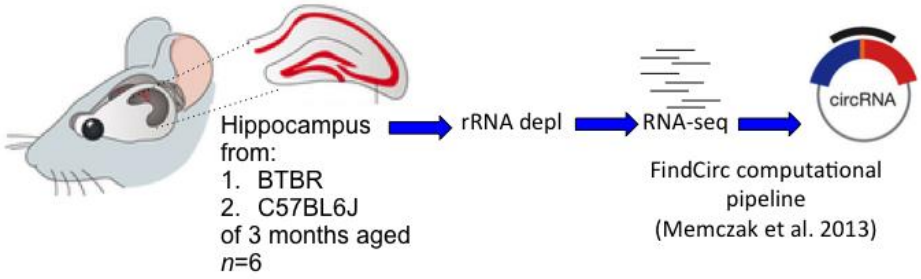
To comprehensively characterize the transcriptional landscape of the hippocampus of BTBR mice compared to B6 control mice, we explored the gene and miRNA expression profiles. Gene ontology and pathway enrichment analyses allowed us to investigate the relevance of the dysregulated BTBR transcriptome profiles in the context of the ASD disease.

RESULTS

1. Identification of circRNAs highly modulated in the hippocampus of BTBR mice

1.1 circRNA expression profile

To comprehensively characterize the circRNA expression profile in the hippocampus of BTBR and B6 mice, we prepared ribosomal-depleted total RNA samples from two independent cohorts of both strains (n=10). RNA sequencing data were obtained from cDNA libraries synthesized with random primers and analyzed by paired-end high depth sequencing. We detected circRNA candidates by applying specific computation pipeline that uncovers the head-to-tail junctions (Fig. 4A) [11]. We identified 4625 unique candidate circRNAs, 3796 of which already annotated in public circRNA databases (circBASE, http://www.circbase.org/) [21]. The majority of head-to-tail junctions were supported by few reads, while a subset of circRNAs was found to be expressed at high levels, as observed for circTulp4 and circHipk3



(Fig. 4B).

Figure 4A. Schematic representation of the experimental procedure applied to perform circRNAs expression profile in the hippocampus of BTBR and B6 mice

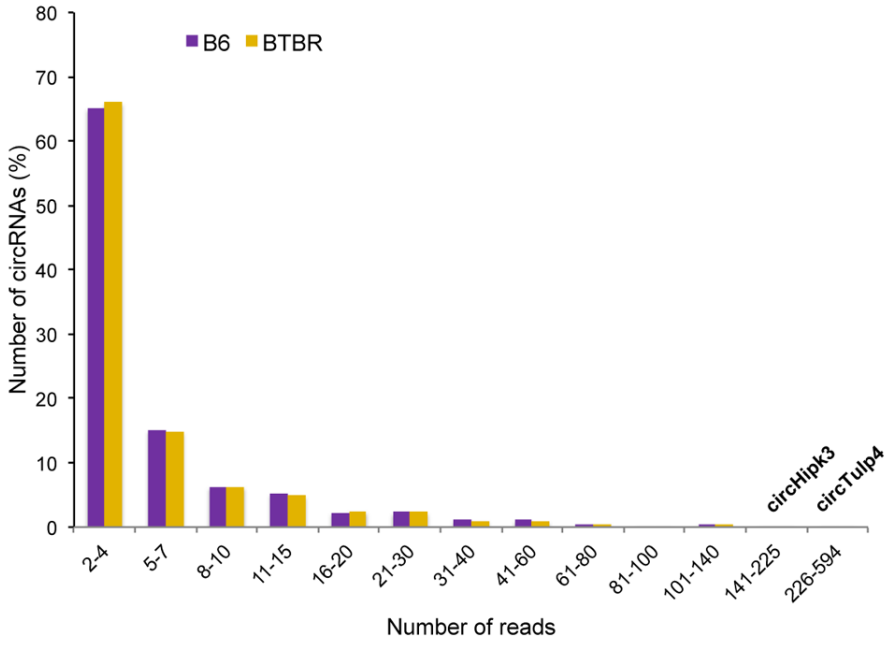


Figure 4B. Histogram showing the abundance of circRNAs detected in the RNA-seq analysis. The majority of hippocampal head-to-tail junctions detected in B6 and BTBR were supported by few reads. Highly expressed Hipk3 and circTulp4 are indicated

We identified 288 high-confidence circRNAs that were supported by at least ten mean reads and showed circular/linear mean ratio higher than 0.5. In line with previous studies on neuronal circRNAs, 93% of the high-confident circRNAs detected in our study derived from protein-coding genes [13], [14]. Similarly, a high percentage of the head-to-tail junctions of selected circRNAs arise from the coding sequence exons (Fig. 5). These features indicate that the majority of the identified hippocampal circRNAs belong to the class of exonic circRNAs.

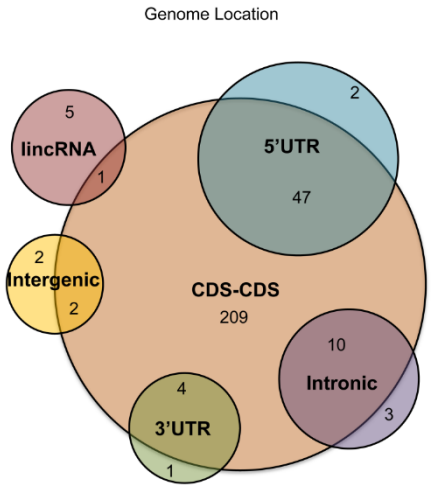


Figure 5. High confidence circRNAs mainly derive from exonic coding regions of host genes

We bioinformatically investigated the enrichment of the selected circRNAs host genes for metabolic or cell signaling pathways. In the Panter pathway analysis, we found significant enrichment for terms related to the metabotropic and ionotropic glutamate receptor pathways (Fig. 6).

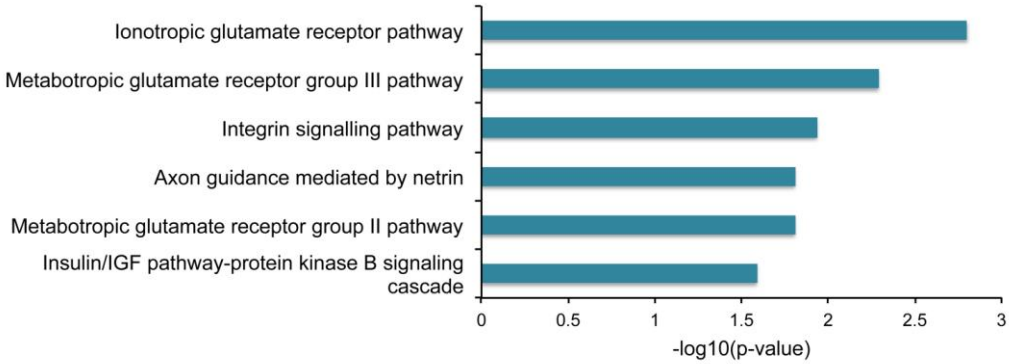


Figure 6. **Panther pathway enrichment analysis of host genes for selected hippocampal circRNAs shows significant enrichment for glutamate receptor signaling and neuronal development and morphogenesis.** GO terms with p value ≤ 0.05 are listed (statistical significance is expressed as −log10 of p values).

We compared the expression profiles of circRNAs in the BTBR and B6 hippocampus and observed a general decreased expression of circRNA in BTBR mice. Specifically, we detected 29 downregulated and 12 upregulated circRNAs (Fig. 7). Among these differentially expressed circRNAs (DEC), we found circRNAs isoforms undetected in either BTBR or B6 hippocampus. Two of them, circMyrip and circWdr49, have been identified for the first time in this study.

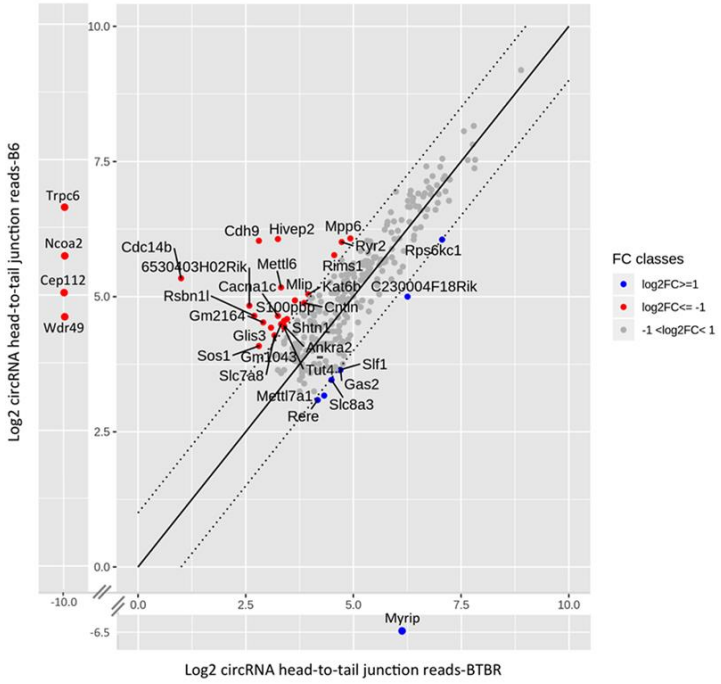


Figure 7. Scatter plot representing the circRNAs differentially expressed in the hippocampus of BTBR (X axis) compared with B6 mice (Y axis). Absolute circRNAs levels are reported. Scale breaks to X and Y axes allow the inclusion in the figure of highly regulated circRNAs isoforms (negative values of X and Y axes).

To assess whether circRNA expression was independent or not from that of their linear counterparts, we analyzed the relative abundance of circular and linear isoforms (circ/linear ratio). The analysis revealed a striking difference in the circ/linear isoforms ratio between BTBR and B6 mice (Fig. 8), suggesting that circRNAs expression changes were independent of those of linear transcripts.

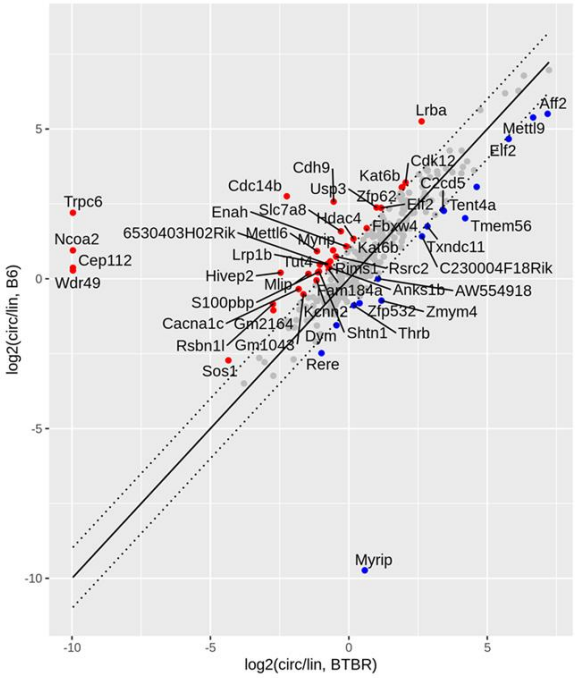


Figure 8. Scatter plot reporting the ratios of circular isoform to the corresponding linear (circ/lin) isoforms

1.2 Validation of Selected BTBR-Regulated circRNAs

To validate with a different technical approach the expression levels of the circRNA candidates, we performed RT-qPCR analysis of selected highly modulated circRNAs identified in the RNA-seq experiments (log2FC ≥ 1 and ≤ -1). By analyzing hippocampal RNA pools derived from BTBR and B6 mice, we validated the differential expression of 12 circRNAs: 9 downregulated and 3 upregulated BTBR circRNAs (Fig. 9). Forward primers specific to the circular isoforms were complementary to the backsplicing junction. Primers for linear isoforms were positioned in exons not included in the circular ones. Expression data obtained by RT-qPCR experiments were consistent with those observed in the RNA-seq analysis, apart from circAuts2 whose expression is not significantly altered in BTBR compared to B6 mice. We confirmed the absence of a deregulated expression of circRyr3 and circTshz2, that we included in our analysis as negative controls. We found an independent and opposite regulation of circular and linear isoforms derived from Hivep2, Zcchc11 and Wdr49 genes, while the majority showed a similar trend in the expression of linear and circular isoforms, this is for instance the case of the downregulated Cdh9 and the upregulated Rmst transcripts.

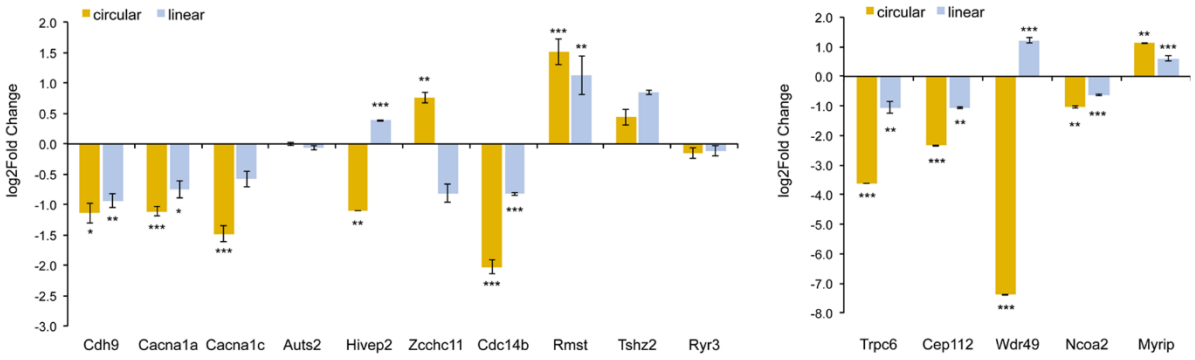


Figure 9. Validation of the differential expression of 12 circRNAs by RT-qPCR. RT-qPCR analysis of circular and linear transcripts was performed on RNA pools (n = 10) from BTBR and B6 mice. Schematic representation of primers used for circular and linear detection are reported. Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).Data are expressed as log2FC of BTBR vs B6 (log2Fold Change = 0). Genes for highly regulated circRNAs are reported in a separate graph on the right.

To summarize, we identified a list of 288 high-confident circRNAs, most of them deriving from protein coding genes. Host genes are enriched in ontogenetic categories related to glutamate receptors, neuronal development and synapse formation. Of the 41 differentially expressed circRNAs in the hippocampus of BTBR mice identified by RNAseq, we were able to confirm the deregulated expression of 12 circRNAs.

2. Characterization of the BTBR DECs produced from the Cdh9 and Rmst genes

We further characterized two circular RNAs differentially expressed in BTBR hippocampus, circCdh9 and circRmst

circCdh9 derives from the Cdh9 gene locus which codifies for a type II cadherin. Cdh9 is implicated in brain development [127]–[130], and its locus is genetically associated with ASD [125], [132]. A circular isoform with an expected spliced length of 1025 nt, has been already identified in several brain regions, including the hippocampus [13] and it is highly conserved between mouse (mmu_circ_0005545) and human (hsa_circ_0128803). To date, it represents the only circular isoform derived from the Cdh9 locus. As previously mentioned, we detected an altered expression of both circular and linear Cdh9 transcripts isoform in the BTBR hippocampus.

To confirm the backpslicing junction sequence, we performed RT-PCR analysis by using two divergent primers designed on the predicted exons within the transcript (Fig. 10). By Sanger sequencing of PCR products, we validated both the head-to-tail junction, encompasses exon 2 and 6, and the exons included within them (Fig. 11A).

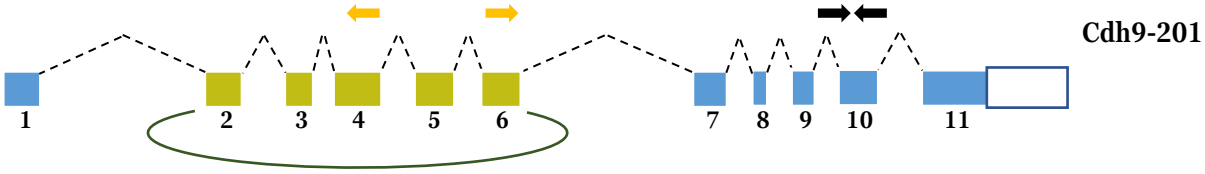


Figure 10. Schematic representation of mouse Cdh9 genomic locus. Divergent primers utilized for circCdh9 analysis are reported in yellow. Primers used for mRNA detection are shown in black

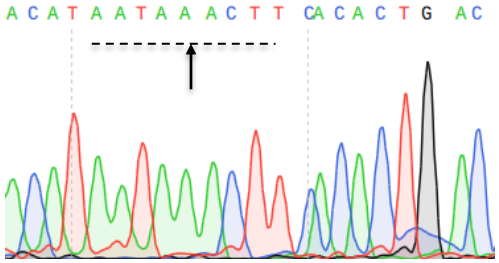


Figure 11. Chromatogram of the circCdh9 head-to-tail junction sequence from back-splicing. Black arrow and the dashed line indicate the head-to-tail junction

The Rmst gene locus codifies for the lncRmst, a key regulator of neurogenesis processes [135], [152], and several circular isoforms [136]. In our RNA-seq analysis, we identified a circRmst isoform of 1.5 kb (circRmst 1.5Kb) differentially expressed in the hippocampus of BTBR mice. circRmst 1.5 Kb has been previously described in two studies [13], [14] that showed its neuronal cytoplasmic expression and localization in synaptic compartments. circRmst 1.5 Kb derives from two non-canonical splicing events: a backpslicing and a trans-splicing reaction, involving exonic sequences of two different linear isoforms, Rmst-205 and Rmst-209 transcripts (Ensembl database annotation, http://www.ensembl.org/). By combining several divergent primer pairs to PCR amplify the entire molecule, we sequenced the backsplicing junction and the full-length sequence of circRmst 1.5 kb (Fig. 12). Our sequence analysis confirmed the head to tail junction sequence between the exons 4 of Rmst-209 and Rmst-205 transcripts (Fig. 13). By analyzing the full length sequence of circRmst, we found that exon 8 is excluded from the transcript and that the trans-splicing event occurs between the exon 12 and exon 3 of Rmst-209 and Rmst-205 transcripts, respectively. Moreover, we found a high degree of circRmst sequence conservation between mouse and human.

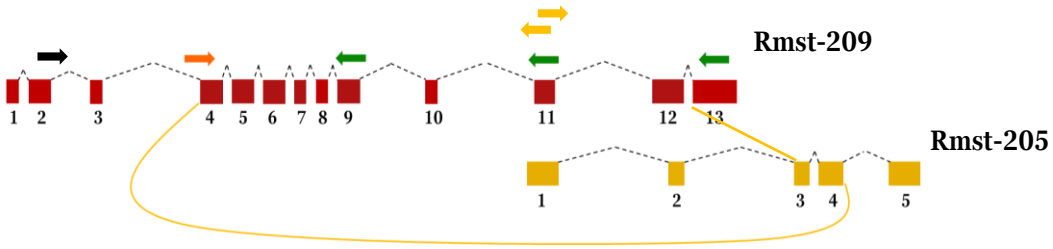


Figure 12. **Schematic representation of mouse Rmst-209 and Rmst-205 transcripts.** Divergent primers utilized for circRmst analysis are reported in yellow.The orange forward primer was used in combination with several reverse primers, shown in green, to detect the full sequence of circRmst. The forward primer used for mRNA detection is shown in black

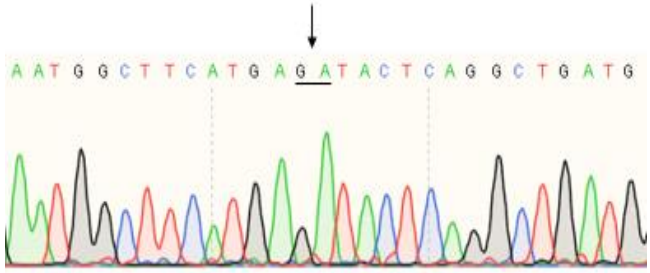


Figure 13. Chromatogram of the circRmst head-to-tail junction sequence from back-splicing. Black arrow and line indicate the head-to-tail junction

2.1 circCdh9 and circRmst expression analysis in BTBR ASD-related brain regions

Hippocampus, as well as the cerebral cortex and the cerebellum, are considered relevant brain regions in ASD [153]–[155]. Therefore, we decided to investigate the expression of circCdh9 and circRmst in the cerebellum and prefrontal cortex of BTBR and B6 mice, and in parallel, we analyzed the expression of their linear counterparts.

We observed that the levels of both circular and linear Cdh9 RNA isoforms were similarly altered in all the BTBR brain regions analyzed (Fig. 14A). Conversely, the circRmst upregulation appeared to be hippocampus specific, while the linear isoform resulted significantly upregulated in the cerebellum of BTBR mice (Fig. 14B).

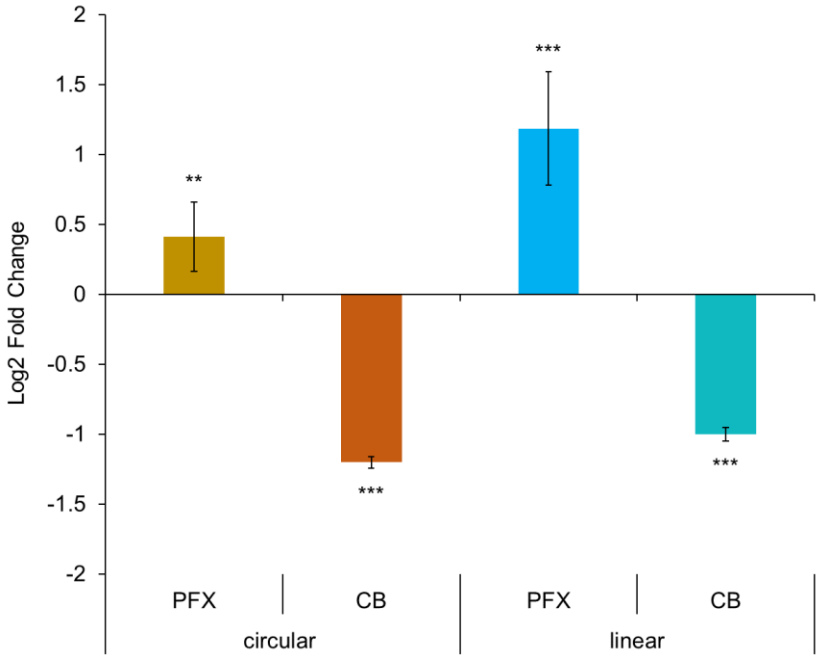


Figure 14A. Expression profile analysis of circular and linear Cdh9 transcripts in the different brain regions of the BTBR mouse model. RT-qPCR analysis was performed on RNA pools (n = 6) from different brain regions of BTBR and B6 mice (prefrontal cortex, PFX; cerebellum, CB). Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of BTBR vs B6 (log2Fold Change = 0)

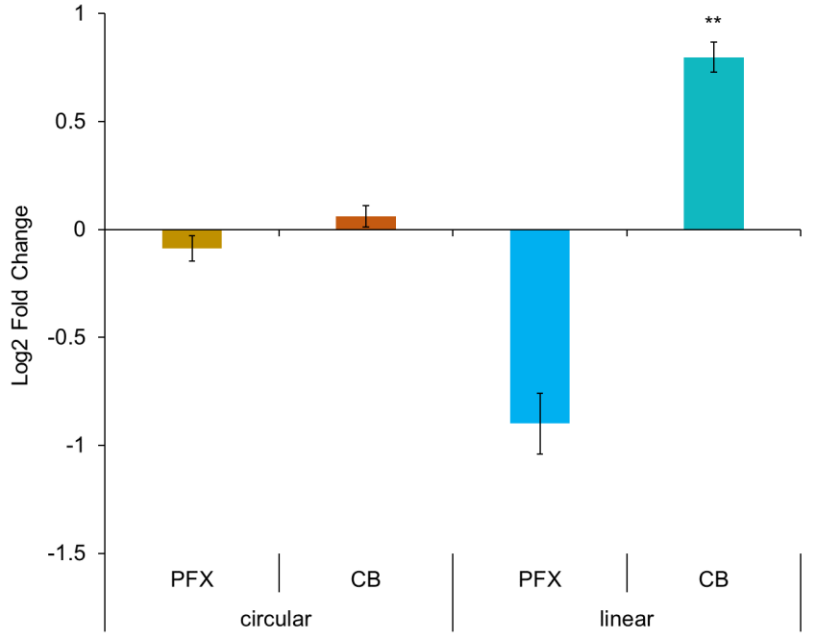


Figure 14B. Expression profile analysis of circular and linear Rmst transcripts in the different brain regions of the BTBR mouse model. RT-qPCR analysis was performed on RNA pools (n = 6) from different brain regions of BTBR and B6 mice (prefrontal cortex, PFX; cerebellum, CB). Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of BTBR vs B6 (log2Fold Change = 0)

In conclusion, we characterized two BTBR-related circRNAs, circCdh9 and circRmst, in term of sequence and expression in ASD-related brain regions. Finally, the circCdh9 downregulation in the cerebellum of BTBR mice equally was observed in the hippocampus, while the only Rmst linear isoform exhibited an altered expression in the same brain region.

3. Expression studies of circCdh9 and circRmst during brain development, neuronal differentiation and synaptic plasticity

3.1 circCdh9 and circRmst expression at early and late developmental stages of CD1 mouse brain

Previous studies demonstrated the critical roles of circCdh9 and circRmst host genes in regulating synapse formation[127]–[130] and neurogenesis [135], [136], [152], respectively. Moreover, experimental evidence showed that the expression of some circRNAs is dynamically regulated during neuronal development [13], [14]. Therefore, we decided to explore the expression profile of circCdh9 and circRmst during mouse brain development. The expression of circular and linear counterparts was studied in the hippocampus, the cerebellum and the prefrontal cortex of CD1 mice, across four brain developmental stages: late embryonic development (E17) and three different postnatal developmental stages (P0, P10, P30).

In the cerebellum, circCdh9 and Cdh9 mRNA exhibited a strong downregulation in postnatal life compared to E17 (Fig 19). In the hippocampus, we observed an opposite trend of expression for both isoforms, with an upregulation at P10 and P30. In the prefrontal cortex, in general, we observed a slight variation of circCdh9 and Cdh9 mRNA levels, with a mild increase at the early postnatal stage (P0) (Fig. 15A).

The analysis of the expression profile of circRmst and the linear isoform lncRmst revealed a general decrease in their expression level during postnatal life, starting at P0 in the prefrontal cortex and the cerebellum at P10 in the hippocampus (Fig 20). Interestingly, we observed a mild increase of circRmst at the latest developmental stage (P30) (Fig. 15B).

In overall, we observed temporal and region-specific dynamic changes in circRNAs expression. Both circular and linear transcripts of Cdh9 exhibited similar expression profiles. Remarkably, the expression level of circCdh9 is higher than of its linear counterpart.

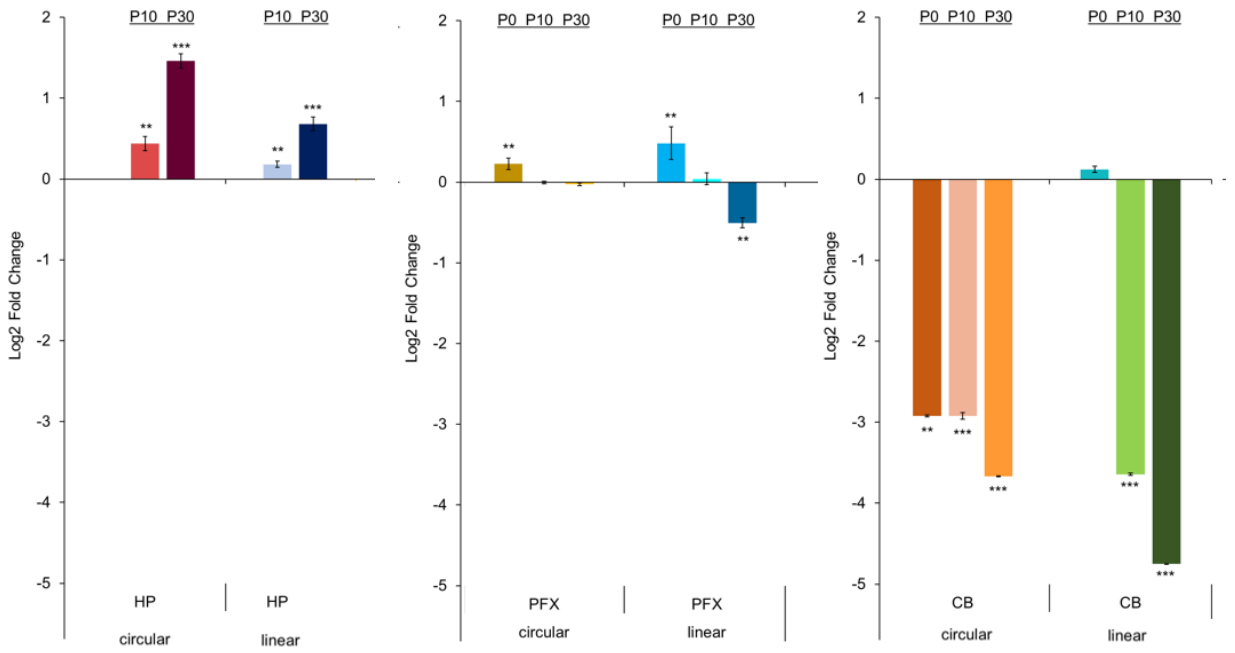


Figure 15A. Expression profile analysis of circular and linear Cdh9 transcripts during mouse brain development. RT-qPCR analysis was performed on RNA pools (n = 3) from hippocampus (HP), prefrontal cortex (PFX), cerebellum (CB). Six to nine technical replicates were performed for each gene product analyzed (two-sided Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of P0, P10, P30 vs E17 for PFX and CB expression analysis, vs P0 for HP expression analysis (log2Fold Change = 0)

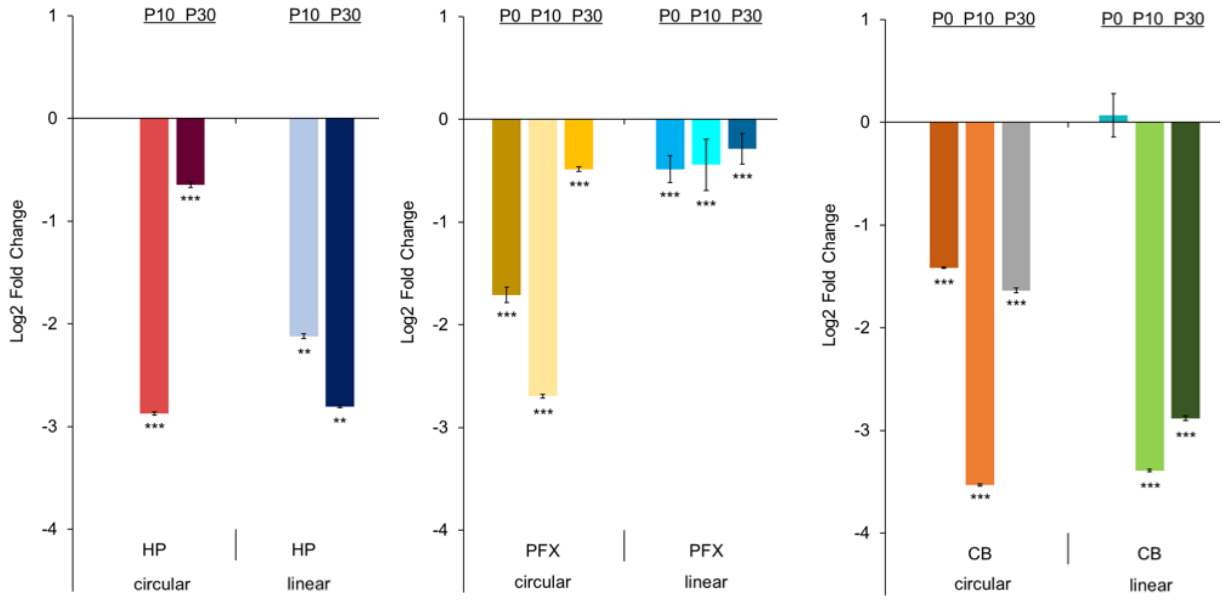


Figure 15B. Expression profile analysis of circular and linear Rmst transcripts during mouse brain development. RT-qPCR analysis was performed on RNA pools (n = 3) from hippocampus (HP), prefrontal cortex (PFX), cerebellum (CB). Six to nine technical replicates were performed for each gene product analyzed (two-sided Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of P0, P10, P30 vs E17 for PFX and CB expression analysis, vs P0 for HP expression analysis (log2Fold Change = 0)

3.2 circCdh9 and circRmst expression during cortical neurons differentiation

To evaluate the expression of circCdh9 and cirRmst during neuronal differentiation we analyzed RNA levels in primary cultures of mouse cortical neurons. We obtained cortical neurons from mouse embryos cortex (E17) and we maintained in culture till day 21 (DIV21). RNA was collected at different times of in vitro culture (DIV4, DIV11, DIV 21). As indicated by RT-qPCR experiments (Fig. 16A), the expression of Cdh9 transcripts shows a bell-shape trend, with an increase from DIV4 to DIV11 and a downregulation from DIV11 to DIV21. As far as concerning circRmst, we observed a strong upregulation during neuronal differentiation. Similarly, but to a lesser extent, an increase in the expression of lncRmst was observed (Fig. 16B).

To conclude, during neuronal differentiation, we detected different expression patterns of circCdh9 and circRmst. Interestingly, while Cdh9 transcripts show similar regulation during neuron maturation, completely distinct expression patterns were observed for lincRmst and circRmst

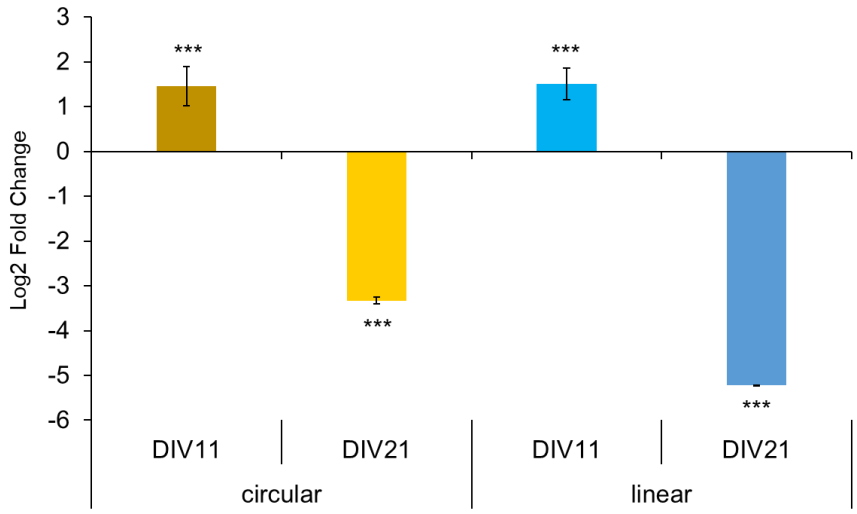


Figure 16A. Expression levels of circular and linear Cdh9 transcripts during neuronal differentiation. RT-qPCR analysis was performed on RNA samples of mouse cortical neurons at DIV4, DIV11, and DIV21. Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ are referred to the comparison of DIV21 or DIV 11 vs DIV4). Data are expressed as log2FC of DIV 11 and DIV 21 vs DIV 4(log2Fold Change = 0)

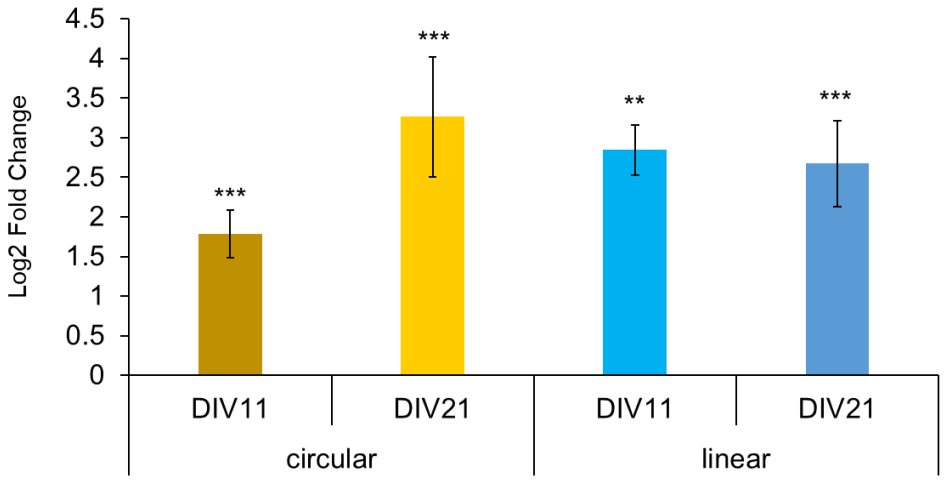


Figure 16B. Expression levels of circular and linear Rmst transcripts during neuronal differentiation. RT-qPCR analysis was performed on RNA samples of mouse cortical neurons at DIV4, DIV11, and DIV21. Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ are referred to the comparison of DIV21 or DIV 11 vs DIV4). Data are expressed as log2FC of DIV 11 and DIV 21 vs DIV 4(log2Fold Change = 0)

3.3 circCdh9 and circRmst expression in a specific form of homeostatic plasticity

Some circRNAs have been demonstrated to respond to modulated neuronal activation and synaptic plasticity [14], [87], [156]. Therefore, we decided to examine the expression levels of circCdh9 and circRmst following the induction of a specific form of homeostatic plasticity, defined as synaptic scaling. [157], [158]. By treating cortical neurons at DIV 10 with 1μM tetrodotoxin (TTX) or 20μM bicuculline (BIC) for 24 hr, synaptic up- or down-scaling are induced, respectively. As a result of RT-qPCR analysis, we observed that circCdh9 slightly responded to synaptic down-scaling, while no changes in the expression have been detected in TTX-treated neurons. The Cdh9 mRNA showed a different pattern of expression, where both synaptic up- and down-scaling led to a reduction of its expression levels (Fig. 17A). Likewise, circRmst expression was moderately regulated by both manipulations and a similar regulation was observed for Rmst linear isoform, with a comparable and marked increase in the expression (Fig. 17B).

To assess whether circCdh9 and circRmst responded to activity-dependent synaptic plasticity mechanisms, we induced synaptic scaling by treating cortical neurons with TTX or BIC. Both circCdh9 and circRmst slightly responded to the induction of homeostatic scaling, suggesting that they might not be directly involved in regulating synaptic plasticity events.

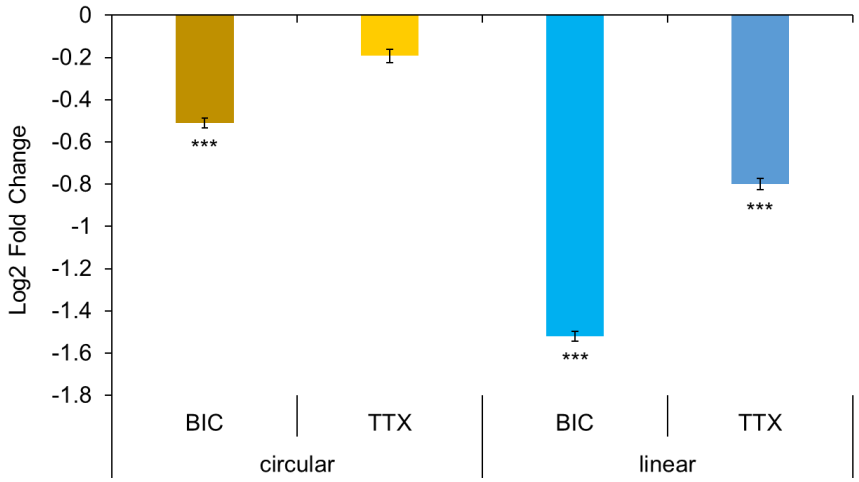


Figure 17A. Expression study of circular and linear Cdh9 transcripts in synaptic scaling. RT-qPCR analysis was performed on RNA samples of three independent biological replicates of cultured cortical neurons from CD1 mice treated with 1mM TTX or 20 mM bicuculline for 24 hr at 10 days in vitro. Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t-test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of TTX- or bicuculline-treated neurons vs untreated neurons (log2Fold Change = 0).

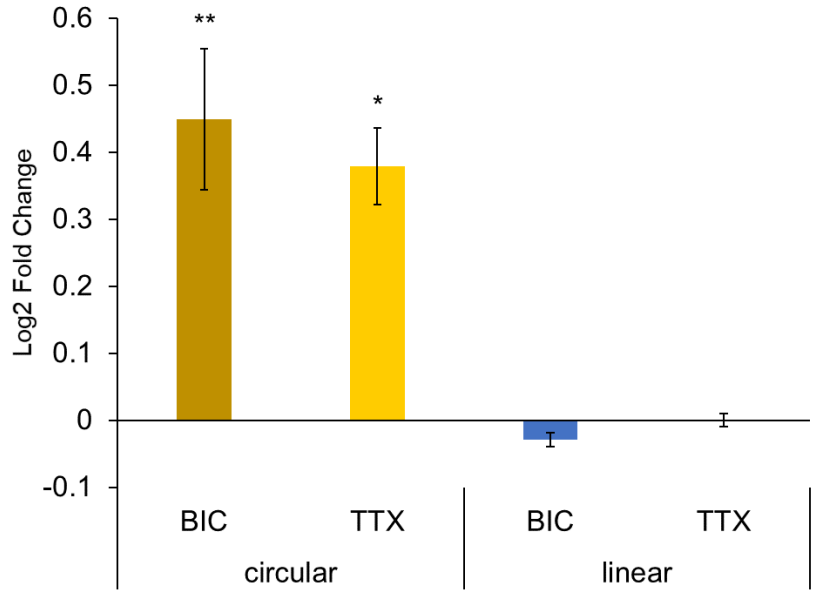


Figure 17B. Expression study of circular and linear Rmst transcripts in synaptic scaling. RT-qPCR analysis was performed on RNA samples of three independent biological replicates of cultured cortical neurons from CD1 mice treated with 1mM TTX or 20 mM bicuculline for 24 hr at 10 days in vitro. Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t-test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of TTX- or bicuculline-treated neurons vs untreated neurons (log2Fold Change = 0).

4. Identification of genes differentially expressed in the BTBR hippocampus

We investigated the transcriptome profile of coding and non-coding genes in BTBR and B6 mice by applying to our RNA-seq raw data a specific computational pipeline for gene detection. We identified a total of 459 differentially expressed genes (DEGs), of which 192 were upregulated, and 268 were downregulated in the hippocampus of BTBR mice compared to B6 mice (log2FC ≥ 0.58 and ≤ − 0.58) (Fig. 18). The analysis of biotypes revealed that the majority of DEGs derives from protein-coding genes, while a minor fraction originated from lncRNAs and processed transcripts (Fig. 19).

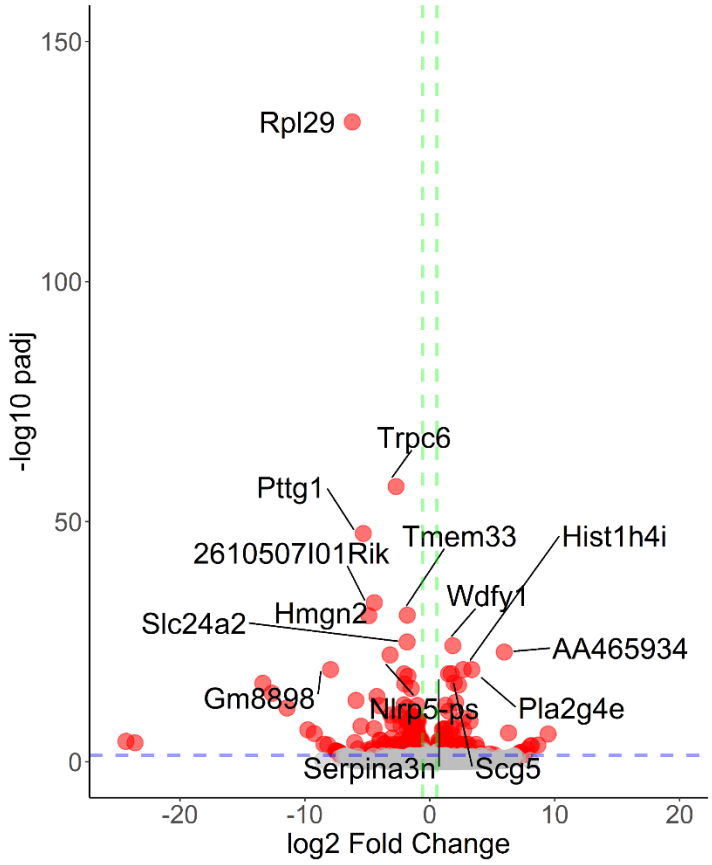


Figure 18. Volcano plot showing genes differentially expressed in the hippocampus of BTBR compared with B6 mice detected in the RNA-seq analysis. The negative log (base 10) of adjust p values (padj), is plotted on the Y axis, and the log of the Fold Change (base 2) is plotted on the X axis. Each dot on the graph represents one gene, red dots represent genes that are significantly differently expressed in the hippocampus of BTBR vs B6 mice (log2FC ≥ 0.58 and ≤− 0.58, q value <0.05).

We interrogated the EnrichR bioinformatic tool (https://maayanlab.cloud/Enrichr/)[159] to determine if our DEG list was enriched for ontology categories or pathways related to the ASD

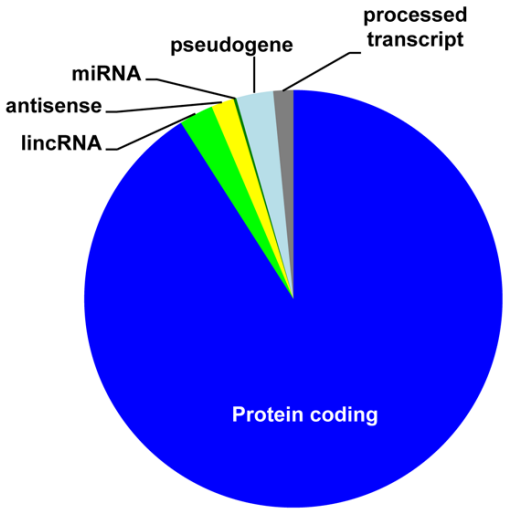


Figure 19. The pie chart shows the most represented biotypes among DEGs. Differentially expressed genes in the BTBR hippocampus were mostly protein coding. Top 15 hippocampal differentially expressed genes in BTBR, in comparison with B6 mice

phenotype. Interestingly, among the over-represented GO categories, we found terms related to chromatin remodeling, active transmembrane transport and heparan sulfate metabolism (Fig 20A-B). Moreover, BTBR DEGs were significantly enriched for Kegg pathway terms related to the tryptophan metabolism and the GABAergic synapse pathway (Fig 21).

Then, to evaluate the relevance of identified DEGs in the context of human ASD, we analyzed a list of 1019 ASD-associated human genes released from the SFARI database (https://gene.sfari.org/). We found that 35 of the differentially expressed genes in the BTBR hippocampus are associated with autism in human with different scores (Table 1). Among them, there are adhesion molecules, protocadherins (Pcdh9, Pcdha9 and Pcdh11x), cadherins (Cdh22 and Cdh9), and ion channels (Trpc6 and Cacna1h).

Finally, the comparison of the DEC and DEG lists showed that only 6 genes were differentially expressed in both circular and linear isoforms, including Cdh9 and Trpc6 genes (Table2). Interestingly both genes are associated with ASD in human (see Table 1). This analysis suggests an overall independent regulation of circular and linear transcripts.

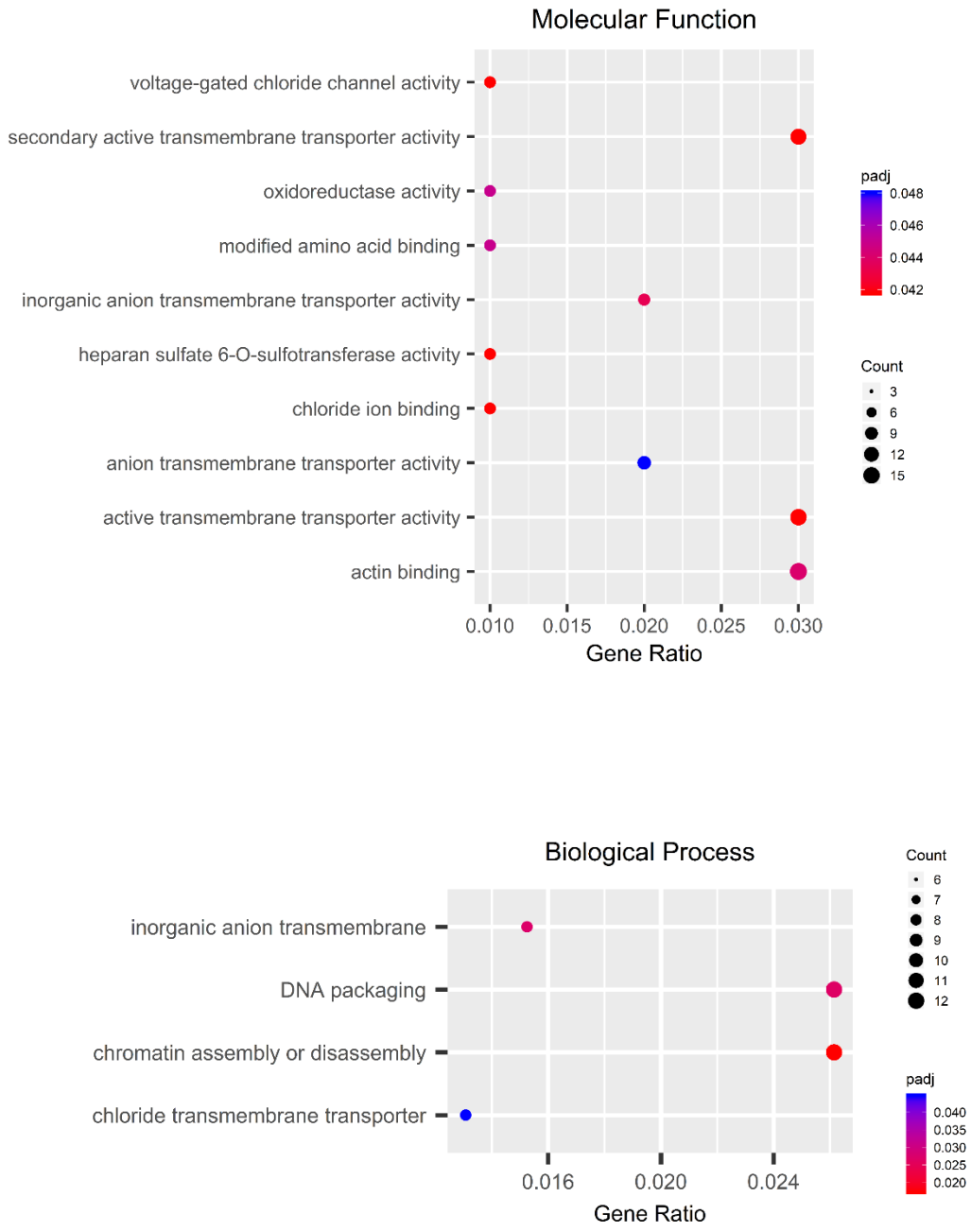


Figure 20A-B. Over-represented gene ontology categories for BTBR DEGs. The figure shows the most significantly enriched ontology terms according to molecular function (upper anel) and biological process (lower panel)

Kegg Pathway		
Term	**Pathway ID**	**P-value**
Tryptophan metabolism	hsa00380	0.0003
Systemic lupus erythematosus	hsa05322	0.0011
Base excision repair	hsa03410	0.0066
Alcoholism	hsa05034	0.0227
Peroxisome	hsa04146	0.0421
Arachidonic acid metabolism	hsa00590	0.0539
GABAergic synapse	hsa0472	0.0519

Figure 21. Kegg pathway analysis of BTBR DEGs. Only neuronal-related terms are reported

Gene Name	Sfari score	log2FC BTBR	Gene description
GATM	S	-0.64	Glycine Amidinotransferase
SLC45A1	S	0.75	Solute Carrier Family 45 Member 1
PACS1	S	0.64	Phosphofurin Acidic Cluster Sorting Protein 1
CACNA1H	2	0.66	Voltage-Gated Calcium Channel Subunit Alpha Cav3.2
ACHE	3	0.66	Acetylcholinesterase (Cartwright Blood Group)
AHDC1	3	1.04	AT-Hook DNA-Binding Motif-Containing Protein 1
DAPP1	3	-1.17	Dual Adaptor Of Phosphotyrosine And 3-Phosphoinositides 1
MFRP	3	-2.33	Membrane Frizzled-Related Protein
SLC12A5	3	0.62	Solute Carrier Family 12 Member 5
TRPC6	3	-2.70	Transient Receptor Potential Cation Channel Subfamily C Member 6
ATP1A3	4	0.59	ATPase Na+/K+ Transporting Subunit Alpha 3
ARHGEF9	4	-1.09	Cdc42 Guanine Nucleotide Exchange Factor 9
CD38	4	-1.08	ADP-Ribosyl Cyclase 1
FABP5	4	-0.63	Fatty Acid Binding Protein 5
CDH22	4	1.54	Cadherin 22
CDH9	4	-0.87	Cadherin 9
CX3CR1	4	-0.70	C-X3-C Motif Chemokine Receptor 1
PCDH11X	4	0.74	Protocadherin 11 X-Linked
PCDH9	4	-0.03	Protocadherin 9
PCDHA9	4	0.94	Protocadherin Alpha 9
PTGS2	4	-0.42	Prostaglandin-Endoperoxide Synthase 2
SPP2	4	7.52	Secreted Phosphoprotein 2
TDO2	4	-0.97	Tryptophan 2,3-Dioxygenase
TSHZ3	4	1.08	Teashirt Zinc Finger Homeobox 3
ZBTB16	4	0.82	Zinc Finger And BTB Domain Containing 16
DOCK10	5	-0.67	Dedicator Of Cytokinesis 10
ARHGAP33	5	0.99	Rho GTPase Activating Protein 33
CBLN1	5	1.90	Cerebellin 1 Precursor
FOLH1	5	0.76	Folate Hydrolase 1
GABRA5	5	-0.64	Gamma-Aminobutyric Acid Type A Receptor Alpha5 Subunit
RFWD2	5	-0.65	RING-Type E3 Ubiquitin Transferase
ARHGAP15	6	NA	Rho GTPase Activating Protein 15
PDE4A	no score assigned	0.77	Phosphodiesterase 4A
RNPS1	no score assigned	-0.72	RNA Binding Protein With Serine Rich Domain 1
SLC24A2	no score assigned	-1.82	Solute Carrier Family 24 Member 2

Table 1. Table showing the 35 genes differentially regulated in the BTBR hippocampus and associated with ASD in humans with variable scores, according to SFARI database

Gene symbol	Gene name	mRNA log2FC	circRNA log2FC	Sfari gene score
Trpc6	Transient receptor potential cation channel subfamily C member 6	−2.7	−13.3	3
Cep112	Centrosomal protein 112	−1.9	−11.7	-
Cds1	CDP-diacylglycerol synthase 1	0.7	0.4	-
Ndst3	N-deacetylase and N-sulfotransferase 3	0.7	0.6	-
Cdh9	Cadherin 9	−0.9	−3.3	4
Asb3	Ankyrin repeat and SOCS box containing 3	−0.7	−0.6	-

Table 2. BTBR differentially expressed genes as circular and mRNA isoforms

4.1 Validation of the selected ASD-related and highly modulated BTBR genes

Among the 15 genes with the highest and most significant modulation (Log2FC ≤-1.5 and ≥1.5 Table 3), we selected 11 genes with known neuronal function and involved in autism relevant pathway. By RT-qPCR analysis on hippocampal RNA pools of BTBR and B6 mice, we confirmed a significant differential expression of Pla2g4E, Hmgn2, Pttg1 and Tmem33 genes, while we did not observe a significant deregulated expression for Rpl29 and Wdfy1 genes. Furthermore, by RT-qPCR we validated the differential expression of genes with a strong association with ASD, Arghef9 and Tshz3 and Tdo2 (SFARI score 4), as well as Slc24a2 genes (no SFARI score assigned). We did not validate the differential expression of Cdh22 (Fig. 22).

Gene Name	log2FC	padj	Name	Biotype
Rpl29	-6.2	5.66E-134	ribosomal protein L29	protein_coding
Trpc6	-2.7	4.98E-58	transient receptor potential cation channel, subfamily C, member 6	protein_coding
Pttg1	-5.3	2.87E-48	pituitary tumor-transforming gene 1	protein_coding
2610507I01Rik	-4.4	7.69E-34	RIKEN cDNA 2610507I01 gene	lincRNA
Tmem33	-1.8	3.08E-31	transmembrane protein 33	protein_coding
Hmgn2	-4.9	3.79E-31	high mobility group nucleosomal binding domain 2	protein_coding
Slc24a2	-1.8	1.10E-25	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	protein_coding
Wdfy1	1.8	7.07E-25	WD repeat and FYVE domain containing 1	protein_coding
AA465934	6.0	1.42E-23	expressed sequence AA465934	lincRNA
Nlrp5-ps	-3.2	5.65E-23	NLR family, pyrin domain containing 5, pseudogene	protein_coding
Gm8898	-8.0	6.30E-20	zinc finger protein 966	protein_coding
Pla2g4e	3.4	6.54E-20	phospholipase A2, group IVE	protein_coding
Hist1h4i	2.7	6.54E-20	histone cluster 1, H4i	protein_coding
Scg5	1.7	4.58E-19	secretogranin V	protein_coding
Serpina3n	1.5	4.75E-19	serine (or cysteine) peptidase inhibitor, clade A, member 3N	protein_coding

Table 3. Top 15 hippocampal differentially expressed genes in BTBR, in comparison with B6 mice

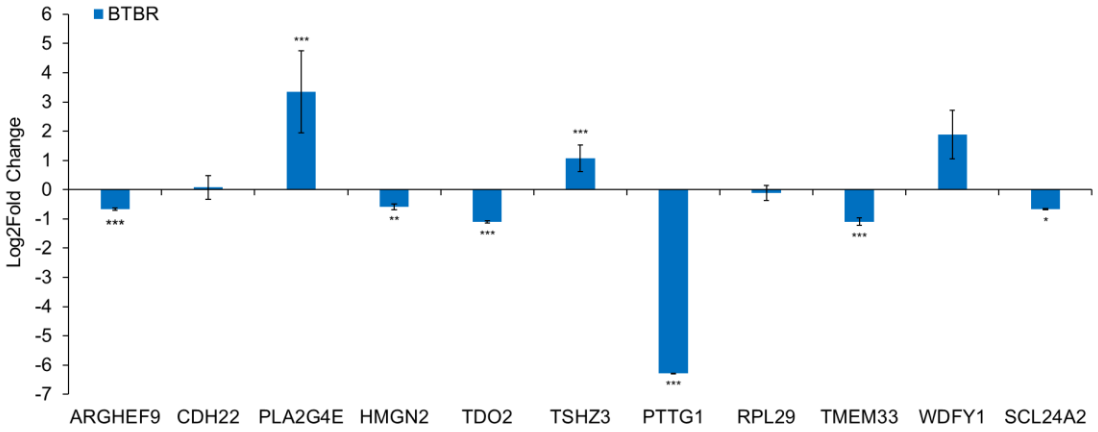


Figure 22. RT-qPCR analysis of selected DEGs . We confirmed the differential expression of 8 genes highly modulated in the BTBR hippocampus and ASD-related**.** Six to nine technical replicates were performed for each gene product analyzed (two-sided unpaired Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Data are expressed as log2FC of BTBR vs B6 (log2Fold Change = 0).

In conclusion, by RNA-seq analysis, we identified 459 genes highly modulated in the hippocampus of the BTBR mice, 35 of them annotated in the SFARI database since strongly associated with ASD in human. By RT-qPCR we validated the differential expression of 8 DEGs, with well-characterized neuronal function and a known association with ASD in human. The bioinformatic analysis of DEGs revealed enrichment for molecular processes and pathways related to autism. Moreover, comparing our DEC and DEG lists, we found only 6 genes deregulated in both circular and linear transcripts.

5. microRNA expression profile in the BTBR hippocampus

We decided to investigate the miRNA expression profile in the hippocampus of BTBR mice, compared to control B6 mouse hippocampus. We prepared total RNA samples from 4 single animals of BTBR and B6 mouse strains. We identified 18 significantly deregulated miRNAs in the hippocampus of BTBR mice with a False Discovery Rate (FDR) < 0.1, 13 of which were upregulated, 5 were downregulated (Table 4).
By using the Mienturnet tool (http://userver.bio.uniroma1.it/apps/mienturnet/) [160], we identified predicted targets of BTBR differentially expressed miRNAs (DEmiRNAs) (Table 5). Mienturnet allows an interaction analysis of mRNA data sets with an input miRNAs list and therefore enable the identification of RNA targeted by multiple miRNAs. Interestingly, we identified potential DEmiRNA-regulated genes, some of them able to interact with multiple deregulated miRNAs. In particular, Zeb2 is targeted by 4 upregulated miRNAs in the BTBR hippocampus.
Then, we interrogated the Autism KnowledgeBase database (http://db.cbi.pku.edu.cn/autismkb_v2/) [161] to examine the genetic association of predicted

target mRNAs with ASD. Among DEmiRNA predicted targets, we identified ASD risk genes, Rnf166, Plxnc1 and Ints6l, Zeb1, and the above-mentioned Zeb2.

To evaluate the potential regulatory function of miRNAs differentially expressed in BTBR mice, we applied to our RNA-seq raw data, a specific computational pipeline to assess the mRNAs expression profile in the BTBR hippocampus. We detected a total of 2524 transcripts highly deregulated in the BTBR mice (log2FC ≥ 0.58 and ≤ -0.58) (Fig. 23), compared to B6 mice, and among them we identified 3 Zeb2 transcripts, whose downregulation correlate with the previous finding of significative interaction with 4 upregulated miRNAs. We computationally validated the predicted interactions with miR429-3p, miR200a-3p, miR200c-3p and miR183-5p, since we observed the presence of their seed sequences in the 3'UTRs of the Zeb2 transcripts corresponding to the Zeb2-202 and Zeb2-212 isoforms (Fig. 24). We did not detect any predicted miRNAs binding sequences in the 3'UTR of the downregulated Zeb2-214 isoform.

microRNA	log2FC	PValue	FDR
mmu-miR-200c-3p	3.579212	1.55E-21	6.54E-19
mmu-miR-200a-3p	2.130819	3.99E-08	8.42E-06
mmu-miR-183-5p	3.919517	5.44E-07	7.65E-05
mmu-miR-451a	1.382968	3.50E-05	0.003033
mmu-miR-429-3p	2.657901	3.59E-05	0.003033
mmu-miR-1298-5p	-3.59777	4.77E-05	0.003352
mmu-miR-100-5p	-1.12364	0.000116	0.007002
mmu-miR-448-3p	-3.53509	0.000223	0.011777
mmu-miR-344d-3p	-1.03058	0.000391	0.01706
mmu-miR-204-3p	-1.78043	0.000404	0.01706
mmu-miR-182-5p	2.116532	0.000862	0.033064
mmu-miR-383-5p	0.887757	0.000949	0.033378
mmu-miR-144-5p	1.309465	0.001694	0.05498
mmu-miR-34a-5p	1.339062	0.002368	0.071382
mmu-miR-486a-5p	1.194204	0.00367	0.093496
mmu-miR-486b-5p	1.194205	0.003691	0.093496
mmu-miR-218-1-3p	2.010943	0.003766	0.093496
mmu-miR-148a-3p	1.147207	0.004053	0.095022

Table 4. 18 differentially expressed miRNAs in the hippocampus of BTBR compared to B6 mice. The color code indicates the upregulated (red) and downregulated (green) miRNAs

Gene Symbol	p-value	FDR	Odd ratio	Number of interactions	microRNA 1	microRNA 2	microRNA 3	microRNA 4
Flt1	6.02E-05	0.01366	0.03125	3	mmu-miR-200c-3p	mmu-miR-200a-3p	mmu-miR-429-3p	
Rnf166	6.02E-05	0.01366	0.03125	3	mmu-miR-344d-3p	mmu-miR-429-3p	mmu-miR-200c-3p	
Zeb1	0.000165	0.025021	0.041667	3	mmu-miR-429-3p	mmu-miR-200a-3p	mmu-miR-200c-3p	
Fam129c	0.000678	0.065854	0.023438	2	mmu-miR-486b-5p	mmu-miR-486a-5p		
Ikzf5	0.001015	0.065854	0.072917	3	mmu-miR-429-3p	mmu-miR-200c-3p	mmu-miR-344d-3p	
Plxnc1	0.000805	0.065854	0.067708	3	mmu-miR-344d-3p	mmu-miR-200c-3p	mmu-miR-429-3p	
Zeb2	0.00095	0.065854	0.121094	4	mmu-miR-429-3p	mmu-miR-200a-3p	mmu-miR-200c-3p	mmu-miR-183-5p
Ints6l	0.001344	0.07627	0.03125	2	mmu-miR-429-3p	mmu-miR-200c-3p		
Pkib	0.001533	0.077321	0.083333	3	mmu-miR-344d-3p	mmu-miR-429-3p	mmu-miR-200c-3p	
Aim2	0.004578	0.098961	0.054688	2	mmu-miR-486a-5p	mmu-miR-486b-5p		
Fzd1	0.003299	0.098961	0.046875	2	mmu-miR-183-5p	mmu-miR-344d-3p		
Gm7609	0.004578	0.098961	0.054688	2	mmu-miR-486b-5p	mmu-miR-486a-5p		
Hopx	0.003299	0.098961	0.046875	2	mmu-miR-1298-5p	mmu-miR-344d-3p		
Rassf10	0.003299	0.098961	0.046875	2	mmu-miR-200c-3p	mmu-miR-429-3p		
Rdh10	0.00222	0.098961	0.039063	2	mmu-miR-200c-3p	mmu-miR-429-3p		
Shc4	0.004578	0.098961	0.054688	2	mmu-miR-486a-5p	mmu-miR-486b-5p		
Smurf2	0.003299	0.098961	0.046875	2	mmu-miR-344d-3p	mmu-miR-200a-3p		
Tmem56	0.004578	0.098961	0.054688	2	mmu-miR-344d-3p	mmu-miR-200a-3p		
Tns3	0.004578	0.098961	0.054688	2	mmu-miR-486b-5p	mmu-miR-486a-5p		
Zcchc9	0.003299	0.098961	0.046875	2	mmu-miR-486a-5p	mmu-miR-486b-5p		
Zfp644	0.004578	0.098961	0.054688	2	mmu-miR-344d-3p	mmu-miR-451a		
Cnnm3	0.005135	0.10432	0.125	3	mmu-miR-486b-5p	mmu-miR-486a-5p	mmu-miR-34a-5p	

Table 5. Target enrichment analysis of miRNA differentially expressed in the BTBR hippocampus. The bioinformatic interaction analysis has been performed by using the Mienturnet tool

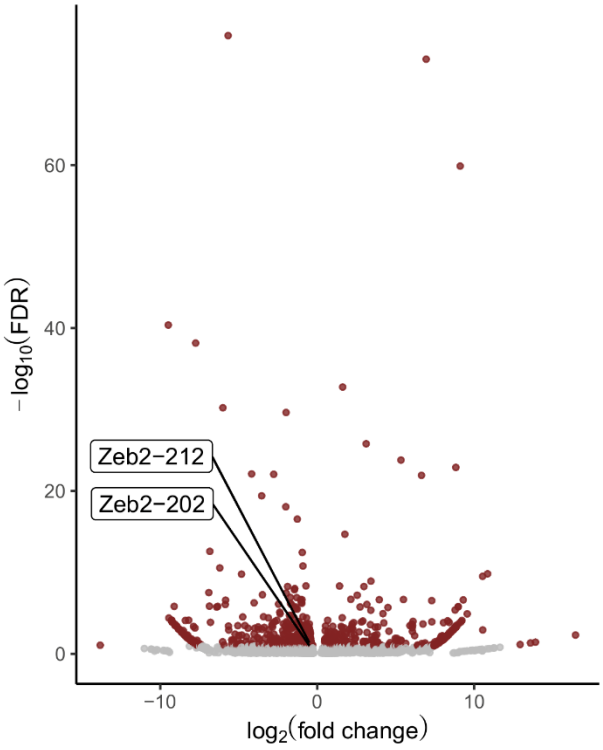


Figure 23. Volcano plot showing transcripts differentially expressed in the hippocampus of BTBR compared with B6 mice detected in the RNA-seq analysis.

The downregulated Zeb2 transcripts corresponding to the Zeb2-202 and Zeb2-214 isoforms, are highlighted. The negative log (base 10) of adjust false discovery rate (FDR), is plotted on the Y axis, and the log of the Fold Change (base 2) is plotted on the X axis. Each dot on the graph represents one single transcript, dark red dots represent transcript that are significantly differently expressed in the hippocampus of BTBR vs B6 mice (log2FC ≥ 0.58 and ≤− 0.58, q value <0.05).

Figure 24. Graphic representation of the upregulated miRNAs targeting the Zeb2 3’UTR. The Zeb2-202 and Zeb-212 isoforms share the same 3’UTR sequence

5.1 Validation of the differentially expressed miRNAs by RT-qPCR analysis

We decided to select DEmiRNA from RNAseq for further analysis by mean of the following criteria: 1) co-regulation of putative targets (number of interactions >1); 2) association of target genes with neuronal and ASD-related pathways. By RT-qPCR analysis, we were able to confirm the deregulated expression of miR-429, miR-200c, miR-183, miR-34a, miR-451 and miR-182 (Fig. 25). As it is known in the literature, miR-200a and miR-200b are co-transcriptionally regulated [162]–[165]. Therefore, we decided to evaluate the expression of miR-200b, although it did not show significant deregulation in the RNA-seq analysis. Surprisingly, miR-200b exhibited the most substantial upregulation among the miRNA differentially expressed. Conversely, we did not observe significant expression changes for miR-200a and miR-100.

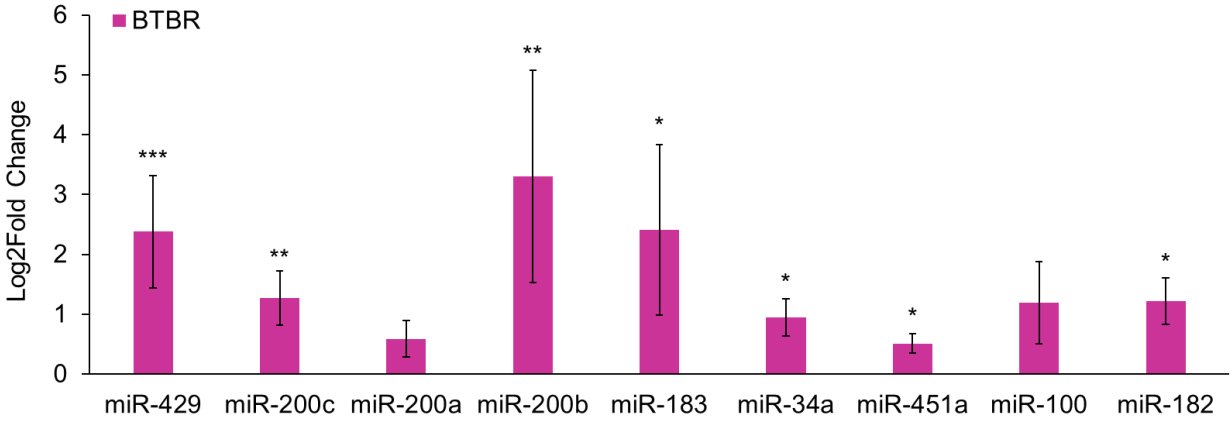


Figure 25. RT-qPCR analysis of 7 differentially expressed miRNAs in the BTBR hippocampus, compared with B6 mice. RT-qPCR analysis was performed on single RNA preparations (n=4) from BTBR and B6 mice. Six to nine technical replicates were performed for each miRNA analyzed (two-sided unpaired Student’s t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).Data are expressed as log2FC of BTBR vs B6 (log2Fold Change = 0).

In conclusion, we explored for the first time the miRNA profile in the BTBR hippocampus, and we identified 18 significantly deregulated miRNAs. We performed a DEmiRNA-target enrichment analysis, and among the predicted gene targets, we identified relevant autism-risk genes. By RT-qPCR analysis on selected DEmiRNAs, we confirmed the expression levels of 6 highly modulated BTBR miRNAs.

DISCUSSION

ASD is a neurodevelopmental disease, with unknown etiology. Despite the high degree of genetic heterogeneity, autism-risk genes exert crucial roles in the activity-dependent signaling networks, which regulate synaptic development and transmission [110], [116]. The recent definition of ASD as a synaptic defect disease relies on the hypothesis that autism-related mutations convey onto common molecular pathways that are involved in the neural circuit formation or maintenance of excitatory and inhibitory ratio of neurotransmission [110], [116], [166]. Recent studies indicate altered expression levels of lncRNAs and miRNAs in ASD postmortem brain tissues [119]–[122], suggesting the potential contribution of non-coding RNAs in the pathophysiology of this disease.

CircRNAs represent a novel class of non-coding RNAs, ubiquitously expressed in the eukaryotic organisms [15]. They are highly enriched in the mammalian brain, particularly at the synapses. Dynamic changes in localization and abundance of circRNAs have been observed during neuronal differentiation, synaptic activity and in aging brain [11], [14], [89]. All these findings suggest that circRNA may play key roles in the nervous system, especially in regulating neuronal development and synaptic functions. Moreover, studies have reported dysregulated expression levels of circRNAs in several neurological conditions, such as Parkinson's disease and Alzheimer's disease [97], [98], [167], [168]. Although there are few examples of neuronal circRNAs with known functions, their ability to act as miRNAs or RBPs sponges represent the better-characterized circRNA molecular function [11], [12], [62], [64]–[66], [70]. Thus, circRNAs might exert potential regulatory functions in the pathogenesis and progression of neuronal disorders.

In the present study, we investigated the expression profile of circRNAs in the hippocampus of BTBR mouse model, to assess the potential contribution of these molecules in the pathophysiology of autism. We identified 288 high-confidence circRNAs mainly derived from host genes enriched in metabolic pathways, such as glutamate receptors, axon guidance, synapse formation and functions. These results are consistent with previous experimental evidence indicating that ASD associated genes converge on pathways related to the assembly of neural circuits and neuronal signaling. We discovered 41 circRNAs highly modulated in the BTBR mice, compared with B6 control mice. We identified interesting circRNAs candidates, and the differential expression of 12 dysregulated circRNAs was validated by RT-qPCR [123]. In-depth characterization was performed on two selected circRNAs: the BTBR downregulated and upregulated circCdh9 and circRmst, respectively.

circCdh9 host gene codifies for a type II cadherin, and it has been found to play critical functions in neural developmental processes [127]–[130]. Moreover, recent studies showed a genetic association of Cdh9 gene locus with ASD [125], [132]. Cdh9 expression has been demonstrated to be regulated during mouse development of ASD-relevant brain regions [133], [134]. The lncRmst has been characterized as a co-transcriptional factor of SOX2 and a fundamental regulator of neurogenesis [152]. Several Rmst circular isoforms have been identified [136], one of which has been found highly enriched in neuronal compartments and its expression changes has been observed during neuronal development and differentiation [13], [14]. We have demonstrated an

altered expression of circCdh9 and circRmst in the BTBR hippocampus, further support the emerging studies that indicate the putative involvement of circRNAs in neurodevelopmental processes [13], [14] These observations prompted us to set up expression studies of the circular and linear isoforms derived from Cdh9 and Rmst gene loci, hypothesizing that they might be implicated in the etiology of ASD.

We characterized the molecular structure of circCdh9 and circRmst 1.5kb by using divergent primers which allowed us to confirm the predicted backsplicing junction sequence and their exonic composition.
Interestingly, we observed an exon skipping event occurring in the exon 8 of circRmst.

Since previous studies identified the cerebellum and the cerebral cortex as ASD-relevant brain regions [153]–[155], we asked whether the expression changes observed in the BTBR hippocampus were similarly appreciable in the BTBR prefrontal cortex and cerebellum. In the cerebellum, both circCdh9 and Cdh9 mRNA are downregulated as in the hippocampus, while we did not observe significant circRmst expression changes in the cerebellum and the cerebral cortex. These data suggest that the expression levels of these circular and linear transcripts might be differentially affected by brain region-specific mechanisms of gene expression dysregulation.

Recent studies have shown that circRNAs are tightly regulated during neurodevelopment [13], [14]. Therefore, we decided to study the expression of circCdh9 and circRmst at different stages of mouse brain development, analyzing in parallel the linear cognate transcripts. We observed distinct patterns of expression of both circCdh9 and circRmst in all the brain regions examined (hippocampus, cerebellum and prefrontal cortex), suggesting that they might exert specific functions in the main processes of neurodevelopment. The expression of circCdh9 and Cdh9 mRNA increases in the HP development, from day 10 of postnatal life. Cadherin-9 protein is involved in the formation of specific hippocampal synapses [130]. Moreover, mouse developmental studies have shown that the P10 postnatal stage corresponds to the formation of synapses [169]. Therefore, our observation of Cdh9 mRNA up-regulation is in line with those findings. Similarly to the hippocampus, in the prefrontal cortex we observed a significant, although not dramatic up-regulation of Cdh9 transcripts. An opposite three-fold down-regulation of Cdh9 circular transcript and mRNA discovered in the cerebellum from embryonal to postnatal life might be related to a specific developmental program of this brain region.

The general parallel regulation of Cdh9 circular isoform and mRNA during brain development might be related to transcriptional regulation of the Cdh9 gene locus. However, considering that the circular transcript from Cdh9 locus is the dominant isoform, we might hypothesize a specific molecular function of the circular isoform, whose deregulation might be implicated in a developmental disease.

Conversely, both circular and linear isoforms of Rmst were downregulated in the hippocampus from P0 to P10, suggesting a specific biological function of Rmst transcripts at this developmental stage. We were able to analyze the expression of Rmst transcripts at E17 prefrontal cortex and cerebellum, finding that in these brain regions from embryonal to postnatal life (P0) there is a dramatic downregulation of circRmst and lncRmst. Therefore, our observations point to a role of

Rmst transcripts in the late embryonal and early postnatal brain developmental processes. Interestingly, similar expression patterns were observed in all brain regions studied, suggesting a role of Rmst in typical brain developmental processes.

Transcriptomic studies of circRNAs expression profiles in several mouse and human cell lines, highlighted their dynamic expression changes during early neuronal cell specification [13], [14]. To better characterize circCdh9 and circRmst during neuron differentiation, we decided to analyze their expression in vitro differentiation of cortical neuron primary culture. Primary culture of embryonal neuron represents an interesting model of neuronal differentiation, where from day 0 to day 21 the extrusion and maturation of neurite processes are observed, with the development of dendrite arborization and axon processes able to establish active synaptic contacts. During in vitro cortical neuron differentiation, we observed in mature DIV21 neurons an up-regulation of circCdh9 and Cdh9 mRNA expression which recalls what it has been observed during the prefrontal cortex maturation in vivo. We can hypothesize that changes on Cdh9 transcripts are related to the ongoing synapse maturation and synaptic network establishment, in both brain embryonic and postnatal stages and in vitro differentiation. On the contrary, the observed dramatic increase in the expression of circRmst during neuronal differentiation is distinct to the regulation of lncRmst whose levels were slightly higher in mature neurons compared the still immature ones. As observed for circCdh9 the expression changes of circRmst recapitulate what has been observed during postnatal life. In overall, these data support the hypothesis of a distinct role of circRmst and lncRmst in the processes of neuronal maturation.

Emerging evidence has shown that circRNAs abundance is modulated by neuronal activity [14], [87], [156]. Moreover, in ASD deficits in the homeostatic regulation of synaptic strengths have been identified, indicating that homeostatic scaling is crucial for proper neuronal functions and maintaining a balance between excitation and inhibition [110], [116], [166]. To evaluate a possible regulation of circCdh9 and circRmst expression by synaptic scaling, we studied their expression in primary mature cortical neurons (DIV10) treated with TTX or BIC. TTX is a pharmacological inhibitor of voltage-gated sodium channels, BIC is an antagonist of GABA-A receptors, inducing synaptic up-scaling and down-scaling, respectively. The synaptic up-scaling led to a marked reduction of Cdh9 mRNA expression, while in our experiments the expression of circCdh9 did not exhibit significant changes. A significant down-regulation was observed for both circCdh9 and mRNA upon synaptic down-scaling induced by BIC treatment. Conversely, circRmst was significantly up-regulated upon induction of homeostatic plasticity by both drug treatments. Although we detected modest expression changes, these results suggest that the expression of both circCdh9 and circRmst is regulated during neuronal plasticity events.

To examine the coding and non-coding transcriptomic landscape of BTBR mice, we analyzed the gene expression profiles in the hippocampus of BTBR and B6 mice. A total of 459 differentially expressed genes have been identified and bioinformatic analysis revealed BTBR DEGs enrichment

for terms related to ASD-relevant pathways. We identified many BTBR DEGs associated with anion transport activity and GABAergic synapse functions. These results are in line with one of the hypothesized mechanisms leading to ASD development, consisting of imbalance in the excitatory and inhibitory neurotransmission. [166]. As previously mentioned, many ASD associated mutations uncover in genes codifying for proteins with synaptic functions [113], [114], [116], [117]. The tryptophan metabolism pathway was found as the most significative affected process in the BTBR hippocampus. Serotonin is a tryptophan metabolite, and recent evidence reported serotonergic abnormalities in ASD [170]. Furthermore, alteration in tryptophan metabolism has been observed in the intestine of BTBR mice [171]. These data indicate convergence in the serotoninergic system deficiency observed in ASD patients and BTBR central nervous system.

We obtained a list of 15 highly modulated genes and we were able to confirme by RT-qPCR analysis the differential expression of Hmgn2, Pla2g4e, Pttg1 and Tmem33. Relevant-ASD functions have been observed for Hmgn2 (High Mobility Group Nucleosomal Binding Domain 2) and Pla2g4e (Phospholipase A2 Group IVE) involved in neuronal developmental processes and glutamatergic and serotoninergic transmission, respectively. Furthermore, we were able to confirm altered expression in the BTBR hippocampus of genes codifying for proteins with neuronal-specific function, component of ASD-associated pathways and included in the SFARI database (Arhgef9, Slc24a2, Tdo2, Tshz3). In order to evaluate whether changes in the circular transcriptome parallel changes in the gene expression profile, we combined our DEG and DEC lists. Interestingly, we identified only 6 genes, whose expression was altered in the BTBR hippocampus as both circular and total linear gene products. Among those genes, Cdh9 and Trpc6 have to be mentioned since their implication in neuronal circuit formation and development, and their association with ASD. Congruently with previous finding [13], we observed that circRNAs expression is independent of that of their host genes, suggesting different mechanisms of gene expression regulation at the transcriptional and post-transcriptional level.

To explore the potential contribution of small non-coding RNAs in ASD pathophysiology, we performed a miRNAs expression profile in the hippocampus of BTBR mice. By small RNA-seq, we identified 18 highly dysregulated miRNAs in BTBR compared to B6 mice. We were able to confirm by RT-qPCR the altered expression of miR-429, miR-200c, miR-183, miR-34a, miR-451. Interestingly, miR-200b exhibited the most robust upregulation, although it was not observed significantly altered in the RNA-seq. By bioinformatic analysis we identified mRNAs potentially target by multiple BTBR-regulated miRNA. In particular, the mRNA of Zeb2 contains in its 3'UTR binding sequences for the BTBR differentially expressed miRNAs miR-429, miR-200c, miR-183 and miR-200a. Several studies reported the essential role of Zeb2 in regulating neurogenic and gliogenic processes during neuronal development [172], [173]. Moreover, deletions in the Zeb2 gene locus have been associated with the Mowat Wilson syndrome, a rare disease characterized by severe mental retardation and phenotypical and behavioral similarities with ASD patients [174]–[176]. Intriguingly, in our mRNAs expression profile, we observed 3 significantly downregulated Zeb2 transcripts, and by computation analysis, we detected the predicted seed sequences of the upregulated BTBR miRNAs in Zeb2-202 and Zeb2-214 isoforms.

In conclusion, the observed anticorrelation of Zeb2 transcript expression and the interacting miRNAs might be indicative of a mechanism of post-transcriptional regulation of Zeb2 gene expression by BTBR-associated miRNAs, potentially relevant in the context of ASD.

PERSPECTIVES

Our study indicates that circCdh9 and circRmst, whose espression has been found to be altered in the hippocampus of BTBR mice, are tightly regulated during neuronal development and differentiation. Their potential role in both processes will be examined by genetic manipulation in *in vitro* system. These experiments would allow us to explore the role of circCdh9 and circRmst in the pathophysiology of ASD.

Interestingly, we observed a dysregulation in the expression of miRNAs interacting with ASD-related gene targets as Zeb2. Experimental validation of the post-transcriptional regulation of Zeb2 mRNA by BTBR-deregulated miRNAs will be performed. Moreover, the in vivo expression of artificial miRNA decoys in the BTBR hippocampus, acting as competitor inhibitors of miRNA upregulated in the ASD mice, would allow us to evaluate their role in the context of disease

MATERIAL AND METHODS

Tissue Collection and RNA Isolation

Total RNA was extracted from single mouse tissues, using Qiazol and miRNeasy spin column (Qiagen), with DNase1 on-column treatment, according to the manufacturer's protocols (Qiagen). RNA concentration was determined by the NanoDrop 1000 analysis (Thermo Fisher Scientific). RNA quality was assessed by gel electrophoresis and by measuring 260/280 and 260/230 absorbance ratios.

For mouse brain developmental expression studies, hippocampus, prefrontal cortex and cerebellum from CD1 male mice have been obtained in collaboration with Arianna Rinaldi, Assistant Professor in the Department of Biology and Biotechnology "Charles Darwin" and Centre for Research in Neurobiology D.Bovet, Sapienza University of Rome.

RT-qPCR

Validation of circRNAs and genes expression profile

RNA pools were reversed transcribed using the SuperScript III (Thermo Fisher Scientific) and random primers, at 50 °C for 1 h. Quantitative PCR (qPCR) was performed using SYBR Green (SensiMix™SYBR Hi-ROX Kit, Bioline) with appropriate primers. For circRNAs, one primer was designed to anneal at the circular junction whereas the other was within the exonic sequence. For linear transcripts, both primers were designed to amplify a sequence not included in the circRNA. Relative quantification of gene expression was conducted with the Applied Biosystems 7500 Real-Time PCR System. Gapdh mRNAs was used as internal controls. RT-qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method. BTBR and B6 were compared by independent sample t-test, with significance set at $p \leq 0.05$.
List of primers used in RT-qPCR validation analysis of selected circRNAs and their linear counterparts.

circRNAs	**Forward**	**Reverse**
circCdh9	CAACATAATAAACTTCACACTGACCAA	GATTGCCAGCTCCATCTCCT
circCacna1c	CCAACTCCAACCTGGTTCCAA	GAGTAGGGATGTGCTCGGGA
circHivep2	ACAGCCAGCCAGTAGGATTG	ACCTGGATACCTGCTTCTGG
circCacna1a	GATTCTGGGTCCTCAAGCACT	CTTGTCTCCCTGCTCCTGAA
circAuts2	CCTAAGGGACTCAGACAAAAGATG	TTCCTTGGTCAGGGGAGTCTG
circZcchc11	ATTTTGTTTGCTTGCCTAAAGTAG	AGAAGTCTTGGGCTCTTCC
circCdc14b	GTGTTATATGAGACTCGCGGGT	GATCACCTCGGCCATCAG
circRmst	AATGGCTTCATGAGATACTCAGG	AGACAATCTGGGCCATGTCT
circTrpc6	CAGTTTGTGGCTCATCCAAAC	CTGCAAGGAGCACACCAGTA
circCep112	TCAAGGTTATTGCTGACATGGA	GTCCTTTGCAGCTCTCTGCT
circWdr49	TAGGCTGGGAGAGGTTTTGA	GGCCAATAAAGCAAGCTGAT
circNcoa2	AGCAAGATTTTCCTCGCATC	GCGGATCTGCTTCACAGTTT
circMyrip	ACCGGCTAAGAGGATGGATT	AGGAGGTACAAGCCCAGACA
circTshz2	CATTCAAGTGGTATGCCCAGG	CCTGTCCCAGTGTCATTGCTG
circRyr3	GTCAGGAAATCAAGGAGAAATTAGAT	TTTTGGCATCTTGTGCTTTG
Linear mRNAs	**Forward**	**Reverse**
Cdh9	TTTGTGTCGTCATCCTGCTTAC	TCCTTAATGTGCCGATGTCA
Cacna1c	GCCTGCGACATGACAATAGAG	CCCTGCAGTTCACAAAAGGT
Hivep2	AAGCAGTTTGCACAGATGAAG	TCCGTGAATCTCTCCTTTCC
Cacna1a	CTGGGCTGTCATCAAACCTG	CAGGAGGGCAAAGACAACG
Auts2	CAACACTGTTCTTGTAAACAAAGATCC	CAGTCCTGGCTCTTCTCCTG
Zcchc11	GTTGTGGTGTGCAAAGATCG	GCACAGCTTAGCCCAATAGC
Cdc14b	TTTGTTTTGCCATTCTCTACAGC	GGTCCAAAATCCGCATAGAA
Rmst	GAATGGCTTCATGAGATGTTAGTC	GGAAAGGTGTTTTTCCTCGAA
Trpc6	GGTGCGGAAGATGCTAGAAG	GCTTGTCGCTAACCTTTTGC
Cep112	ACCATACGGAGACCATCCAG	CCAGCTCCAATTTTTCTTGG
Wdr49	CTGGTGTCATGTGGAGCATC	CAGAATCTCCAGTGGCAAGG
Ncoa2	TGCACTACTGCGCTATTTGC	GCTCACCTCCTCCTTCACAG
Myrip	CGAGGAGTTGATCGCAGAGT	TGGTCTGTCCCAAGAGGAAA
Tshz2	CGAAGACTCTCTACAGAAACCTCTCG	CTGTTGATGGCTGTGGTTACG
Ryr3	TATGCCCCTTGCTATGAAGC	AAGAGGCTGGGAGAGGAAC

List of primers used in RT-qPCR validation analysis of selected genes

Genes	Forward	Reverse
Cdh22	CAGTGTGGCCGAGCTGTC	ACACCTGTCTTGGGGTCCA
Slc24a2	CCACTCGCTGAAGAACTTGG	CAGCCCCATTCTGCCTCT
Tshz3	CAGCAGCCTATGTTTCCGAT	GCCGGTGAGTTCTGGTAGC
Tdo2	CTTCAGAGAATACCTGTCTCCAGC	ACAGCTCATTGTAGTCTCCTCCA
Arhgef9	ACCTGGACCCCAACTCAGAC	GTAGCCCTCGCAAATGTCCT
Hmgn2	GTTGTCTGCTAAACCTGCTCCTC	TTGGCATCTCCATTTTCTGC
Pla2g4e	CACTCAACCCAGAGGGCAT	GCATGAACCTCCAGGCAAG
Rpl29	ACAACCAGTCCCGCAAATG	TGTTGGCCTGCATCTTCTTC
Tmem33	CCTCTTCTTGGGTTGCATGA	CAGGAACGCTCTGCTCAACT
Pttg1	CAACCAAAACAGCCGACCT	CTCAAAGTCTAGAGGATTGAAAGGG
Wdfy1	CAAGGCCACCATGACAGAGT	GTCGCTTTCTAACCACTGAGGAG

Endogenous control	Forward	Reverse
Gapdh	ACTTGAAGGGTGGAGCCAAA	TCATGAGCCCTTCCACAATG

Validation of miRNAs expression profile

RNA from single animal preparations (n=4) was reverse transcribed using miScript the miScript Reverse Transcription Kit (QIAGEN). The qPCR detection of miRNAs was performed using miScript SYBR-Green Kit (QIAGEN) on a 7300 Real-Time PCR System. Data were analyzed using the comparative 2−ΔΔCt method. U6 snRNA was used as an internal control. BTBR and B6 were compared by independent sample t-test, with significance set at $p \leq 0.05$.

Sanger Sequencing

circCdh9 and circRmst nucleotide sequences were detected on PCR amplification products obtained with divergent primers designed on linear transcripts. Mouse BTBR and B6 hippocampal cDNAs were used as templates. The purified PCR product was directly sequenced using the Sanger sequencing method. B-actin mRNA was used as endogenous controls
List of primers used for the analysis of backspling junctions

circRNAs	Forward	Reverse
circCdh9	CTCATGTCTGGCAATGCAGT	AAGATGTGAAGGAGGGGAGC
circRmst	CTGGAATGGCCAAGGCTGTAG	AGGGACAATCAGTCACCCG
Endogenous control	**Forward**	**Reverse**
B-actin	TGTGACGTTGACATCCGTAAAGA	CTGCATCCTGTCAGCAATGC

Deep Sequencing of RNA

Two hippocampal RNA pools of different animal cohorts of BTBR and B6 mice were prepared, pool 1 (n = 6) and pool 2 (n = 4). Single hippocampal RNA preparations from BTBR and B6 mice (n=4) were used for miRNA expression profile. RNA-seq was performed on an Illumina Hiseq 2500 sequencing system using TruSeq Stranded Total RNA Library Prep kit with Ribo-Zero treatment (Illumina)

Bioinformatic analysis

Ensembl database (http://www.ensembl.org/)and circBASE database (http://www.circbase.org/) were interrogated to obtain information about genes and circRNAs of interest.

Gene functional annotation analysis was performed using the KEGG Orthology- Based Annotation System-KOBAS v. 3.0 (https://www.genome.jp/kegg/pathway/) and EnrichR web services (https://maayanlab.cloud/Enrichr/)

Human ASD-association studies were performed using SFARI database (https://gene.sfari.org/). and Autism KnowledgeBase database (http://db.cbi.pku.edu.cn/autismkb_v2/)

Prediction analysis of the miRNA targets was performed using the Mienturnet bioinformatic tool (MicroRNA ENrichment TURned NETwork) which performs both statistical and network-based analyses for detecting miRNA-target interactions.

Primary cell culture

Cortical neurons were obtained from the cerebral cortex of CD1 mouse embryos (E17). Neurons were cultured in complete Neurobasal Medium supplemented with B27 (ThermoFisher Scientific). Cells were grown at the humidified atmosphere of 5% CO2 t 37°C.
For circRNAs expression analysis during neuronal differentiation, RNA was collected from three biological replicates of cortical neurons maintained for 4, 11 and 21 days in vitro.
Synaptic scaling was performed by treating cortical neurons at 10 days in vitro with either 1 μM tetrodotoxin (TTX) or 20 μM Bicuculline (BIC). RNA was collected from three independent biological replicates 24 hours after the pharmacological treatments.

REFERENCES

[1] H. L. Sanger, G. Klotzt, D. Riesnert, H. J. Gross, and K. Albrecht, "Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures," *Proc. NatI. Acad. Sci. USA*, vol. 73, no. 11, pp. 3852–3856, 1976.

[2] J. M. Nigro *et al.*, "Scrambled exons," *Cell*, vol. 64, no. 3, pp. 607–613, 1991, doi: 10.1016/0092-8674(91)90244-S.

[3] C. Cocquerelle, P. Daubersies, M. A. Majerus, J. P. Kerckaert, and B. Bailleul, "Splicing with inverted order of exons occurs proximal to large introns," *EMBO J.*, vol. 11, no. 3, pp. 1095–1098, 1992, doi: 10.1002/j.1460-2075.1992.tb05148.x.

[4] B. Capel *et al.*, "Circular transcripts of the testis-determining gene Sry in adult mouse testis," *Cell*, vol. 73, no. 5, pp. 1019–1030, 1993, doi: 10.1016/0092-8674(93)90279-Y.

[5] R. A. Dubin, M. A. Kazmi, and H. Ostrer, "Inverted repeats are necessary for circularization of the mouse testis Sry transcript," *Gene*, vol. 167, no. 1–2, pp. 245–248, 1995, doi: 10.1016/0378-1119(95)00639-7.

[6] P. G. Zaphiropoulos, "Circular RNAs from transcripts of the rat cytochrome P450 2C24 gene: Correlation with exon skipping," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 13, pp. 6536–6541, 1996, doi: 10.1073/pnas.93.13.6536.

[7] A. Surono, Y. Takeshima, T. Wibawa, M. Ikezawa, I. Nonaka, and M. Matsuo, "Circular dystrophin RNAs consisting of exons that were skipped by alternative splicing," 1999.

[8] R. J. Dixon, I. C. Eperon, L. Hall, and N. J. Samani, "A genome-wide survey demonstrates widespread non-linear mRNA in expressed sequences from multiple species," *Nucleic Acids Res.*, vol. 33, no. 18, pp. 5904–5913, Oct. 2005, doi: 10.1093/nar/gki893.

[9] H. H. Al-Balool *et al.*, "Post-transcriptional exon shuffling events in humans can be evolutionarily conserved and abundant," *Genome Res.*, vol. 21, no. 11, pp. 1788–1799, Nov. 2011, doi: 10.1101/gr.116442.110.

[10] J. Salzman, C. Gawad, P. L. Wang, N. Lacayo, and P. O. Brown, "Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types," *PLoS One*, vol. 7, no. 2, 2012, doi: 10.1371/journal.pone.0030733.

[11] S. Memczak *et al.*, "Circular RNAs are a large class of animal RNAs with regulatory potency," *Nature*, vol. 495, no. 7441, pp. 333–338, 2013, doi: 10.1038/nature11928.

[12] W. R. Jeck *et al.*, "Circular RNAs are abundant, conserved, and associated with ALU repeats," *RNA*, vol. 19, no. 2, pp. 141–157, Feb. 2013, doi: 10.1261/rna.035667.112.

[13] A. Rybak-Wolf *et al.*, "Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed," *Mol. Cell*, vol. 58, no. 5, pp. 870–885, Dec. 2014, doi: 10.1016/j.molcel.2015.03.027.

[14] X. You *et al.*, "Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity," *Nat. Neurosci.*, vol. 18, no. 4, pp. 603–610, 2015, doi: 10.1038/nn.3975.

[15] P. L. Wang *et al.*, "Circular RNA is expressed across the eukaryotic tree of life," *PLoS One*, vol. 9, no. 3, 2014, doi: 10.1371/journal.pone.0090859.

[16] S. Djebali *et al.*, "Landscape of transcription in human cells," *Nature*, vol. 489, no. 7414, pp. 101–108, Sep. 2012, doi: 10.1038/nature11233.

[17] M. J. Hangauer, I. W. Vaughn, and M. T. McManus, "Pervasive Transcription of the

Human Genome Produces Thousands of Previously Unidentified Long Intergenic Noncoding RNAs," *PLoS Genet.*, vol. 9, no. 6, p. 1003569, Jun. 2013, doi: 10.1371/journal.pgen.1003569.
[18] Y. Gao, J. Wang, and F. Zhao, "CIRI: An efficient and unbiased algorithm for de novo circular RNA identification," *Genome Biol.*, vol. 16, no. 1, pp. 1–16, 2015, doi: 10.1186/s13059-014-0571-3.
[19] K. Wang *et al.*, "MapSplice: Accurate mapping of RNA-seq reads for splice junction discovery," *Nucleic Acids Res.*, vol. 38, no. 18, pp. 1–14, 2010, doi: 10.1093/nar/gkq622.
[20] Y. C. Liu *et al.*, "CircNet: A database of circular RNAs derived from transcriptome sequencing data," *Nucleic Acids Res.*, vol. 44, no. D1, pp. D209–D215, 2016, doi: 10.1093/nar/gkv940.
[21] P. Glažar, P. Papavasileiou, and N. Rajewsky, "CircBase: A database for circular RNAs," *RNA*, vol. 20, no. 11, pp. 1666–1670, Nov. 2014, doi: 10.1261/rna.043687.113.
[22] Q. Liu *et al.*, "Circular RNA Related to the Chondrocyte ECM Regulates MMP13 Expression by Functioning as a MiR-136 'Sponge' in Human Cartilage Degradation," *Sci. Rep.*, vol. 6, no. March, pp. 1–11, 2016, doi: 10.1038/srep22572.
[23] D. B. Dudekula, A. C. Panda, I. Grammatikakis, S. De, K. Abdelmohsen, and M. Gorospe, "Circinteractome: A web tool for exploring circular RNAs and their interacting proteins and microRNAs," *RNA Biol.*, vol. 13, no. 1, pp. 34–42, Jan. 2016, doi: 10.1080/15476286.2015.1128065.
[24] S. Li *et al.*, "ExoRBase: A database of circRNA, lncRNA and mRNA in human blood exosomes," *Nucleic Acids Res.*, vol. 46, no. D1, pp. D106–D112, Jan. 2018, doi: 10.1093/nar/gkx891.
[25] X. Meng, D. Hu, P. Zhang, Q. Chen, and M. Chen, "CircFunBase: A database for functional circular RNAs," *Database*, vol. 2019, Feb. 2019, doi: 10.1093/database/baz003.
[26] L. Szabo, J. Salzman, B. Informatics, T. Program, and S. Cancer, "Detecting circular RNAs: bioinformatic and experimental challenges," vol. 17, no. 11, pp. 679–692, 2016, doi: 10.1038/nrg.2016.114.Detecting.
[27] R. Rigatti, J. H. Jia, N. J. Samani, and I. C. Eperon, "Exon repetition: A major pathway for processing mRNA of some genes is allele-specific," *Nucleic Acids Res.*, vol. 32, no. 2, pp. 441–446, 2004, doi: 10.1093/nar/gkh197.
[28] W. R. Jeck and N. E. Sharpless, "Detecting and characterizing circular RNAs," *Nature Biotechnology*, vol. 32, no. 5. Nature Publishing Group, pp. 453–461, 2014, doi: 10.1038/nbt.2890.
[29] X. Li, L. Yang, and L. L. Chen, "The Biogenesis, Functions, and Challenges of Circular RNAs," *Mol. Cell*, vol. 71, no. 3, pp. 428–442, 2018, doi: 10.1016/j.molcel.2018.06.034.
[30] H. Suzuki, Y. Zuo, J. Wang, M. Q. Zhang, A. Malhotra, and A. Mayeda, "Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing," *Nucleic Acids Res.*, vol. 34, no. 8, p. e63, 2006, doi: 10.1093/nar/gkl151.
[31] R. Ashwal-Fluss *et al.*, "CircRNA Biogenesis competes with Pre-mRNA splicing," *Mol. Cell*, vol. 56, no. 1, pp. 55–66, Oct. 2014, doi: 10.1016/j.molcel.2014.08.019.
[32] S. Starke *et al.*, "Exon circularization requires canonical splice signals," *Cell Rep.*, vol. 10, no. 1, pp. 103–111, Jan. 2015, doi: 10.1016/j.celrep.2014.12.002.
[33] D. Liang *et al.*, "The Output of Protein-Coding Genes Shifts to Circular RNAs When the

Pre-mRNA Processing Machinery Is Limiting," *Mol. Cell*, vol. 68, no. 5, pp. 940-954.e3, Dec. 2017, doi: 10.1016/j.molcel.2017.10.034.
[34] W. Chen and E. Schuman, "Circular RNAs in Brain and Other Tissues: A Functional Enigma," *Trends Neurosci.*, vol. 39, no. 9, pp. 597–604, 2016, doi: 10.1016/j.tins.2016.06.006.
[35] T. Shen, M. Han, G. Wei, and T. Ni, "An intriguing RNA species—perspectives of circularized RNA," *Protein and Cell*, vol. 6, no. 12. Higher Education Press, pp. 871–880, Dec. 01, 2015, doi: 10.1007/s13238-015-0202-0.
[36] J. Salzman, R. E. Chen, M. N. Olsen, P. L. Wang, and P. O. Brown, "Cell-Type Specific Features of Circular RNA Expression," *PLoS Genet.*, vol. 9, no. 9, p. e1003777, Sep. 2013, doi: 10.1371/journal.pgen.1003777.
[37] Y. Zhang *et al.*, "Circular Intronic Long Noncoding RNAs," *Mol. Cell*, vol. 51, no. 6, pp. 792–806, 2013, doi: 10.1016/j.molcel.2013.08.017.
[38] A. Ivanov *et al.*, "Analysis of intron sequences reveals hallmarks of circular RNA biogenesis in animals," *Cell Rep.*, vol. 10, no. 2, pp. 170–177, Jan. 2015, doi: 10.1016/j.celrep.2014.12.019.
[39] S. Kelly, C. Greenman, P. R. Cook, and A. Papantonis, "Exon Skipping Is Correlated with Exon Circularization," *J. Mol. Biol.*, vol. 427, no. 15, pp. 2414–2417, 2015, doi: 10.1016/j.jmb.2015.02.018.
[40] X. O. Zhang, H. Bin Wang, Y. Zhang, X. Lu, L. L. Chen, and L. Yang, "Complementary sequence-mediated exon circularization," *Cell*, vol. 159, no. 1, pp. 134–147, Sep. 2014, doi: 10.1016/j.cell.2014.09.001.
[41] D. Liang and J. E. Wilusz, "Short intronic repeat sequences facilitate circular RNA production," *Genes Dev.*, vol. 28, no. 20, pp. 2233–2247, 2014, doi: 10.1101/gad.251926.114.
[42] T. Xia *et al.*, "Long noncoding RNA associated-competing endogenous RNAs in gastric cancer," *Sci. Rep.*, vol. 4, no. 1, pp. 1–7, Aug. 2014, doi: 10.1038/srep06088.
[43] S. J. Conn *et al.*, "The RNA binding protein quaking regulates formation of circRNAs," *Cell*, vol. 160, no. 6, pp. 1125–1134, 2015, doi: 10.1016/j.cell.2015.02.014.
[44] L. Errichelli *et al.*, "FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons," *Nat. Commun.*, vol. 8, pp. 1–11, 2017, doi: 10.1038/ncomms14741.
[45] M. C. Kramer *et al.*, "Combinatorial control of Drosophila circular RNA expression by intronic repeats, hnRNPs, and SR proteins," *Genes Dev.*, vol. 29, no. 20, pp. 2168–2182, Oct. 2015, doi: 10.1101/gad.270421.115.
[46] H. R. Koh, L. Xing, L. Kleiman, and S. Myong, "Repetitive RNA unwinding by RNA helicase A facilitates RNA annealing," *Nucleic Acids Res.*, vol. 42, no. 13, pp. 8556–8564, 2014, doi: 10.1093/nar/gku523.
[47] T. Aktaş *et al.*, "DHX9 suppresses RNA processing defects originating from the Alu invasion of the human genome," *Nature*, vol. 544, no. 7648, pp. 115–119, Apr. 2017, doi: 10.1038/nature21715.
[48] X. Wen *et al.*, "NF90 Exerts Antiviral Activity through Regulation of PKR Phosphorylation and Stress Granules in Infected Cells," *J. Immunol.*, vol. 192, no. 8, pp. 3753–3764, Apr. 2014, doi: 10.4049/jimmunol.1302813.
[49] J. U. Guo, V. Agarwal, H. Guo, and D. P. Bartel, "Expanded identification and characterization of mammalian circular RNAs," *Genome Biol.*, vol. 15, no. 7, pp. 1–14,

2014, doi: 10.1186/s13059-014-0409-z.
[50] T. B. Hansen *et al.*, “MiRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA,” *EMBO J.*, vol. 30, no. 21, pp. 4414–4422, 2011, doi: 10.1038/emboj.2011.359.
[51] M. Piwecka *et al.*, “Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function,” *Science (80-.).*, vol. 357, no. 6357, pp. 1–14, 2017, doi: 10.1126/science.aam8526.
[52] B. Kleaveland, C. Y. Shi, J. Stefano, and D. P. Bartel, “A Network of Noncoding Regulatory RNAs Acts in the Mammalian Brain,” *Cell*, vol. 174, no. 2, pp. 350-362.e17, 2018, doi: 10.1016/j.cell.2018.05.022.
[53] A. Ok Hyun Park *et al.*, “Endoribonucleolytic Cleavage of m 6 A-Containing RNAs by RNase P/MRP Complex,” *Mol. Cell*, vol. 74, pp. 494-507.e8, 2019, doi: 10.1016/j.molcel.2019.02.034.
[54] C. X. Liu *et al.*, “Structure and Degradation of Circular RNAs Regulate PKR Activation in Innate Immunity,” *Cell*, vol. 177, no. 4, pp. 865-880.e21, 2019, doi: 10.1016/j.cell.2019.03.046.
[55] C. Preußer *et al.*, “Selective release of circRNAs in platelet-derived extracellular vesicles,” *J. Extracell. Vesicles*, vol. 7, no. 1, 2018, doi: 10.1080/20013078.2018.1424473.
[56] E. Lasda and R. Parker, “Circular RNAs co-precipitate with extracellular vesicles: A possible mechanism for circrna clearance,” *PLoS One*, vol. 11, no. 2, pp. 1–11, 2016, doi: 10.1371/journal.pone.0148407.
[57] S. Werfel, S. Nothjunge, T. Schwarzmayr, T. M. Strom, T. Meitinger, and S. Engelhardt, “Characterization of circular RNAs in human, mouse and rat hearts,” *J. Mol. Cell. Cardiol.*, vol. 98, pp. 103–107, Sep. 2016, doi: 10.1016/j.yjmcc.2016.07.007.
[58] Z. Li *et al.*, “Exon-intron circular RNAs regulate transcription in the nucleus,” *Nat. Struct. Mol. Biol.*, vol. 22, no. 3, pp. 256–264, Mar. 2015, doi: 10.1038/nsmb.2959.
[59] C. Huang, D. Liang, D. C. Tatomer, and J. E. Wilusz, “A length-dependent evolutionarily conserved pathway controls nuclear export of circular RNAs,” *Genes Dev.*, vol. 32, no. 9–10, pp. 639–644, May 2018, doi: 10.1101/gad.314856.118.
[60] V. M. Conn *et al.*, “A circRNA from SEPALLATA3 regulates splicing of its cognate mRNA through R-loop formation,” *Nat. Plants*, vol. 3, no. 5, pp. 1–5, Apr. 2017, doi: 10.1038/nplants.2017.53.
[61] N. Chen *et al.*, “A novel FLI1 exonic circular RNA promotes metastasis in breast cancer by coordinately regulating TET1 and DNMT1,” *Genome Biol.*, pp. 1–14, 2018.
[62] T. B. Hansen *et al.*, “Natural RNA circles function as efficient microRNA sponges,” *Nature*, vol. 495, no. 7441, pp. 384–388, 2013, doi: 10.1038/nature11993.
[63] K. Abdelmohsen *et al.*, “Identification of HuR target circular RNAs uncovers suppression of PABPN1 translation by CircPABPN1,” *RNA Biol.*, vol. 14, no. 3, pp. 361–369, 2017, doi: 10.1080/15476286.2017.1279788.
[64] Y. Chen *et al.*, “Circular RNA circAGO2 drives cancer progression through facilitating HuR-repressed functions of AGO2-miRNA complexes,” *Cell Death Differ.*, vol. 26, no. 7, pp. 1346–1364, 2019, doi: 10.1038/s41418-018-0220-6.
[65] L. M. Holdt *et al.*, “Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans,” *Nat. Commun.*, vol. 7, no. 1, pp. 1–14, Aug. 2016, doi: 10.1038/ncomms12429.

[66] P. Xia *et al.*, “A Circular RNA Protects Dormant Hematopoietic Stem Cells from DNA Sensor cGAS-Mediated Exhaustion,” *Immunity*, vol. 48, no. 4, pp. 688-701.e7, 2018, doi: 10.1016/j.immuni.2018.03.016.
[67] N. Wu *et al.*, “Translation of yes-associated protein (YAP) was antagonized by its circular RNA via suppressing the assembly of the translation initiation machinery,” *Cell Death Differ.*, vol. 26, no. 12, pp. 2758–2773, 2019, doi: 10.1038/s41418-019-0337-2.
[68] A. Nan *et al.*, “Circular RNA circNOL10 Inhibits Lung Cancer Development by Promoting SCLM1-Mediated Transcriptional Regulation of the Humanin Polypeptide Family,” *Adv. Sci.*, vol. 6, no. 2, Jan. 2019, doi: 10.1002/advs.201800654.
[69] Y. Zeng *et al.*, “A circular RNA binds to and activates AKT phosphorylation and nuclear localization reducing apoptosis and enhancing cardiac repair,” *Theranostics*, vol. 7, no. 16, pp. 3842–3855, 2017, doi: 10.7150/thno.19764.
[70] W. W. Du, W. Yang, E. Liu, Z. Yang, P. Dhaliwal, and B. B. Yang, “Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2,” *Nucleic Acids Res.*, vol. 44, no. 6, pp. 2846–2858, 2016, doi: 10.1093/nar/gkw027.
[71] W. W. Du *et al.*, “Induction of tumor apoptosis through a circular RNA enhancing Foxo3 activity,” *Cell Death Differ.*, vol. 24, no. 2, pp. 357–370, 2017, doi: 10.1038/cdd.2016.133.
[72] Y. Gao and F. Zhao, “Computational Strategies for Exploring Circular RNAs,” *Trends Genet.*, vol. 34, no. 5, pp. 389–400, 2018, doi: 10.1016/j.tig.2017.12.016.
[73] C. Y. Chen and P. Sarnow, “Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs,” *Science (80-.).*, vol. 268, no. 5209, pp. 415–417, Apr. 1995, doi: 10.1126/science.7536344.
[74] Y. Wang and Z. Wang, “Efficient backsplicing produces translatable circular mRNAs,” *Rna*, vol. 21, no. 2. pp. 172–179, 2015, doi: 10.1261/rna.048272.114.
[75] R. A. Wesselhoeft, P. S. Kowalski, and D. G. Anderson, “Engineering circular RNA for potent and stable translation in eukaryotic cells,” *Nat. Commun.*, vol. 9, no. 1, pp. 1–10, Dec. 2018, doi: 10.1038/s41467-018-05096-6.
[76] Y. Yang *et al.*, “Novel Role of FBXW7 Circular RNA in Repressing Glioma Tumorigenesis,” *J. Natl. Cancer Inst.*, vol. 110, no. 3, pp. 304–315, 2018, doi: 10.1093/jnci/djx166.
[77] N. R. Pamudurti *et al.*, “Translation of CircRNAs,” *Mol. Cell*, vol. 66, no. 1, pp. 9-21.e7, 2017, doi: 10.1016/j.molcel.2017.02.021.
[78] I. Legnini *et al.*, “Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis,” *Mol. Cell*, vol. 66, no. 1, pp. 22-37.e9, 2017, doi: 10.1016/j.molcel.2017.02.017.
[79] M. Zhang *et al.*, “A peptide encoded by circular form of LINC-PINT suppresses oncogenic transcriptional elongation in glioblastoma,” *Nat. Commun.*, vol. 9, no. 1, pp. 1–17, Dec. 2018, doi: 10.1038/s41467-018-06862-2.
[80] M. Zhang *et al.*, “A novel protein encoded by the circular form of the SHPRH gene suppresses glioma tumorigenesis,” *Oncogene*, vol. 37, no. 13, pp. 1805–1814, Mar. 2018, doi: 10.1038/s41388-017-0019-9.
[81] Y. Yang *et al.*, “Extensive translation of circular RNAs driven by N 6 -methyladenosine,” *Cell Res.*, vol. 27, no. 5, pp. 626–641, May 2017, doi: 10.1038/cr.2017.31.
[82] C. Zhou *et al.*, “Genome-Wide Maps of m6A circRNAs Identify Widespread and Cell-Type-Specific Methylation Patterns that Are Distinct from mRNAs,” *Cell Rep.*, vol. 20,

no. 9, pp. 2262–2276, 2017, doi: 10.1016/j.celrep.2017.08.027.
[83] X. Chen, P. Han, T. Zhou, X. Guo, X. Song, and Y. Li, "CircRNADb: A comprehensive database for human circular RNAs with protein-coding annotations," *Sci. Rep.*, vol. 6, no. September, pp. 1–6, 2016, doi: 10.1038/srep34985.
[84] J. O. Westholm *et al.*, "Genome-wide Analysis of Drosophila Circular RNAs Reveals Their Structural and Sequence Properties and Age-Dependent Neural Accumulation," *Cell Rep.*, vol. 9, no. 5, pp. 1966–1980, 2014, doi: 10.1016/j.celrep.2014.10.062.
[85] M. T. Venø *et al.*, "Spatio-temporal regulation of circular RNA expression during porcine embryonic brain development," *Genome Biol.*, vol. 16, no. 1, 2015, doi: 10.1186/s13059-015-0801-3.
[86] A. Gokool, F. Anwar, and I. Voineagu, "The Landscape of Circular RNA Expression in the Human Brain," *Biol. Psychiatry*, vol. 87, no. 3, pp. 294–304, Feb. 2020, doi: 10.1016/j.biopsych.2019.07.029.
[87] E. Mahmoudi, D. Kiltschewskij, C. Fitzsimmons, and M. J. Cairns, "Depolarization-Associated CircRNA Regulate Neural Gene Expression and in Some Cases May Function as Templates for Translation," *Cells*, vol. 9, no. 1, p. 25, 2019, doi: 10.3390/cells9010025.
[88] M. Cortés-López *et al.*, "Global accumulation of circRNAs during aging in Caenorhabditis elegans," *BMC Genomics*, vol. 19, no. 1, pp. 1–12, 2018, doi: 10.1186/s12864-017-4386-y.
[89] H. Gruner, M. Cortés-López, D. A. Cooper, M. Bauer, and P. Miura, "CircRNA accumulation in the aging mouse brain," *Sci. Rep.*, vol. 6, no. 1, pp. 1–14, Dec. 2016, doi: 10.1038/srep38907.
[90] E. Mahmoudi and M. J. Cairns, "Circular RNAs are temporospatially regulated throughout development and ageing in the rat," *Sci. Rep.*, pp. 1–12, 2019, doi: 10.1038/s41598-019-38860-9.
[91] Z. Liu, Y. Ran, C. Tao, S. Li, J. Chen, and E. Yang, "Detection of circular RNA expression and related quantitative trait loci in the human dorsolateral prefrontal cortex," *Genome Biol.*, pp. 1–16, 2019.
[92] K. Charizanis *et al.*, "Muscleblind-like 2-Mediated Alternative Splicing in the Developing Brain and Dysregulation in Myotonic Dystrophy," *Neuron*, vol. 75, no. 3, pp. 437–450, 2012, doi: 10.1016/j.neuron.2012.05.029.
[93] H. Deng, K. Gao, and J. Jankovic, "The role of FUS gene variants in neurodegenerative diseases," *Nature Reviews Neurology*, vol. 10, no. 6. Nature Publishing Group, pp. 337–348, 2014, doi: 10.1038/nrneurol.2014.78.
[94] K. Gao *et al.*, "Genetic analysis of the fused in sarcoma gene in Chinese Han patients with Parkinson's disease," *Park. Relat. Disord.*, vol. 20, no. 1, pp. 119–121, Jan. 2014, doi: 10.1016/j.parkreldis.2013.09.010.
[95] T. L. Lauriat *et al.*, "Developmental expression profile of Quaking, a candidate gene for schizophrenia, and its target genes in human prefrontal cortex and hippocampus shows regional specificity," *J. Neurosci. Res.*, vol. 86, no. 4, pp. 785–796, 2008, doi: 10.1002/jnr.21534.
[96] Z. Zhao *et al.*, "CircRNA disease: A manually curated database of experimentally supported circRNA-disease associations," *Cell Death Dis.*, vol. 9, no. 5, pp. 4–5, 2018, doi: 10.1038/s41419-018-0503-3.
[97] M. Zhao *et al.*, "Altered expression of circular RNAs in Moyamoya disease," *J. Neurol. Sci.*, vol. 381, no. 6, pp. 25–31, 2017, doi: 10.1016/j.jns.2017.08.011.

[98] W. J. Lukiw, “Circular RNA (circRNA) in Alzheimer’s disease (AD),” *Frontiers in Genetics*, vol. 4, no. DEC. 2013, doi: 10.3389/fgene.2013.00307.
[99] J. H. Nie, T. X. Li, X. Q. Zhang, and J. Liu, “Roles of non-coding RNAs in normal human brain development, brain tumor, and neuropsychiatric disorders,” *Non-coding RNA*, vol. 5, no. 2, 2019, doi: 10.3390/ncrna5020036.
[100] Z. Shi *et al.*, “The circular RNA ciRS-7 promotes APP and BACE1 degradation in an NF-κB-dependent manner,” *FEBS J.*, vol. 284, no. 7, pp. 1096–1109, Apr. 2017, doi: 10.1111/febs.14045.
[101] S. Zhang, D. Zhu, H. Li, H. Li, C. Feng, and W. Zhang, “Characterization of circRNA-Associated-ceRNA Networks in a Senescence-Accelerated Mouse Prone 8 Brain,” *Mol. Ther.*, vol. 25, no. 9, pp. 2053–2061, 2017, doi: 10.1016/j.ymthe.2017.06.009.
[102] Z. Wang *et al.*, “Identifying circRNA-associated-ceRNA networks in the hippocampus of Aß1-42-induced Alzheimer’s disease-like rats using microarray analysis,” *Aging (Albany. NY).*, vol. 10, no. 4, pp. 775–788, Apr. 2018, doi: 10.18632/aging.101427.
[103] Y. J. Chen *et al.*, “Genome-wide, integrative analysis of circular RNA dysregulation and the corresponding circular RNAmicroRNA-mRNA regulatory axes in autism,” *Genome Res.*, vol. 30, no. 3, pp. 375–391, 2020, doi: 10.1101/gr.255463.119.
[104] R. T. Zhao *et al.*, “Circular ribonucleic acid expression alteration in exosomes from the brain extracellular space after traumatic brain injury in mice,” *J. Neurotrauma*, vol. 35, no. 17, pp. 2056–2066, Sep. 2018, doi: 10.1089/neu.2017.5502.
[105] Y. Li *et al.*, “Circular RNA is enriched and stable in exosomes: A promising biomarker for cancer diagnosis,” *Cell Research*, vol. 25, no. 8. Nature Publishing Group, pp. 981–984, Aug. 06, 2015, doi: 10.1038/cr.2015.82.
[106] Y. Wang *et al.*, “Exosomal circRNAs: Biogenesis, effect and application in human diseases,” *Mol. Cancer*, vol. 18, no. 1, pp. 1–10, 2019, doi: 10.1186/s12943-019-1041-z.
[107] S. Saeedi, S. Israel, C. Nagy, and G. Turecki, “The emerging role of exosomes in mental disorders,” *Translational Psychiatry*, vol. 9, no. 1. Nature Publishing Group, pp. 1–11, Dec. 01, 2019, doi: 10.1038/s41398-019-0459-9.
[108] J. He, M. Ren, H. Li, L. Yang, X. Wang, and Q. Yang, “Exosomal circular RNA as a biomarker platform for the early diagnosis of immune-mediated demyelinating disease,” *Front. Genet.*, vol. 10, no. SEP, pp. 1–15, 2019, doi: 10.3389/fgene.2019.00860.
[109] L. Iparraguirre, M. Muñoz-Culla, I. Prada-Luengo, T. Castillo-Triviño, J. Olascoaga, and D. Otaegui, “Circular RNA profiling reveals that circular RNAs from ANXA2 can be used as new biomarkers for multiple sclerosis,” *Hum. Mol. Genet.*, vol. 26, no. 18, pp. 3564–3572, 2017, doi: 10.1093/hmg/ddx243.
[110] D. H. Ebert and M. E. Greenberg, “Activity-dependent neuronal signalling and autism spectrum disorder,” *Nature*, vol. 493, no. 7432. Nature, pp. 327–337, Jan. 17, 2013, doi: 10.1038/nature11860.
[111] H. Y. Zoghbi and M. F. Bear, “Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities,” *Cold Spring Harb. Perspect. Biol.*, vol. 4, no. 3, Mar. 2012, doi: 10.1101/cshperspect.a009886.
[112] American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders*. American Psychiatric Association, 2013.
[113] T. Bourgeron, “From the genetic architecture to synaptic plasticity in autism spectrum disorder,” *Nature Reviews Neuroscience*, vol. 16, no. 9. Nature Publishing Group, pp. 551–563, Aug. 20, 2015, doi: 10.1038/nrn3992.

[114] G. Huguet, E. Ey, and T. Bourgeron, “The genetic landscapes of autism spectrum disorders,” *Annual Review of Genomics and Human Genetics*, vol. 14. Annu Rev Genomics Hum Genet, pp. 191–213, Aug. 2013, doi: 10.1146/annurev-genom-091212-153431.
[115] C. Betancur, “Etiological heterogeneity in autism spectrum disorders: More than 100 genetic and genomic disorders and still counting,” *Brain Research*, vol. 1380. Brain Res, pp. 42–77, Mar. 22, 2011, doi: 10.1016/j.brainres.2010.11.078.
[116] C. A. Doll and K. Broadie, “Impaired activity-dependent neural circuit assembly and refinement in autism spectrum disorder genetic models,” *Front. Cell. Neurosci.*, vol. 8, no. FEB, Feb. 2014, doi: 10.3389/fncel.2014.00030.
[117] S. J. Sanders, “First glimpses of the neurobiology of autism spectrum disorder,” *Current Opinion in Genetics and Development*, vol. 33. Elsevier Ltd, pp. 80–92, Aug. 01, 2015, doi: 10.1016/j.gde.2015.10.002.
[118] R. M. Smith and W. Sadee, “Synaptic signaling and aberrant RNA splicing in autism spectrum disorders,” *Frontiers in Synaptic Neuroscience*, vol. 3, no. JAN. Frontiers Media SA, 2011, doi: 10.3389/fnsyn.2011.00001.
[119] N. N. Parikshak *et al.*, “Genome-wide changes in lncRNA, splicing, and regional gene expression patterns in autism,” *Nature*, vol. 540, no. 7633, pp. 423–427, Dec. 2016, doi: 10.1038/nature20612.
[120] M. N. Ziats and O. M. Rennert, “Identification of differentially expressed microRNAs across the developing human brain,” *Mol. Psychiatry*, vol. 19, no. 7, pp. 848–852, 2014, doi: 10.1038/mp.2013.93.
[121] M. N. Ziats and O. M. Rennert, “Aberrant Expression of Long Noncoding RNAs in Autistic Brain,” *J. Mol. Neurosci.*, vol. 49, no. 3, pp. 589–593, Mar. 2013, doi: 10.1007/s12031-012-9880-8.
[122] B. Stamova, B. P. Ander, N. Barger, F. R. Sharp, and C. M. Schumann, “Specific Regional and Age-Related Small Noncoding RNA Expression Patterns Within Superior Temporal Gyrus of Typical Human Brains Are Less Distinct in Autism Brains,” *J. Child Neurol.*, vol. 30, no. 14, pp. 1930–1946, Dec. 2015, doi: 10.1177/0883073815602067.
[123] S. Gasparini *et al.*, “Differential Expression of Hippocampal Circular RNAs in the BTBR Mouse Model for Autism Spectrum Disorder,” *Mol. Neurobiol.*, vol. 57, no. 5, pp. 2301–2313, Feb. 2020, doi: 10.1007/s12035-020-01878-6.
[124] M. Stamou *et al.*, “A Balanced Translocation in Kallmann Syndrome Implicates a Long Noncoding RNA, RMST, as a GnRH Neuronal Regulator,” *J. Clin. Endocrinol. Metab.*, vol. 105, no. 3, Jan. 2020, doi: 10.1210/clinem/dgz011.
[125] K. Wang *et al.*, “Common genetic variants on 5p14.1 associate with autism spectrum disorders,” *Nature*, vol. 459, no. 7246, pp. 528–533, May 2009, doi: 10.1038/nature07999.
[126] D. Ma *et al.*, “A genome-wide association study of autism reveals a common novel risk locus at 5p14.1,” *Ann. Hum. Genet.*, vol. 73, no. 3, pp. 263–273, 2009, doi: 10.1111/j.1469-1809.2009.00523.x.
[127] J. D. Jontes, “The cadherin superfamily in neural circuit assembly,” *Cold Spring Harb. Perspect. Biol.*, vol. 10, no. 7, pp. 1–19, 2018, doi: 10.1101/cshperspect.a029306.
[128] M. Takeichi, “The cadherin superfamily in neuronal connections and interactions,” *Nat. Rev. Neurosci.*, vol. 8, no. 1, pp. 11–20, 2007, doi: 10.1038/nrn2043.
[129] H. Togashi, K. Abe, A. Mizoguchi, K. Takaoka, O. Chisaka, and M. Takeichi, “Cadherin regulates dendritic spine morphogenesis,” *Neuron*, vol. 35, no. 1, pp. 77–89, 2002, doi:

10.1016/S0896-6273(02)00748-1.
[130] M. E. Williams *et al.*, "Cadherin-9 regulates synapse-specific differentiation in the developing hippocampus," *Neuron*, vol. 71, no. 4, pp. 640–655, Aug. 2011, doi: 10.1016/j.neuron.2011.06.019.
[131] F. M. Oeschger *et al.*, "Gene expression analysis of the embryonic subplate," *Cereb. Cortex*, vol. 22, no. 6, pp. 1343–1359, 2012, doi: 10.1093/cercor/bhr197.
[132] Y. C. Lin, J. A. Frei, M. B. C. Kilander, W. Shen, and G. J. Blatt, "A subset of autism-associated genes regulate the structural stability of neurons," *Front. Cell. Neurosci.*, vol. 10, no. NOV2016, pp. 1–35, 2016, doi: 10.3389/fncel.2016.00263.
[133] K. Lefkovics, M. Mayer, K. Bercsényi, G. Szabó, and Z. Lele, "Comparative analysis of type II classic cadherin mRNA distribution patterns in the developing and adult mouse somatosensory cortex and hippocampus suggests significant functional redundancy," *J. Comp. Neurol.*, vol. 520, no. 7, pp. 1387–1405, 2012, doi: 10.1002/cne.22801.
[134] C. Wang, Y. H. Pan, Y. Wang, G. Blatt, and X. B. Yuan, "Segregated expressions of autism risk genes Cdh11 and Cdh9 in autism-relevant regions of developing cerebellum," *Mol. Brain*, vol. 12, no. 1, pp. 1–13, 2019, doi: 10.1186/s13041-019-0461-4.
[135] S. Y. Ng, R. Johnson, and L. W. Stanton, "Human long non-coding RNAs promote pluripotency and neuronal differentiation by association with chromatin modifiers and transcription factors," *EMBO J.*, vol. 31, no. 3, pp. 522–533, 2012, doi: 10.1038/emboj.2011.459.
[136] O. G. Izuogu *et al.*, "Analysis of human ES cell differentiation establishes that the dominant isoforms of the lncRNAs RMST and FIRRE are circular," *BMC Genomics*, vol. 19, no. 1, Apr. 2018, doi: 10.1186/s12864-018-4660-7.
[137] J. L. Silverman *et al.*, "Sociability and motor functions in Shank1 mutant mice," *Brain Res.*, vol. 1380, pp. 120–137, Mar. 2011, doi: 10.1016/j.brainres.2010.09.026.
[138] K. Radyushkin *et al.*, "Neuroligin-3-deficient mice: Model of a monogenic heritable form of autism with an olfactory deficit," *Genes, Brain Behav.*, vol. 8, no. 4, pp. 416–425, Jun. 2009, doi: 10.1111/j.1601-183X.2009.00487.x.
[139] M. D. Shahbazian *et al.*, "Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3," *Neuron*, vol. 35, no. 2, pp. 243–254, Jul. 2002, doi: 10.1016/S0896-6273(02)00768-7.
[140] M. Lacaria, C. Spencer, W. Gu, R. Paylor, and J. R. Lupski, "Enriched rearing improves behavioral responses of an animal model for CNV-based autistic-like traits," *Hum. Mol. Genet.*, vol. 21, no. 14, pp. 3083–3096, Jul. 2012, doi: 10.1093/hmg/dds124.
[141] R. F. Kooy *et al.*, "Transgenic mouse model for the fragile X syndrome," *Am. J. Med. Genet.*, vol. 64, no. 2, pp. 241–245, Aug. 1996, doi: 10.1002/(SICI)1096-8628(19960809)64:2<241::AID-AJMG1>3.0.CO;2-X.
[142] S. S. Moy and J. J. Nadler, "Advances in behavioral genetics: Mouse models of autism," *Mol. Psychiatry*, vol. 13, no. 1, pp. 4–26, 2008, doi: 10.1038/sj.mp.4002082.
[143] H. G. McFarlane, G. K. Kusek, M. Yang, J. L. Phoenix, V. J. Bolivar, and J. N. Crawley, "Autism-like behavioral phenotypes in BTBR T+tf/J mice," *Genes, Brain Behav.*, vol. 7, no. 2, pp. 152–163, 2008, doi: 10.1111/j.1601-183X.2007.00330.x.
[144] K. Z. Meyza *et al.*, "The BTBR T+tf/J mouse model for autism spectrum disorders-in search of biomarkers," *Behav. Brain Res.*, vol. 251, pp. 25–34, 2013, doi: 10.1016/j.bbr.2012.07.021.
[145] M. L. Scattoni, S. U. Gandhy, L. Ricceri, and J. N. Crawley, "Unusual repertoire of

vocalizations in the BTBR T+tf/J mouse model of autism," *PLoS One*, vol. 3, no. 8, pp. 48–52, 2008, doi: 10.1371/journal.pone.0003067.
[146] M. L. Scattoni, A. Martire, G. Cartocci, A. Ferrante, and L. Ricceri, "Reduced social interaction, behavioural flexibility and BDNF signalling in the BTBR T+tf/J strain, a mouse model of autism," *Behav. Brain Res.*, vol. 251, pp. 35–40, 2013, doi: 10.1016/j.bbr.2012.12.028.
[147] J. L. Silverman *et al.*, "Low stress reactivity and neuroendocrine factors in the BTBR T+tf/J mouse model of autism," *Neuroscience*, vol. 171, no. 4, pp. 1197–1208, 2010, doi: 10.1016/j.neuroscience.2010.09.059.
[148] D. Wahlsten, P. Metten, and J. C. Crabbe, "Survey of 21 inbred mouse strains in two laboratories reveals that BTBR T/+ tf/tf has severely reduced hippocampal commissure and absent corpus callosum," *Brain Res.*, vol. 971, no. 1, pp. 47–54, May 2003, doi: 10.1016/S0006-8993(03)02354-0.
[149] D. T. Stephenson *et al.*, "Histopathologic characterization of the BTBR mouse model of autistic-like behavior reveals selective changes in neurodevelopmental proteins and adult hippocampal neurogenesis," *Mol. Autism*, vol. 2, no. 1, 2011, doi: 10.1186/2040-2392-2-7.
[150] M. Wang *et al.*, "Integrated analysis of miRNA and mRNA expression profiles in the brains of BTBR mice," *Int. J. Dev. Neurosci.*, vol. 80, no. 3, pp. 221–233, 2020, doi: 10.1002/jdn.10019.
[151] C. M. Daimon *et al.*, "Hippocampal transcriptomic and proteomic alterations in the BTBR mouse model of autism spectrum disorder," *Front. Physiol.*, vol. 6, no. NOV, pp. 1–17, 2015, doi: 10.3389/fphys.2015.00324.
[152] S. Y. Ng, G. K. Bogu, B. S. Soh, and L. W. Stanton, "The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis," *Mol. Cell*, vol. 51, no. 3, pp. 349–359, Aug. 2013, doi: 10.1016/j.molcel.2013.07.017.
[153] D. R. Hampson and G. J. Blatt, "Autism spectrum disorders and neuropathology of the cerebellum," *Frontiers in Neuroscience*, vol. 9, no. NOV. Frontiers Research Foundation, p. 420, Nov. 06, 2015, doi: 10.3389/fnins.2015.00420.
[154] A. M. D'Mello and C. J. Stoodley, "Cerebro-cerebellar circuits in autism spectrum disorder," *Front. Neurosci.*, vol. 9, no. NOV, p. 408, Nov. 2015, doi: 10.3389/fnins.2015.00408.
[155] Z. Ouhaz, H. Fleming, and A. S. Mitchell, "Cognitive functions and neurodevelopmental disorders involving the prefrontal cortex and mediodorsal thalamus," *Frontiers in Neuroscience*, vol. 12, no. FEB. Frontiers Media S.A., p. 33, Feb. 06, 2018, doi: 10.3389/fnins.2018.00033.
[156] A. J. Zimmerman *et al.*, "A psychiatric disease-related circular RNA controls synaptic gene expression and cognition," *Mol. Psychiatry*, pp. 1–16, Jan. 2020, doi: 10.1038/s41380-020-0653-4.
[157] G. G. Turrigiano, K. R. Leslie, N. S. Desai, L. C. Rutherford, and S. B. Nelson, "Activity-dependent scaling of quantal amplitude in neocortical neurons," *Nature*, vol. 391, no. 6670, pp. 892–896, Feb. 1998, doi: 10.1038/36103.
[158] T. C. Thiagarajan, M. Lindskog, and R. W. Tsien, "Adaptation to synaptic inactivity in hippocampal neurons," *Neuron*, vol. 47, no. 5, pp. 725–737, Sep. 2005, doi: 10.1016/j.neuron.2005.06.037.
[159] E. Y. Chen *et al.*, "Enrichr: Interactive and collaborative HTML5 gene list enrichment analysis tool," *BMC Bioinformatics*, vol. 14, Apr. 2013, doi: 10.1186/1471-2105-14-128.

[160] V. Licursi, F. Conte, G. Fiscon, and P. Paci, “MIENTURNET: An interactive web tool for microRNA-target enrichment and network-based analysis,” *BMC Bioinformatics*, vol. 20, no. 1, p. 545, Nov. 2019, doi: 10.1186/s12859-019-3105-x.
[161] C. Yang *et al.*, “AutismKB 2.0: A knowledgebase for the genetic evidence of autism spectrum disorder,” *Database*, vol. 2018, no. 2018, p. 106, Jan. 2018, doi: 10.1093/database/bay106.
[162] J. L. Attema *et al.*, “Identification of an Enhancer That Increases miR-200b~200a~429 Gene Expression in Breast Cancer Cells,” *PLoS One*, vol. 8, no. 9, p. e75517, Sep. 2013, doi: 10.1371/journal.pone.0075517.
[163] A. Jauhari and S. Yadav, “MiR-34 and MiR-200: Regulator of Cell Fate Plasticity and Neural Development,” *NeuroMolecular Medicine*, vol. 21, no. 2. Humana Press Inc., pp. 97–109, Jun. 01, 2019, doi: 10.1007/s12017-019-08535-9.
[164] S.-M. Park, A. B. Gaur, E. Lengyel, and M. E. Peter, “The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2,” 2008, doi: 10.1101/gad.1640608.
[165] A. Bendoraite *et al.*, “Regulation of miR-200 family microRNAs and ZEB transcription factors in ovarian cancer: Evidence supporting a mesothelial-to-epithelial transition,” *Gynecol. Oncol.*, vol. 116, no. 1, pp. 117–125, Jan. 2010, doi: 10.1016/j.ygyno.2009.08.009.
[166] B. Zikopoulos and H. Barbas, “Altered neural connectivity in excitatory and inhibitory cortical circuits in autism,” *Front. Hum. Neurosci.*, vol. 7, no. SEP, pp. 1–24, 2013, doi: 10.3389/fnhum.2013.00609.
[167] U. Dube *et al.*, “An atlas of cortical circular RNA expression in Alzheimer disease brains demonstrates clinical and pathological associations,” *Nat. Neurosci.*, vol. 22, no. 11, pp. 1903–1912, 2019, doi: 10.1038/s41593-019-0501-5.
[168] Z. Feng, L. Zhang, S. Wang, and Q. Hong, “Circular RNA circDLGAP4 exerts neuroprotective effects via modulating miR-134-5p/CREB pathway in Parkinson’s disease,” *Biochem. Biophys. Res. Commun.*, vol. 522, no. 2, pp. 388–394, 2020, doi: 10.1016/j.bbrc.2019.11.102.
[169] V. S. Chen, J. P. Morrison, M. F. Southwell, J. F. Foley, B. Bolon, and S. A. Elmore, “Histology Atlas of the Developing Prenatal and Postnatal Mouse Central Nervous System, with Emphasis on Prenatal Days E7.5 to E18.5,” *Toxicol. Pathol.*, vol. 45, no. 6, pp. 705–744, Aug. 2017, doi: 10.1177/0192623317728134.
[170] C. L. Muller, A. M. J. Anacker, and J. Veenstra-VanderWeele, “The serotonin system in autism spectrum disorder: From biomarker to animal models,” *Neuroscience*, vol. 321. Elsevier Ltd, pp. 24–41, 2016, doi: 10.1016/j.neuroscience.2015.11.010.
[171] G. G. Gould *et al.*, “Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT1A and cannabinoid CB1 receptors in adolescent BTBR mice,” *Psychoneuroendocrinology*, vol. 39, no. 1, pp. 158–169, Jan. 2014, doi: 10.1016/j.psyneuen.2013.09.003.
[172] S. V. Hegarty, A. M. Sullivan, and G. W. O’Keeffe, “Zeb2: A multifunctional regulator of nervous system development,” *Progress in Neurobiology*, vol. 132. Elsevier Ltd, pp. 81–95, Sep. 01, 2015, doi: 10.1016/j.pneurobio.2015.07.001.
[173] G. L. McKinsey *et al.*, “Dlx1&2-Dependent Expression of Zfhx1b (Sip1, Zeb2) Regulates the Fate Switch between Cortical and Striatal Interneurons,” *Neuron*, vol. 77, no. 1, pp. 83–98, Jan. 2013, doi: 10.1016/j.neuron.2012.11.035.

[174] M. P. Adam *et al.*, “Clinical features and management issues in Mowat-Wilson syndrome,” *Am. J. Med. Genet. Part A*, vol. 140, no. 24, pp. 2730–2741, Dec. 2006, doi: 10.1002/ajmg.a.31530.
[175] L. Garavelli and P. C. Mainardi, “Mowat-Wilson syndrome,” *Orphanet J. Rare Dis.*, vol. 2, no. 1, p. 42, Dec. 2007, doi: 10.1186/1750-1172-2-42.
[176] I. Ivanovski *et al.*, “Phenotype and genotype of 87 patients with Mowat–Wilson syndrome and recommendations for care,” *Genet. Med.*, vol. 20, no. 9, pp. 965–975, Sep. 2018, doi: 10.1038/gim.2017.221.

LIST OF PUBLICATIONS

S. Gasparini *et al.*, "Differential Expression of Hippocampal Circular RNAs in the BTBR Mouse Model for Autism Spectrum Disorder," *Mol. Neurobiol.*, vol. 57, no. 5, pp. 2301–2313, Feb. 2020, doi: 10.1007/s12035-020-01878-6.

S. Gasparini *et al.*, "The Secret Garden of Neuronal circRNAs," *Cells*., 2020 Aug; 9(8): 1815. doi: 10.3390/cells9081815