Expression Profile of Long Non-Coding RNAs during Early Postnatal Development of Mouse Spinal Cord

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Abstract: Long non-coding RNAs (lncRNAs) are a diverse class of transcripts that are >200 nucleotides long and lack significant protein-coding potential. LncRNAs are emerging as major regulators of gene expression networks in various physiological and pathological processes. Interestingly, many lncRNAs show tissue-specific expression, for example, in the nervous system. Although lncRNAs have been suggested to play key roles in the brain, most functions of neural lncRNAs remain poorly understood. In order to provide a catalog of lncRNA changes that occur in spinal cord during early postnatal development, RNA from mouse spinal cord was sequenced at different time points in the first week after birth (postnatal day 1 and postnatal day 7). Two hundred and ninety-six differentially expressed lncRNAs (FDR < 0.05) were identified in the resulting dataset. Altered transcripts were associated with several biological processes including myelination, neural differentiation, and glial cell development. PCR validation confirmed differential expression of select lncRNAs (i.e., Cerox1, IncOL3, Neat1, and Sox2ot). Additionally, analysis of circular RNAs (circRNAs), another class of non-coding RNA with regulatory potency, pointed out a number of circRNAs associated with spinal cord development. These data can be used as a resource for future studies on transcriptional changes during early postnatal nervous system development and studies of disorders that affect the spinal cord, e.g., spinal muscular atrophy.

Keywords: spinal cord; postnatal development; RNA sequencing; long non-coding RNA; myelination; neurodevelopment; splicing; circular RNA; spinal muscular atrophy

1. Introduction

Development of the mammalian central nervous system (CNS) is a tightly regulated process that involves dramatic morphological and functional changes. Dynamic gene expression patterns play an important part in coordinating these changes at the molecular level. To ensure proper CNS formation, gene expression is precisely controlled throughout an ordered series of developmental events. The importance of accurate control of gene expression during neural development is illustrated by the finding that disruptions of developmental gene networks are associated with multiple neurological and psychiatric diseases [1–3]. Various proteins are known to mediate neural circuit integration through specialized signaling pathways [4]. Interestingly, previous studies have indicated that in addition to proteins, non-coding RNAs (ncRNAs) are important players in CNS development and function [5–7]. Only ~1–2% of the human genome actually encodes proteins, whereas a major part of the genome is transcribed into ncRNAs [8]. ncRNAs are highly diverse in structure and function and fully characterizing the non-coding transcriptome of the nervous system will be instrumental for a more comprehensive understanding of the biological significance of neural ncRNAs.

Long non-coding RNAs (lncRNAs) are a group of ncRNA molecules that are more than 200 nucleotides long and have virtually no protein-coding capacity (although it has been reported that

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micropeptides can potentially be translated from a number of IncRNAs) [9–11]. LncRNAs often show highly specific spatiotemporal expression patterns and are particularly abundant in the brain [12]. Although initially assumed to be junk transcripts and pseudogene remnants, a growing number of studies suggest that LncRNAs play central roles in the regulation of neural development and function [13–15]. For example, LncRNAs may regulate glial–neuronal lineage specification of neural stem cells [16,17] and they can influence synaptic function [18]. Additionally, LncRNAs have been associated with neuronal aging and several neural disorders [14,19]. The exact functions and mechanisms of action of most neural LncRNAs, however, remain unknown.

The spinal cord is a particularly interesting region for studies of neurodevelopment, because the physiology and neural connectivity within the mature spinal cord have been very well defined, providing a clear end point for analyses of neural circuit formation [20,21]. In the present study, transcriptional changes in mouse spinal cord were mapped during early postnatal development. For this purpose, RNA was isolated from whole mouse spinal cord at postnatal day 1 (PND 1) and postnatal day 7 (PND 7), and sequenced to look at expression changes over time, with special emphasis on LncRNAs. These time points were chosen because they represent a critical period in postnatal spinal cord development during which myelination starts and the onset of early degenerative disease of the spinal cord occurs. Importantly, non-polyadenylated (poly(A)-minus) transcripts were included in this analysis. Many RNAs do not carry a poly(A) tail and would therefore be missed when using a sequencing approach that utilizes poly(A) selection. For example, circular RNAs (circRNAs) are a class of poly(A) minus RNA that could also be analyzed in these data. CircRNAs are characterized by a covalently closed loop structure formed by a “backsplcing” event wherein a downstream 5′ splice site is joined to an upstream 3′ splice site in a reversed orientation [22,23]. New and improved techniques for identifying, characterizing, and manipulating circRNAs have enabled the study of these molecules in unprecedented detail [22–24]. CircRNAs are highly abundant in the nervous system [25], are dynamically regulated during development [26], and have been demonstrated to exert important biological functions [22,23]. For example, the circRNA cIRS-7/Cdr1as is part of a ncRNA regulatory network in the brain [27] and loss of cIRS-7/Cdr1as results in a behavioral phenotype in mice [28]. The circRNA circSIC45a4 appears to be required for maintaining the pool of neural progenitors in brain [29]. Additionally, neural circRNAs are enriched at synapses and are altered by homeostatic plasticity [30]. Here, an overview of developmentally regulated circRNAs in postnatal spinal cord is provided. The resulting dataset can be used as a resource for future studies on spinal cord development and for studies of disorders that affect the spinal cord.

2. Results

2.1. Transcriptome Analysis of PND 7 vs. PND 1 Mouse Spinal Cord Identifies Gene Expression Changes Associated with Myelination and Neural Maturation

RNA isolated from “early” (PND 1, n = 4) and “late” (PND 7, n = 4) murine spinal cord was sequenced to measure expression changes during early postnatal development. Principal component analysis showed clear separation of PND 1 and PND 7 expression clusters (Figure 1).
Figure 1. Overview of the RNA-sequencing experiment. (a) Schematic overview of the RNA-seq pipeline. (b) Principal component analysis (PCA) plot of the RNA-seq results. (c) Volcano plot of altered transcripts. Plotted are -log10 (p-values) vs. log2 (fold change). Orange: <0.05 adjusted p-value, Red: <0.05 adjusted p-value and >1 log2 (fold change). Vertical lines indicate +/- 1 log2 (fold change).

Over 900 differentially expressed protein-coding transcripts (>2 fold, FDR <0.05) were present in the RNA-seq data (Table S1) and gene ontology (GO) term enrichment analysis revealed that altered transcripts were associated with biological processes such as myelination, neuron projection development, and oligodendrocyte differentiation (Figure 2).

Figure 2. Altered expression of protein-coding genes in mouse spinal cord during early postnatal development (PND 7 vs. PND 1). (a) Heat map of top 200 significantly altered protein-coding transcripts. (b) Volcano plot of significantly altered protein-coding transcripts. Plotted are -log10 (p-
values) vs. log2 (fold change). Top 20 altered transcripts are indicated with gene symbols (c) Gene ontology (GO) enrichment analysis shows expression changes related to biological processes including myelination, neuron projection development, and oligodendrocyte differentiation (FDR < 0.05).

Among the most significantly changed RNAs were transcripts involved in myelination (e.g., Gjc3↑, Mag↑, Mal↑, Pllp↑, Pllp↑, Ugt8a↑) and neuronal differentiation (e.g., Aatk↑, Dcx↓, Gpr37l1↑, Mapt↓, Plxb3↑, Sox11↓).

Alternative exon usage was also evaluated and it was found that there was differential usage of exons in 14 transcripts (FDR < 0.0001) in PND 7 vs. PND 1 spinal cord (Table 1) (results with less stringent selection [FDR < 0.1] are provided in Table S2). Out of these 14 transcripts, 11 were also differentially expressed. A considerable number of the transcripts identified in the exon usage analysis were linked to cytoskeletal organization (e.g., Actb, Ank3, Cdc42, Eef1a1, Map2, Map6, Sptb).
Table 1. Alternative exon usage in postnatal day 7 (PND 7) vs. postnatal day 1 (PND 1) spinal cord (FDR < 0.00001).

| Gene ID          | Mgi_Symbol | Description                                                                 | Chr | Start    | End      | Total_Exons | Exon_Changes |
|------------------|------------|-----------------------------------------------------------------------------|-----|----------|----------|-------------|--------------|
| ENSMUSG00000006699 | Cdc42      | cell division cycle 42 (Source:MGI Symbol;Acc:MGI:106211)                   | chr4| 137319696| 137357720| 8           | 1            |
| ENSMUSG00000015222 | Map2       | microtubule-associated protein 2 (Source:MGI Symbol;Acc:MGI:97175)          | chr1| 66175273 | 66442583 | 68          | 1            |
| ENSMUSG00000017466 | Timp2      | tissue inhibitor of metalloproteinase 2 (Source:MGI Symbol;Acc:MGI:98753)  | chr11| 118301069| 118355740| 7           | 1            |
| ENSMUSG00000019923 | Zwint      | ZW10 interactor (Source:MGI Symbol;Acc:MGI:1289227)                        | chr10| 72654845 | 72674964 | 20          | 1            |
| ENSMUSG00000021061 | Sptb       | spectrin beta, erythrocytic (Source:MGI Symbol;Acc:MGI:98387)              | chr12| 76580488 | 76710547 | 42          | 1            |
| ENSMUSG00000021196 | Pfkp       | phosphofructokinase, platelet (Source:MGI Symbol;Acc:MGI:1891833)         | chr13| 6579768  | 6648777  | 50          | 1            |
| ENSMUSG00000029580 | Actb       | actin, beta (Source:MGI Symbol;Acc:MGI:87904)                             | chr5 | 142903115| 142906754| 30          | 2            |
| ENSMUSG00000032356 | Rasgrf1    | RAS protein-specific guanine nucleotide-releasing factor 1 (Source:MGI Symbol;Acc:MGI:99694) | chr9 | 89909908 | 90026977 | 34          | 1            |
| ENSMUSG00000037458 | Azin1      | antizyme inhibitor 1 (Source:MGI Symbol;Acc:MGI:1859169)                    | chr15| 38487430 | 38519266 | 31          | 1            |
| ENSMUSG00000037742 | Eef1a1     | eukaryotic translation elongation factor 1 alpha 1 (Source:MGI Symbol;Acc:MGI:1096881) | chr9 | 78478449 | 78489151 | 24          | 1            |
| ENSMUSG00000040785 | Ttc3       | tetratricopeptide repeat domain 3 (Source:MGI Symbol;Acc:MGI:1276539)      | chr16| 94370618 | 94522117 | 109         | 1            |
| ENSMUSG00000041607 | Mbp        | myelin basic protein (Source:MGI Symbol;Acc:MGI:96925)                     | chr18| 82475146 | 82585637 | 31          | 1            |
| ENSMUSG00000055407 | Map6       | microtubule-associated protein 6 (Source:MGI Symbol;Acc:MGI:1201690)       | chr7 | 99267447 | 99337137 | 13          | 1            |
| ENSMUSG00000069601 | Ank3       | ankyrin 3, epithelial (Source:MGI Symbol;Acc:MGI:88026)                    | chr10| 69533772 | 70027438 | 128         | 1            |
2.2. Differential Expression of lncRNAs in PND 7 vs. PND Mouse Spinal Cord

Expression of lncRNAs was examined in mouse spinal cord at PND 1 (n = 4) and PND 7 (n = 4). Differential expression analysis of lncRNAs uncovered a number of changes that occurred during postnatal development (Figure 3a,b). Two hundred and ninety-six altered lncRNAs (FDR < 0.05) were identified in the PND 7 vs. PND 1 data (Table S1) and RT-qPCR validations were performed for select lncRNAs, i.e., 1700047M11Rik (oligodendrocyte-restricted lncRNA 3 or lncOL3), Sox2 overlapping transcript (Sox2ot), 2810468N07Rik (cytoplasmic endogenous regulator of oxidative phosphorylation 1 or Cerox1), and nuclear enriched abundant transcript 1 (Neat1) (Table 2). PCR experiments confirmed up-regulated expression of the candidate lncRNAs (lncOL3 FC 6.16, not plotted; Cerox1 FC 4.98; Sox2ot FC 2.97; Neat1 FC 1.99) (Figure 3c). Increased expression of Cerox1 was particularly interesting, because this lncRNA was recently identified as a post-transcriptional regulator of mitochondrial protein production and energy metabolism [31] (Figure 3d).

Figure 3. Long non-coding RNAs (lncRNAs) expression changes in mouse spinal cord during early postnatal development (PND 7 vs. PND 1). (a) Heat map of all significantly altered lncRNAs in mouse spinal cord (FDR < 0.05). (b) Volcano plot of significantly altered lncRNAs. Plotted are -log10 (p-values) vs. log2 (fold change). Top 20 altered lncRNAs are highlighted (c) RT-qPCR verification of select altered lncRNAs (Cerox1, Sox2ot, and Neat1) (values are mean ± SEM). Each dot represents a unique PND 7 vs PND 1 comparison (average of triplicate values). (d) Cerox1 has been shown to influence expression mitochondrial proteins by sequestering microRNA (miRNA) miR-488-3p, thereby regulating activity of the electron transport chain (ETC).
Table 2. Selected differentially expressed lncRNAs in PND 7 vs. PND 1 spinal cord (FDR < 0.05).

| LncRNA          | Description                                           | Log2foldchange | Padj       | Foldchange (qPCR) | Reference |
|-----------------|-------------------------------------------------------|----------------|------------|-------------------|-----------|
| Neat1           | Structural determinant of paraspeckles; oligodendrocyte differentiation | 1.411085184   | 1.15 × 10^{-21} | 1.99              | [16,32,33] |
| 2810468N07Rik   | Regulator of mitochondrial oxidative phosphorylation  | 2.207508806   | 4.93 × 10^{-34} | 4.98              | [31]      |
| 1700047M11Rik   | Oligodendrocyte lineage; minimal effect on promoting oligodendrocyte differentiation | 2.906835561   | 2.80 × 10^{-63} | 6.16              | [34]      |
| Sox2ot          | Negative regulator of neural progenitor proliferation | 1.819081372   | 8.47 × 10^{-52} | 2.97              | [35]      |

2.3. CircRNA Expression Changes Associated with Early Postnatal Development of Mouse Spinal Cord

CircRNAs are a class of poly(A) minus ncRNA characterized by a covalently closed loop structure. CircRNAs lack 5'-3' polarity and are resistant to exoribonuclease-mediated degradation (Figure 4a) (circRNAs are not resistant to endoribonuclease-mediated decay (Supplementary File 1)). RNA from mouse spinal cord was sequenced at PND 1 (n = 4) and PND 7 (n = 4) to explore circRNA expression (mice for circRNA analysis were different from those used for 2.1 and 2.2). 5461 circRNAs were discovered. The number of linear reads vs. the number of circular reads at each backsplice junction was plotted, showing that most circRNAs were made from transcripts that predominantly produce linear transcript (above line), while some circRNAs were expressed at higher levels than the linear isoform (below line) (Figure 4b).

![Figure 4](image-url)

**Figure 4.** Circular RNAs (circRNAs) expression changes in mouse spinal cord during early postnatal development (PND 7 vs. PND 1). (a) CircRNAs lack free 5' and 3' ends and are resistant to exoribonuclease-mediated degradation. (b) Plotted is the number of linear reads vs. the number of circular reads at each backsplice junction. Most circRNAs are made from transcripts that primarily produce linear transcript (above line). Some circRNAs were expressed at higher levels than the linear isoform (below line).

In general, circRNA expression did not seem to follow circ-to-linear ratio, indicating that circRNAs are not tightly dependent on host expression. Due to the low number of reads for circRNAs, few significantly changed circRNAs (adjusted p-value < 0.05) were detected (Table S3).
circRNAs were detected as significantly differentially expressed between the two developmental stages. A circRNA originating from the breast carcinoma amplified sequence 1 (Bcas1) locus was the most significant hit from this screen (circBcas1 ↑) (Table 3).

Table 3. Altered circRNAs in PND 7 vs. PND 1 spinal cord (FDR < 0.05).

| CircRNA          | CircBase ID | Chrom | Spliced_Len | Unspliced_Len | Log2FoldChange | Padj     |
|------------------|-------------|-------|-------------|---------------|----------------|---------|
| circ_00296_Bcas1 | mmu_circ_0009627 | chr2 | 546         | 9230          | 4.062045179    | 0.00818747 |
| circ_00212_intergenic | . | chr9 | 2286        | 46830         | 6.452673201    | 0.00818747 |
| circ_00022_Eif2  | mmu_circ_0001125 | chr3 | 484         | 17986         | -1.00480207    | 0.00818747 |
| circ_00481_St7   | mmu_circ_0001444 | chr6 | 569         | 10120         | 5.048381199    | 0.00818747 |
| circ_00048_Sergef| mmu_circ_0001566 | chr7 | 951         | 44950         | 1.134067666    | 0.017309759 |
| circ_00006_Fat3  | mmu_circ_0001746 | chr9 | 3306        | 3306          | 0.577957579    | 0.024065145 |
| circ_00051_Epb41l| mmu_circ_0000808 | chr17 | 416         | 2525          | 1.060147922    | 0.042563095 |
| circ_00573_Dcp2  | mmu_circ_0007361 | chr18 | 374         | 551           | 4.586006583    | 0.044531922 |
| circ_00466_Kansl1| mmu_circ_0008912 | chr1  | 1120        | 1120          | 4.725088678    | 0.044531922 |

3. Discussion

In the present study, RNA-seq analysis was performed to comprehensively map expression changes in mouse spinal cord during a critical period of postnatal development (PND 1 to PND 7). This resulted in a differential gene expression signature enriched for myelination and neural maturation processes. This is consistent with known CNS development, e.g., myelination starts in the spinal cord of mice after birth [36]. Developmental RNA expression data are also in line with high resolution in situ hybridization image data from the Allen Developing Mouse Brain Atlas (https://developingmouse.brain-map.org). Additionally, exon usage analysis showed that there was differential usage of exons in 14 transcripts (FDR < 0.0001). A number of these transcripts were related to the cytoskeleton, e.g., Actb, Ank3, Cdc42, Eef1a1, Map2, Map6, and Sptb. These changes in cytoskeletal transcripts are not surprising, as the structural organization and dynamic remodeling of the neuronal cytoskeleton critically contribute to morphological and functional changes in developing neurons [37].

The main aim of this study was to identify differentially expressed lncRNAs during postnatal spinal cord development. Two hundred and ninety-six significantly altered lncRNAs were found in the sequencing data and four of these were validated with RT-qPCR, i.e., 1700047M11Rik (lncOL3), Sox2ot, 2810468N07Rik (Cerox1), and Neat1. All of the selected lncRNAs were up-regulated in PND 7 vs. PND 1 spinal cord. Differential expression of these particular lncRNAs is biologically plausible. For example, lncOL3 is highly expressed in (myelinating) oligodendrocytes (https://www.brainrnaseq.org) [38] and was shown to exhibit minimal effects on promoting oligodendrocyte differentiation in previous experiments [34]. Sox2ot is an evolutionarily conserved lncRNA that represses neural progenitor proliferation and promotes neuronal differentiation [35]. Neat1 is a structural determinant of paraspeckles [32,33] and robust changes in Neat1 expression have been observed in mouse oligodendrocyte precursors during differentiation [16]. Perhaps most interesting was the marked increase in Cerox1, a lncRNA that functions as a post-transcriptional regulator of mitochondrial complex I catalytic activity through decoying miR-488-3p [31]. Changing energetic demands in the developing spinal cord probably require alterations in mitochondrial
respiration and Cerox1 may contribute to such adjustments. Cerox1 expression is especially high in neuroglia, neural progenitor cells and oligodendrocyte progenitors [31].

Lastly, circRNA expression was determined in spinal cord. This analysis revealed few significantly altered circRNAs in PND 7 vs. PND 1 samples. It takes a lot of circRNAs to get significant adjusted p-values because their detection is based on the number of reads spanning the backsplice junction only, which results in very low expression counts for circRNAs (and therefore low p-values). Validation of non-significant circRNAs could therefore yield positive results. Among the altered circRNAs, circBcas1 was the most significantly changed transcript. Bcas1 is highly expressed in oligodendrocytes and absence of Bcas1 in mice results in hypo-myelination and increased expression of inflammation-related genes in the brain [38–40]. CircBcas1 seems to follow linear Bcas1 expression in spinal cord (Table S1).

Overall, the RNA-seq data strongly link altered RNAs to myelination and oligodendrocytes. Previous work has uncovered important roles for ncRNAs in oligodendrocyte development (e.g., [41,42]), indicating that a complex transcriptional network underlies oligodendrocyte differentiation. The data presented here can be used as a reference for studies on postnatal CNS development and disorders of the spinal cord, like motor neuron disease [43]. Additionally, insights derived from normal development may inform strategies that promote neural regeneration [44,45].

Several limitations should be noted. First, because RNA-seq was performed for only two time points in postnatal development, it is not straightforward to directly compare expression data to other phases of spinal cord development (e.g., embryonic development, adult stage). Second, sample sizes were modest, which may have resulted in insufficient power to identify some transcripts. Third, bulk spinal cord tissue was sequenced and therefore the data do not yield insight into cell type composition and single-cell variation in RNA. Differences in dissection and sampling procedures cannot be excluded (variation in PC2 in Figure 1 is most likely due to variation in dissection procedures). Single-cell transcriptomics of human brain cells revealed that many lncRNAs are abundantly expressed in individual cells and are cell type-specific [46]. More detailed single-cell transcriptomic analyses and localization experiments (using single-molecule RNA fluorescence in situ hybridization) that specify the full complement of cellular subtypes and RNAs within spinal cord are needed (e.g., [47,48]). Also, while postnatal oligodendrocyte precursors from brain and spinal cord display similar transcriptional signatures [49], other cell types can show region-specific expression. Comparing spinal cord data to findings in other parts of the CNS will be required to identify region-specific features. Fourth, due to performing ribosomal RNA depletion and using a particular analysis approach, it was not possible to explore changes in a number of other ncRNAs. Fifth, species differences (i.e., mouse vs. human) were not examined in these data. Many lncRNA sequences are poorly conserved among species and developmental timing is not identical. Consequently, translating these data to human studies may pose a challenge. Finally, the functional significance of the ncRNAs identified in these experiments was not investigated further in the context of spinal cord tissue. Probing the effects and mechanisms of specific lncRNAs, e.g., using CRISPR-based technology for silencing the expression of lncRNAs, will be necessary to fully understand the roles of these RNAs in CNS development and function.

In conclusion, a transcriptional map of postnatal mouse spinal cord is provided. The data show that RNAs associated with myelination, oligodendrocyte lineage, and neuronal maturation are markedly altered in the spinal cord during early postnatal development. Several lncRNAs associated with postnatal spinal cord development are identified.

As mentioned above, the data may be used as a resource for future studies on disorders of the spinal cord. An example of such a disorder is spinal muscular atrophy (SMA). SMA is a monogenetic neurodegenerative disease that is characterized by loss of α-motor neurons in the anterior horn of the spinal cord, resulting in progressive muscle wasting and paralysis [50]. SMA is a leading genetic cause of infant death and is caused by homozygous disruptions of the survival motor neuron 1 (SMN1) gene, resulting in reduced expression of survival motor neuron (SMN) protein [51]. Although the genetic cause of SMA has been known for over 20 years, the pathomechanisms underlying the disease remain poorly understood [52–54]. Previous studies have shown SMN complex to be a versatile
ribonucleoprotein (RNP) assembly hub and have demonstrated the involvement of SMN in various cellular processes, such as small nuclear RNP assembly and pre-mRNA splicing, neuronal transport and localization of RNAs, and control of protein synthesis [52–54]. Protein-protein interaction data support an association of SMN with small nuclear RNP assembly components and translational machinery (Supplementary File 2 and Table S4). RNA-seq experiments performed on spinal cord tissue (PND 1 and PND 5) from a mouse model of SMA revealed that processes such as (i) cell cycle and (ii) axon guidance signaling were affected in these animals [55]. Accordingly, function of the guidance receptor PlexinD1 was found to be altered in SMA experimental models [56]. Interestingly, the SMN-interacting protein fused in sarcoma (FUS) (also implicated in the motor neuron disease amyotrophic lateral sclerosis) [57–59] (Supplementary File 2 and Table S4) was found to influence lncRNA transcription [60] and to regulate circRNA biogenesis in motor neurons [61]. It is surmised that SMN levels can affect both lncRNA expression and circRNA biogenesis and functionality in cells. Indeed, transcriptomics analysis has revealed certain lncRNA abnormalities in SMA mice [62]. Additionally, a critical role for lncRNAs in regulating expression of SMN protein was shown [63,64] and SMN genes were found to generate a vast repertoire of circRNAs [65,66]. Dysregulation of lncRNAs and circRNAs might contribute to neuronal dysfunction in SMA. A detailed comparison between SMA and WT spinal cord would provide further insight into dysregulation of specific lncRNAs in SMA. Of note, no changes in oligodendrocyte development and CNS myelination were seen in a mouse model of severe SMA [67], suggesting that purified cell populations (e.g., motor neurons) may be needed to adequately detect disease-relevant expression differences. Preliminary data showed a decrease in circCDYL in induced pluripotent stem cell-derived motor neurons from SMA type I patients (n = 2) vs. healthy donors (n = 2), while levels of linear CDYL were unaffected in these cells (unpublished results). Future studies should address the role of altered ncRNAs in SMA in more detail, also focusing on crucial sites of pathology, e.g., at axons and neuromuscular junctions. LncRNAs and circRNAs can potentially be used as biomarkers (e.g., to longitudinally assess disease progression and the impact of treatments) and may represent therapeutic targets in disease.

4. Materials and Methods

4.1. Experimental Animals and Tissue Collection

Phenotypically normal (WT) FVB/N mice (The Jackson Laboratory) were used in this study. Mouse pups were euthanized by decapitation at PND 1 (n = 8) and PND 7 (n = 8), and spinal cords were dissected and immediately frozen in liquid nitrogen. When possible, male mice were used. 1 spinal cord was used per replicate. Animal experiments were performed according to institutional guidelines and were approved by the local ethical animal experimentation committee (Dierexperimenten Ethische Commissie; DEC [in Dutch], AVD1150020171565).

4.2. RNA Sequencing Experiments

4.2.1. RNA Extraction

Spinal cord tissue was crushed using a grinder in a pre-chilled mortar to prevent thawing. Total RNA was then extracted from the powdered frozen samples using the RNaseasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Samples were treated with RNase-free DNase I to eliminate genomic DNA contamination. RNA integrity (RIN) was verified on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and only samples with RIN value ≥ 8 were used for RNA sequencing.

4.2.2. RNA-Seq Library Preparation and Sequencing Analysis

RNA was depleted of ribosomal RNA using Ribo-Zero (Illumina, San Diego, CA, USA). Sequencing libraries were generated using ScriptSeq v2 (Illumina). RNA-seq was performed on the HiSeq 4000 system (Illumina, San Diego, CA) using a 100 bp paired-end sequencing protocol. Raw data was filtered and trimmed using Trim Galore. Numbers of filtered reads are provided in
Supplementary File 3. The filtered data was subjected to three analysis pipelines: (i) Gene expression and differential expression, (ii) differential exon usage, and (iii) circRNA discovery and differential expression. Differential expression analysis was performed by mapping the filtered reads to the mouse genome (mm10) using TopHat2 [68]. Next, featureCounts [69] was used to quantify the number of reads mapping to each gene and differential expression was analyzed using DESeq2 [70] in R (https://www.r-project.org). The DESeq2 package was used to calculate normalized expression values, lfcSE, p-values, and adjusted p-values. All plotting was done in R. For GO term enrichment analysis in Figure 2 the Gene Ontology enRICHment anaLYsis and visualizaTION (GOrilla) online tool (http://cbl-gorilla.cs.technion.ac.il) [71,72] was used on a single ranked list of genes (adjusted p-value < 0.05). Differential exon usage was analyzed using the Bioconductor package DEXseq (Bioconductor. Available online: https://bioconductor.org/packages/release/bioc/manuals/DEXSeq/man/DEXSeq.pdf [accessed on 14 May 2020]) [73]. For circRNA detection, both find-circ [74] and CIRCexplorer [75] were used on the filtered RNA-seq data and differential expression analysis was done with DESeq2.

4.3. PCR Experiments

Total RNA was isolated from mouse spinal cord using the miRNeasy kit (Qiagen) according to the manufacturer’s protocol (see Section 4.2.1). RNA quantity was determined using NanoDrop (Thermo Scientific, Rockford, IL, USA) and equal amounts of each sample were used for cDNA synthesis using SuperScript IV Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) with random hexamers according to the manufacturer’s instructions. RT-qPCRs were run on a Quantstudio 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master Mix (ROX) (Roche Diagnostics, Mannheim, Germany). All reactions were run in triplicate. Ct values were determined using QuantStudio Real-Time PCR software. Expression levels were normalized to Gapdh and relative gene expression was determined using the \(2^{-\Delta\Delta Ct}\) method [76]. Several PCR products were analyzed with agarose gel electrophoresis following RNase treatment. For these experiments, RNase R (Epicentre Technologies, Madison, WI, USA) and RNase A (Roche Diagnostics) were used. Primers sequences are provided in Table S5.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/2311-553X/6/2/18/s1. Supplementary File 1: RNase treatments, Supplementary File 2: Smm interactome analysis, Supplementary File 3: Filtered reads, Table S1: Differential RNA expression PND 7_PND 1 spinal cord, Table S2: Alternative exon usage PND 7_PND 1 spinal cord, Table S3: Circular RNA expression PND 7_PND 1 spinal cord, Table S4: Smm interactome data, Table S5: Primer sequences.

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Data availability: Datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Abbreviations

circRNA Circular RNA
CNS Central nervous system
ETC Electron transport chain
FC Fold change
FDR  False discovery rate
FUS  Fused in sarcoma
GO  Gene ontology
lncRNA  Long non-coding RNA
miRNA  MicroRNA
ncRNA  Non-coding RNA
PCA  Principal component analysis
PCR  Polymerase chain reaction
PND  Postnatal day
RIN  RNA integrity
RNA-seq  RNA sequencing
RNase  Ribonuclease
RNP  Ribonucleoprotein
RT-qPCR  Real-time quantitative PCR
SMA  Spinal muscular atrophy
SMN  Survival motor neuron
WT  Wild-type

References
1. Hu, W.F.; Chahrour, M.H.; Walsh, C.A. The diverse genetic landscape of neurodevelopmental disorders. *Annu. Rev. Genom. Hum. Genet.* 2014, 15, 195–213.
2. Van Battum, E.Y.; Brignani, S.; Pasterkamp, R.J. Axon guidance proteins in neurological disorders. *Lancet Neurol.* 2015, 14, 532–546.
3. Geschwind, D.H.; Levitt, P. Autism spectrum disorders: Developmental disconnection syndromes. *Curr. Opin. Neurobiol.* 2007, 17, 103–111.
4. Kolodkin, A.L.; Tessier-Lavigne, M. Mechanisms and molecules of neuronal wiring: A primer. *Cold Spring Harb Perspect Biol* 2011, 3, a001727.
5. Satterlee, J.S.; Barbee, S.; Jin, P.; Krichevsky, A.; Salama, S.; Schratt, G.; Wu, D.-Y. Noncoding RNAs in the brain. *J. Neurosci.* 2007, 27, 11856–11859.
6. Qureshi, I.A.; Mehler, M.F. Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease. *Nat. Rev. Neurosci.* 2012, 13, 528–541.
7. Salta, E.; De Strooper, B. Noncoding RNAs in neurodegeneration. *Nat. Rev. Neurosci.* 2017, 18, 627–640.
8. Mattick, J.S.; Makunin, I.V. Non-coding RNA. *Hum. Mol. Genet.* 2006, 15, R17–R29.
9. Ponting, C.P.; Oliver, P.L.; Reik, W. Evolution and functions of long noncoding RNAs. *Cell* 2009, 136, 629–641.
10. Nagano, T.; Fraser, P. No-nonsense functions for long noncoding RNAs. *Cell* 2011, 145, 178–181.
11. Kopp, F.; Mendell, J.T. Functional classification and experimental dissection of long noncoding RNAs. *Cell* 2018, 172, 393–407.
12. Mercer, T.R.; Dinger, M.E.; Sunkin, S.M.; Mehler, M.F.; Mattick, J.S. Specific expression of long noncoding RNAs in the mouse brain. *Proc. Natl. Acad. Sci. USA* 2008, 105, 716–721.
13. Barry, G. Integrating the roles of long and small non-coding RNA in brain function and disease. *Mol. Psychiatry* 2014, 19, 410–416.
14. Briggs, J.A.; Wolveetang, E.J.; Mattick, J.S.; Rinn, J.L.; Barry, G. Mechanisms of long non-coding RNAs in mammalian nervous system development, plasticity, disease, and evolution. *Neuron* 2015, 88, 861–877.
15. Aprea, J.; Calegari, F. Long non-coding RNAs in corticogenesis: Deciphering the non-coding code of the brain. *EMBO J.* 2015, 34, 2865–2884.
16. Mercer, T.R.; Qureshi, I.A.; Gokhan, S.; Dinger, M.E.; Li, G.; Mattick, J.S.; Mehler, M.F. Long noncoding RNAs in neuronal-glial fate specification and oligodendrocyte lineage maturation. *BMC Neurosci.* 2010, 11, 14–15.
17. Ramos, A.D.; Diaz, A.; Nellore, A.; Delgado, R.N.; Park, K.-Y.; Gonzales-Roybal, G.; Oldham, M.C.; Song, J.S.; Lim, D.A. Integration of genome-wide approaches identifies lncRNAs of adult neural stem cells and their progeny in vivo. *Cell Stem Cell* 2013, 12, 616–628.
18. Keihani, S.; Kluever, V.; Mandad, S.; Bansal, V.; Rahman, R.; Fritsch, E.; Gomes, L.C.; Gärtner, A.; Kügler, S.; Urlaub, H.; et al. The long noncoding RNA neuroLNC regulates presynaptic activity by interacting with the neurodegeneration-associated protein TDP-43. *Sci. Adv.* 2019, 5, eaay2670.

19. Pereira Fernandes, D.; Bitar, M.; Jacobs, F.M.J.; Barry, G. Long non-coding RNAs in neuronal aging. *Noncoding RNA* 2018, 4, 12.

20. Jessell, T.M. Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. *Nat. Rev. Genet.* 2000, 1, 20–29.

21. Brown, A.G. *Organization in the Spinal Cord*; Springer: Berlin, Germany, 1981.

22. Kristensson, L.S.; Andersen, M.S.; Stagsted, L.V.W.; Ebbesen, K.K.; Hansen, T.B.; Kjems, J. The biogenesis, biology and characterization of circular RNAs. *Nat. Rev. Genet.* 2019, 20, 675–691.

23. Xiao, M.-S.; Ai, Y.; Wilusz, J.E. Biogenesis and functions of circular RNAs come into focus. *Trends Cell Biol.* 2020, 30, 226–240.

24. Dieterich, C.; Papantonis, A. *Circular RNAs: Methods and Protocols*; Humana Press: New York, NY, USA, 2018; Vol. 1724.

25. Rybak-Wolf, A.; Stottmeister, C.; Glažar, P.; Jens, M.; Pino, N.; Giusti, S.; Hanan, M.; Behm, M.; Bartok, O.; Ashwal-Fluss, R.; et al. Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol. Cell* 2015, 58, 870–885.

26. Venø, M.T.; Hansen, T.B.; Venø, S.T.; Clausen, B.H.; Grebing, M.; Finsen, B.; Holm, I.E.; Kjems, J. Spatio-temporal regulation of circular RNA expression during porcine embryonic brain development. *Genome Biol.* 2015, 16, 245.

27. Kleaveland, B.; Shi, C.Y.; Stefano, J.; Bartel, D.P. A network of noncoding regulatory RNAs acts in the mammalian brain. *Cell* 2018, 174, 350–362.e17.

28. Piwecka, M.; Glažar, P.; Hernandez-Miranda, L.R.; Memczak, S.; Wolf, S.A.; Rybak-Wolf, A.; Filipchyk, A.; Klononomos, F.; Cerda Jara, C.A.; Fenske, P.; et al. Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. *Science* 2017, 357, eaam8526.

29. Suenkel, C.; Cavalli, D.; Massalini, S.; Calegari, F.; Rajewsky, N. A highly conserved circular RNA is required to keep neural cells in a progenitor state in the mammalian brain. *Cell Rep.* 2020, 30, 2170–2179.e5.

30. You, X.; Vlatkovic, I.; Babic, A.; Will, T.; Epstein, I.; Tushev, G.; Akbalik, G.; Wang, M.; Glock, C.; Quedenau, C.; et al. Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nat. Neurosci.* 2015, 18, 603–610.

31. Sirey, T.M.; Roberts, K.; Haerty, W.; Bedoya-Reina, O.; Rogatti-Granados, S.; Tan, J.Y.; Li, N.; Heather, L.C.; Carter, R.N.; Cooper, S.; et al. The long non-coding RNA Cerox1 is a post transcriptional regulator of mitochondrial complex I catalytic activity. *Elife* 2019, 8, 7154.

32. Clemson, C.M.; Hutchinson, J.N.; Sara, S.A.; Ensminger, A.W.; Fox, A.H.; Chess, A.; Lawrence, J.B. An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. *Mol. Cell* 2009, 33, 717–726.

33. Lin, Y.; Schmidt, B.F.; Brueche, M.P.; McManus, C.J. Structural analyses of NEAT1 IncRNAs suggest long-range RNA interactions that may contribute to paraspeckle architecture. *Nucleic Acids Res.* 2018, 46, 3742–3752.

34. He, D.; Wang, J.; Lu, Y.; Deng, Y.; Zhao, C.; Xu, L.; Shen, Y.; Hu, Y.-C.; Zhou, W.; Lu, Q.R. IncRNA functional networks in oligodendrocytes reveal stage-specific myelination control by an IncOL1/Suz12 complex in the CNS. *Neuron* 2017, 93, 362–378.

35. Knauss, J.L.; Miao, N.; Kim, S.-N.; Nie, Y.; Shi, Y.; Wu, T.; Pinto, H.B.; Donohoe, M.E.; Sun, T. Long noncoding RNA Sox2ot and transcription factor YY1 co-regulate the differentiation of cortical neural progenitors by repressing Sox2. *Cell Death Dis.* 2018, 9, 799–13.

36. Snaidero, N.; Simons, M. Myelination at a glance. *J. Cell. Sci.* 2014, 127, 2999–3004.

37. Menon, S.; Gupton, S.L. Building blocks of functioning brain: Cytoskeletal dynamics in neuronal development. *Int Rev Cell Mol Biol* 2016, 322, 183–245.

38. Zhang, Y.; Chen, K.; Sloan, S.A.; Bennett, M.L.; Scholze, A.R.; O’Keeffe, S.; Phatnani, H.P.; Guarnieri, P.; Caneda, C.; Ruderisch, N.; et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J. Neurosci.* 2014, 34, 11929–11947.

39. Ishimoto, T.; Ninomiya, K.; Inoue, R.; Koike, M.; Uchiyama, Y.; Mori, H. Mice lacking BCAS1, a novel myelin-associated protein, display hypomyelination, schizophrenia-like abnormal behaviors, and upregulation of inflammatory genes in the brain. *Glia* 2017, 65, 727–739.
40. Fard, M.K.; van der Meer, F.; Sánchez, P.; Cantuti-Castelvetri, L.; Mandad, S.; Jäkel, S.; Fornasiero, E.F.; Schmitt, S.; Ehrlich, M.; Starost, L.; et al. BCAS1 expression defines a population of early myelinating oligodendrocytes in multiple sclerosis lesions. Sci. Transl. Med. 2017, 9, eaam7816.

41. Dugas, J.C.; Cuellar, T.L.; Scholze, A.; Ason, B.; Ibrahim, A.; Emery, B.; Zamanian, J.L.; Foo, L.C.; McManus, M.T.; Barres, B.A. Dicer1 and miR-219 are required for normal oligodendrocyte differentiation and myelination. Neuron 2010, 65, 597–611.

42. Dong, X.; Chen, K.; Cuevas-Díaz Durán, R.; You, Y.; Sloan, S.A.; Zhang, Y.; Zong, S.; Cao, Q.; Barres, B.A.; Wu, J.Q. Comprehensive identification of long non-coding RNAs in purified cell types from the brain reveals functional lncRNA in OCP fate determination. PLoS Genet. 2015, 11, e1005669.

43. Chen, K-W.; Chen, J-A. Functional roles of long non-coding RNAs in motor neuron development and disease. J. Biomed. Sci. 2020, 27, 38–14.

44. Baldwin, K.T.; Giger, R.J. Insights into the physiological role of CNS regeneration inhibitors. Front. Mol. Neurosci. 2015, 8, 23.

45. He, Z.; Jin, Y. Intrinsic control of axon regeneration. Neuron 2016, 90, 437–451.

46. Liu, S.J.; Nowakowski, T.J.; Pollen, A.A.; Lui, J.H.; Horibeck, M.A.; Attenello, F.J.; He, D.; Weissman, J.S.; Kriegstein, A.R.; Diaz, A.A.; et al. Single-cell analysis of long non-coding RNAs in the developing human neocortex. Genome Biol. 2016, 17, 67–17.

47. Sathyamurthy, A.; Johnson, K.R.; Matson, K.J.E.; Dobrott, C.J.; Li, L.; Ryba, A.R.; Bergman, T.B.; Kelly, M.C.; Kelley, M.W.; Levine, A.J. Massively parallel single nucleus transcriptional profiling defines spinal cord neurons and their activity during behavior. Cell Rep. 2018, 22, 2216–2225.

48. Rosenberg, A.B.; Roco, C.M.; Muscat, R.A.; Kuchina, A.; Sample, P.; Yao, Z.; Graybuck, L.T.; Peeler, D.J.; Mukherjee, S.; Chen, W.; et al. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. Science 2018, 360, 176–182.

49. Marques, S.; van Bruggen, D.; Vanichkina, D.P.; Floriddia, E.M.; Munguba, H.; Värelo, L.; Giacomello, S.; Falcão, A.M.; Björklund, Å.K.; Hjerling-Neilsen, Å.; Krainer, A.R.; Andresen, B.S. RNA-dependent introns. J. Neurosci. 2019, 39, 8700–8708.

50. Lunn, M.R.; Wang, C.H. Spinal muscular atrophy. Lancet 2008, 371, 2120–2133.

51. Lefebvre, S.; Bürglen, L.; Reboulet, S.; Clermont, O.; Burlet, P.; Viollet, L.; Benichou, B.; Cruaud, C.; Millasseau, P.; Zeviani, M. Identification and characterization of a spinal muscular atrophy-determining gene. Cell 1995, 80, 155–165.

52. Burghes, A.H.M.; Beattie, C.E. Spinal muscular atrophy: Why do low levels of survival motor neuron protein make motor neurons sick? Nat. Rev. Neurosci. 2009, 10, 597–609.

53. Tisdale, S.; Pellizzoni, L. Disease mechanisms and therapeutic approaches in spinal muscular atrophy. J. Neurosci. 2015, 35, 8691–8700.

54. Sumner, C.J.; Paushkin, S.; Ko, C.-P. Spinal Muscular Atrophy: Disease Mechanisms and Therapy; Elsevier/Academic Press: Cambridge, MA, USA, 2017.

55. Doktor, T.K.; Hua, Y.; Andersen, H.S.; Brønen, S.; Liu, Y.H.; Wieckowska, A.; Dembic, M.; Bruun, G.H.; Krainer, A.R.; Andresen, B.S. RNA-sequencing of a mouse model of spinal muscular atrophy reveals tissue-wide changes in splicing of U12-dependent introns. Nucleic Acids Res. 2017, 45, 395–416.

56. Rademacher, S.; Verheijen, B.M.; Hensel, N.; Peters, M.; Bora, G.; Brandes, G.; Vieira de Sá, R.; Heidrich, N.; Fischer, S.; Brinkmann, H.; et al. Metalloprotease-mediated cleavage of PlexinD1 and its sequestration to actin rods in the motoneuron disease spinal muscular atrophy (SMA). Hum. Mol. Genet. 2017, 26, 3946–3959.

57. Yamazaki, T.; Chen, S.; Yu, Y.; Yan, B.; Haertlein, T.C.; Carrasco, M.A.; Tapia, J.C.; Zhai, B.; Das, R.; Lalancette-Hebert, M.; et al. FUS-SM protein interactions link the motor neuron diseases ALS and SMA. Cell Rep. 2012, 2, 799–806.

58. Groen, E.J.N.; Fumoto, K.; Blokhuis, A.M.; Engelen-Lee, J.; Zhou, Y.; van den Heuvel, D.M.A.; Koppers, M.; van Diggelen, P.; van Heest, J.; Demmers, J.A.A.; et al. ALS-associated mutations in FUS disrupt the axonal distribution and function of SMN. Hum. Mol. Genet. 2015, 22, 3690–3704.

59. Sun, S.; Qiu, J.; Fard, M.K.; Scholze, A.; Ason, B.; Ibrahim, A.; Emery, B.; Zamanian, J.L.; Foo, L.C.; McManus, M.T.; Barres, B.A. Dicer1 and miR-219 are required for normal oligodendrocyte differentiation and myelination. Neuron 2010, 65, 597–611.

60. Biscarini, S.; Capauto, D.; Peruzzi, G.; Lu, L.; Colantoni, A.; Santini, T.; Shneider, N.A.; Caffarelli, E.; Laneve, P.; Bozzoni, I. Characterization of the lncRNA transcriptome in mESC-derived motor neurons: Implications for FUS-ALS. Stem Cell Res. 2018, 27, 172–179.
61. Errichelli, L.; Dini Modigliani, S.; Laneve, P.; Colantoni, A.; Legnini, I.; Capauto, D.; Rosa, A.; De Santis, R.; Scarfò, R.; Peruzzi, G.; et al. FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons. *Nat. Commun.* 2017, 8, 14741.

62. Bernabò, P.; Tebaldi, T.; Groen, E.J.N.; Lane, F.M.; Perenthaler, E.; Mattedì, F.; Newbery, H.J.; Zhou, H.; Zucconi, P.; Potrich, V.; et al. In vivo translome profiling in spinal muscular atrophy reveals a role for SMN protein in ribosome biology. *Cell Rep.* 2017, 21, 953–965.

63. d’Ydewalle, C.; Ramos, D.M.; Pyles, N.J.; Ng, S.-Y.; Gorz, M.; Pilato, C.M.; Ling, K.; Kong, L.; Ward, A.J.; Rubin, L.L.; et al. The antisense transcript SMN-AS1 regulates SMN expression and is a novel therapeutic target for spinal muscular atrophy. *Neuron* 2017, 93, 66–79.

64. Woo, C.J.; Maier, V.K.; Davey, R.; Brennan, J.; Li, G.; Brothers, J.; Schwartz, B.; Gordo, S.; Kasper, A.; Okamoto, T.R.; et al. Gene activation of SMN by selective disruption of lncRNA-mediated recruitment of PRC2 for the treatment of spinal muscular atrophy. *Proc. Natl. Acad. Sci. USA* 2017, 114, E1509–E1518.

65. Ottesen, E.W.; Luo, D.; Seo, J.; Singh, N.N.; Singh, R.N. Human survival motor neuron genes generate a vast repertoire of circular RNAs. *Nucleic Acids Res.* 2019, 47, 2884–2905.

66. Pagliarini, V.; Jolly, A.; Bielli, P.; Di Rosa, V.; la Grange, D.; Sette, C. Sam68 binds Alu-rich introns in SMN and promotes pre-mRNA circularization. *Nucleic Acids Res.* 2020, 48, 633–645.

67. O’Meara, R.W.; Cummings, S.E.; De Repentigny, Y.; McFall, E.; Michalski, J.-P.; Deguise, M.-O.; Gibeault, S.; Kohthy, L. Oligodendrocyte development and CNS myelination are unaffected in a mouse model of severe spinal muscular atrophy. *Hum. Mol. Genet.* 2017, 26, 282–292.

68. Liao, Y.; Smyth, G.K.; Shi, W. featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014, 30, 923–930.

69. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014, 15, R36–13.

70. Eden, E.; Lipson, D.; Yogevo, S.; Yakhini, Z. Discovering motifs in ranked lists of DNA sequences. *PLoS Comput. Biol.* 2007, 3, e39.

71. Memczak, S.; Jens, M.; Elefsinioti, A.; Torti, F.; Krueger, J.; Rybak, A.; Maier, L.; Mackowiak, S.D.; Gregersen, L.H.; Munschauer, M.; et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 2013, 495, 333–338.

72. Zhang, X.-O.; Wang, H.-B.; Zhang, Y.; Lu, X.; Chen, L.-L.; Yang, L. Complementary sequence-mediated exon circularization. *Cell* 2014, 159, 134–147.

73. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(Delta Delta C(T)) Method. *Methods* 2001, 25, 402–408.