Circular RNAs in cell differentiation and development
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ABSTRACT
In recent years, circular RNAs (circRNAs) – a novel class of RNA molecules characterized by their covalently closed circular structure – have emerged as a complex family of eukaryotic transcripts with important biological features. Besides their peculiar structure, which makes them particularly stable molecules, they have attracted much interest because their expression is strongly tissue and cell specific. Moreover, many circRNAs are conserved across eukaryotes, localized in particular subcellular compartments, and can play disparate molecular functions. The discovery of circRNAs has therefore added not only another layer of gene expression regulation but also an additional degree of complexity to our understanding of the structure, function and evolution of eukaryotic genomes. In this Review, we summarize current knowledge of circRNAs and discuss the possible functions of circRNAs in cell differentiation and development.

KEY WORDS: Developmental biology, Differentiation, circRNAs, Noncoding RNAs, Stem cells

Introduction
Circular RNAs (circRNAs) are covalently closed single-stranded RNA molecules that were identified many years ago but have only recently attracted the attention of the general scientific community. They were initially described in the 1970s as the genomic constituents of viroids (Sanger et al., 1976). However, until several years later that a connection was made between circRNAs and mRNAs; specifically, it was observed that, upon splicing, not all exons could be found in the order predicted from their positions in genomic DNA (Nigro et al., 1991; Coccuereille et al., 1993; Starke et al., 2015). As they do not possess polyA tails, circRNAs were missed in transcriptomic profiling studies of polyadenylated mRNAs. Moreover, circRNAs were not identified in total RNA-sequencing experiments because ad hoc computational pipelines identifying back-splicing junctions have only recently been developed (Memczak et al., 2013). Finally, although in a few cases circRNAs can be the predominant isoform produced from a gene (Salzman et al., 2012), they are generally poorly expressed and are present at lower levels than their linear counterpart. It was only with the advent of new techniques for deep sequencing of total RNA, together with the development of ad hoc computational methods, that circRNAs came to the fore. They were shown to originate from a large number of mRNA coding genes, to be expressed in many cell types and to be conserved across eukaryotes (Salzman et al., 2012; Jeck et al., 2013; Memczak et al., 2013).

Since their discovery, many studies have been devoted to elucidating the expression profiles of circRNAs during development and cell differentiation. These studies have revealed that circRNAs are generally found in the cytoplasm, are expressed in a tissue- and cell-specific manner, and are highly abundant in the nervous system. In addition, and despite the challenges of assessing circRNA function (see Box 1), potential roles for circRNAs, as well their possible alterations in pathological conditions, have emerged. Here, we provide an overview of this research and summarize the key roles of circRNAs in various developmental contexts.

The biogenesis of circRNAs
As mentioned above, circRNAs are usually generated from genes that also produce linear isofoms. They arise via a process termed back splicing, in which an upstream 3′ splice site is joined to a downstream 5′ splice site, resulting in the junction of the 3′ end of an exon with the 5′ end of the same or upstream exon(s) (Fig. 1). The formation of circRNAs can be enhanced by the inhibition or slowing of canonical pre-mRNA splicing events and even by readthrough transcription (Liang et al., 2019; Wang et al., 2019). Even though canonical splicing is usually more efficient than back splicing, specific inhibition of linear splicing, adjudicated by the fact that circRNAs have a longer half-life than their linear counterparts, may allow circRNAs to become the predominant products of their host genes. The fact that canonical splicing and back splicing can be differently affected could be due to differences in exon definition mechanisms and to the requirement of a different set of splicing regulators (Liang et al., 2017; Wang et al., 2019).

One aspect of circRNAs that has been widely investigated is the identification of cis- and trans-acting factors that control biogenesis. Current research indicates that, similar to alternative splicing, the number of factors that can influence circRNA biogenesis is diverse and complex. Inverted repeats, such as Alu repeats, in the introns flanking the exons to be circularized were the first elements demonstrated to mediate the back-splicing reaction by bringing the splice sites into close proximity (Jeck et al., 2013; Liang and Wilusz, 2014). In some cases, they were shown to be sufficient to trigger back splicing (Zhang et al., 2014). Interestingly, double-stranded RNA sequences produced by Alu elements are particularly susceptible to adenosine-to-inosine (A-I) editing performed by ADAR (adenosine deaminases acting on RNA) enzymes (Kim et al., 2004; Levanon et al., 2004), and A-I editing was found to be enriched in introns surrounding circRNA exons (Ivanov et al., 2015). Because inosine forms Watson–Crick pairing with cytosine instead of thymine, these modifications result in a significant reduction in RNA pairing and a consequent decrease in the production of a subset of circRNAs (Rybalk-Wolf et al., 2015). More recently, it was shown that the interferon-inducible isoform of ADAR (p150) is able to interact with DExH-Box Helicase 9 (DHHX9), an RNA helicase that binds specifically to Alu elements. Loss of DHHX9 leads to an increase in the level and in the number of

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RNA-binding proteins (RBPs) can also regulate exon circularization (Fig. 1). For instance, NF90/NF110 were shown to bind intronic repeats and regulate circRNA production (Li et al., 2017). Unlike DHX9, NF90/NF110 promote circularization by associating with intronic RNA pairs and juxtaposing the circularizing exon(s) (Fig. 1). Another RBP, QKI, was shown to be responsible for the biogenesis of one of the most abundant circRNAs in human mammary epithelial cells and to promote circularization by directly binding to flanking introns (Conn et al., 2015). In Drosophila, the muscleblind (MBL) protein was shown to autoregulate its own production in a negative-feedback loop involving a circRNA (circMBL) originating from the same gene. In fact, although MBL promotes the formation of the circular form at the expense of the linear one, it also remains bound to it, thus lowering the levels of free MBL protein and bringing linear splicing levels back to normal (Ashwal-Fluss et al., 2014). The ALS-associated protein Fused in Sarcoma (FUS) is another RBP that can regulate the production of circRNAs. It has been shown in mouse embryonic stem cells to bind to the neighboring intron region of the exons to be circularized and to regulate the expression of 132 circRNAs without affecting the levels of their linear counterparts (Errichelli et al., 2017). Heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins have also been shown to participate in circRNA biogenesis (Ashwal-Fluss et al., 2014; Kramer et al., 2015; Errichelli et al., 2017), as have specific splicing factors such as ESRP1, which controls the circularization of circBIRC6 by binding to specific sites in the introns flanking the circularizable exon (Yu et al., 2017).

Interestingly, the lariat structure – a splicing intermediate with a branched circular conformation – can act similarly to inverted repeats and RBPs by favoring the circularization of sequences contained within it (Holdt et al., 2018). Although the lariat structure itself cannot account for circularization, exon-retaining lariats occasionally have the ability to produce circRNAs, and longer retained exons were shown to circularize more than shorter ones (Barret et al., 2015).

More recently, RNA modifications have also been shown to play a role in the biogenesis of circRNAs. In particular, for a specific subset of circRNAs, N6-methyladenosine (m6A) deposition at specific sites on primary transcripts was shown to direct the splicing machinery towards the back splicing reaction through a circuitry involving the METTL3 m6A writer and the YTHDC1 m6A reader (Di Timoteo et al., 2020).

**General mechanisms of action**

circRNAs have been found to be broadly expressed, conserved and modulated in response to cellular stimuli, and are sometimes expressed in a tissue-specific fashion (Salzman et al., 2012; Jeck et al., 2013; Memczak et al., 2013). In most cases, circRNAs exhibit cytoplasmic localization, although few details are available about the factors involved and mechanisms through which they are exported in the cytoplasm (Huang et al., 2018; Chen et al., 2019a). Current evidence suggests that circRNAs can actually have biological relevance and specific molecular activities (Fig. 2, Table 1). However, despite the large number of identified species, the characterization of their molecular mechanism of action is in most cases still largely owing due to difficulties in experimentally analyzing circRNA function (see Box 1).

The first example of an active circRNA was CDR1as, which is preferentially expressed in the brain (Hansen et al., 2013a,b; Memczak et al., 2013; Piwecka et al., 2017). CDR1as, which is sometimes referred to as ciRS-7, CDR1-AS or CDR1os, was
initially considered to act as a competing endogenous RNA (ceRNA; Fig. 2A) by sequestering multiple copies of the microRNA miR-7 and interfering with its mRNA-targeting activity (Memczak et al., 2013). However, in a more recent work, the ceRNA activity of CDR1as has been questioned (Piwecka et al., 2017). Following the establishment of a CDR1as knock-out mouse model, it was shown that the loss of CDR1as was related to the dysfunction of excitatory synaptic transmission, and also to a downregulation of miR-7 and an upregulation of its target mRNAs at the molecular level. This finding was in contrast with the previously proposed miRNA-sponge activity of CDR1as and highlighted a more complex role for this circRNA. In particular, the authors proposed that CDR1as may instead stabilize rather than titrate miR-7 and translocate it towards synapses (Piwecka et al., 2017).

Although the role of circRNAs as ceRNAs cannot be generalized, there are several examples to date of circRNAs that alter microRNA activity and impact many cellular functions, primarily those related to cell proliferation (Li et al., 2015a; Zheng et al., 2016; Zhong et al., 2017; Panda, 2018; Zhou et al., 2020; Zang et al., 2020). For instance, another circRNA with multiple miRNA binding sites is Sry, a testis-specific circRNA that acts by sponging miR-138 (Capel et al., 1993; Hansen et al., 2013a).

circRNAs can also act as scaffolds or decoys for RBPs (Fig. 2B). One of the most interesting cases is circFOXO3, which forms a ternary complex with the proteins p21 (CDKN1A) and CDK2; the effect of this assembly is that the function of CDK2 is arrested with the consequent block of cell cycle progression (Du et al., 2016). Through a similar mechanism, circANRIL sequesters PES1, suppressing ribosome biogenesis in smooth muscle and in macrophages (Holdt et al., 2016).

Another example of functional circRNAs is represented by the so-called ‘Exon-Intron’ circRNAs (EIciRNAs; Fig. 2C). Examples of these are circPAIP2 and circEIF3J, which control the expression of their mRNA counterparts through the binding of small nuclear ribonucleoproteins (snRNPs) and the modulation of RNA polymerase II activity (Li et al., 2015b). In particular, it was shown that EIciRNAs can base-pair with U1 snRNA, and that contact with RNA polymerase II can be mediated by U1 snRNP.

Some circRNAs have also been shown to act as templates for cap-independent translation (Fig. 2D), sometimes enhanced by the m6A methyltransferases. These circRNAs can serve as templates for cap-independent translation (m6A).

Fig. 2. Molecular functions of circRNAs. (A) circRNAs can act as competing endogenous RNAs (ceRNAs) by sequestering and/or stabilizing miRNAs. (B) circRNAs can act as scaffolds or decoys for RBPs; the examples of circFOXO3 and circANRIL are shown here. (C) circRNAs can also regulate the transcription of their parental gene, as occurs in the case of circPAIP2 and circEIF3J. RNA Pol-II, RNA polymerase II. (D) Some circRNAs can act as a template for cap-independent translation.
RNA modification (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017; Di Timoteo et al., 2020). These circRNAs harbor an open reading frame (ORF) spanning the back-splicing junction until they reach a downstream termination code. Therefore, a new ORF is produced that differs from that of the corresponding linear counterpart in the codons arising downstream of the back-splicing junction (Fig. 2). However, to date, a coding ability has only been reported for a few circRNA species: circZNF609, which is expressed in human primary myoblasts and controls cell proliferation (Legnini et al., 2017); circMBL, which is produced in the Drosophila head (Pamudurti et al., 2017); circSHPRH, the peptide of which has a suppressive role in glioblastoma (Begum et al., 2018); and circβ-catenin, which has a role in controlling the growth of liver cancer cells through the Wnt pathway (Liang et al., 2019).

Finally, circRNAs have recently been proposed to act as key modulators of the innate immune response (Fig. 3). Endogenously produced circRNAs can be recognized as ‘self’ molecules by cytoplasmic immune receptors (i.e. RIG-I, also known as DDX58) as they are marked by RNA modifications (i.e. m6A) and are bound to specific cellular proteins (Chen et al., 2017, 2019b; Fig. 3A). These self circRNAs can then regulate antiviral responses by competing with viral mRNAs for binding to NF90/NF110 factors (Li et al., 2017), or by inhibiting the activity of protein kinase R (PKR, also known as EIF2AK2), which plays a role in the early stress response.

### Table 1. circRNA functions

| circRNA         | Cell type                                      | Biological function                                      | Molecular function                                      | References                  |
|-----------------|------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|-----------------------------|
| CDR1as          | Neural tissue, PDLSCs, goat satellite muscle stem cells | Neural development, osteoblast differentiation, muscle stem cell differentiation | miR-7 regulation                                       | Memczac et al., 2013; Li et al., 2018b, 2019a |
| SRY             | Testis                                        | Testis development                                        | Protein coding, miR-138 sponge                           | Hansen et al., 2013a        |
| circBIRC6       | hESCs                                         | Pluripotency                                              | miR-34a sponge, miR-145 sponge                           | Yu et al., 2017             |
| circCOR01C      | hESCs                                         | Pluripotency                                              | –                                                        | Yu et al., 2017             |
| circFOX1P1      | MSCs                                          | Bone repair                                               | Suggested miRNA sponge                                   | Cherubini et al., 2019     |
| circHomer1_a    | Rat primary hippocampal neurons               | Synaptogenesis                                            | –                                                        | You et al., 2015           |
| circIGSF11      | Human BMSC-derived osteoblasts                | Osteoblast proliferation                                  | Suggested miR199b-5p sponge                              | Zhang et al., 2019         |
| hsa-circRNA_0074834 | Human BMSC-derived osteoblasts                      | Osteoblast differentiation                                  | Suggested miR942-5p sponge                              | Ouyang et al., 2019        |
| circPOMT1       | Human ADSC-derived osteoblasts                 | Osteoblast proliferation                                  | Suggested hsa-miR-6881-3p sponge                         | Huang et al., 2019b        |
| circMCM3AP      | Human ADSC-derived osteoblasts                 | Osteoblast proliferation                                  | Suggested hsa-miR-6881-3p sponge                         | Huang et al., 2019a,b      |
| circRFWD2       | Human ADSC-derived osteoblasts                 | Osteoblast differentiation                                  | Suggested hsa-miR-6817-5p sponge                         | Huang et al., 2019a        |
| circINO80       | Human ADSC-derived osteoblasts                 | Osteoblast differentiation                                  | Suggested hsa-miR-6817-5p sponge                         | Huang et al., 2019a        |
| mmu_circRNA_013422 | Murine ADSC-derived osteoblasts                   | Osteoblast differentiation                                  | Suggested miR-338-3p sponge                              | Long et al., 2018          |
| mmu_circRNA_22566 | Murine ADSC-derived osteoblasts                    | Osteoblast differentiation                                  | Suggested miR-338-3p sponge                              | Long et al., 2018          |
| circBANP        | PDLSCs                                        | Osteoblast differentiation                                  | Suggested miR-34a and miR-148a sponge                     | Gu et al., 2017            |
| circITCH        | PDLSCs                                        | Osteoblast differentiation                                  | Suggested miR-34a and miR-148a sponge                     | Gu et al., 2017            |
| circQKI         | Human primary myoblasts                        | Myoblast differentiation                                   | –                                                        | Legnini et al., 2017       |
| circBNC2        | Human primary myoblasts                        | Myoblast proliferation                                     | –                                                        | Legnini et al., 2017       |
| circZNF609      | Human primary myoblasts, murine myoblasts       | Myoblast proliferation                                     | Protein coding, suggested miR-194-5p sponge               | Legnini et al., 2017       |
| circFUT10       | Bovine myoblasts                               | Myoblast proliferation                                     | miR-133a sponge                                           | Li et al., 2018a           |
| circLMO7        | Bovine myoblasts                               | Bovine myoblast proliferation                              | Suggested miR-378a-3p sponge                             | Wei et al., 2017           |
| circZNF91       | Human epidermal cells                          | Keratinocyte differentiation                               | miR23b-3p and miR-766-3p sponge                          | Kristensen et al., 2018    |
| circH19         | ADSCs                                         | Adipogenesis inhibition                                    | PTBP1 binding                                            | Zhu et al., 2020           |

= unknown function.
The image contains diagrams illustrating the regulation of innate immune response by circRNAs. The text explains that endogenous circRNAs produced by cellular back splicing do not induce RIG-I activation because they are recognized as ‘self’ molecules (due to the binding of back splicing-coupled proteins, m6A decoration, and m6A-reader recognition). By contrast, circRNAs devoid of this ‘self’ signature can activate RIG-I and hence induce an innate immune response. Upon viral infection, circRNAs are globally degraded and PKR monomers are released and participate in an anti-viral response.

**circRNAs in stemness**

Besides being abundant in undifferentiated human embryonic stem cells (hESCs) and being associated with pluripotency (Yu et al., 2017), specific circRNAs have been shown to promote and maintain hESC pluripotency. For example, circBIRC6 and circCORO1C knockdown impairs pluripotency, whereas the ectopic expression of these circRNAs in hESCs promotes pluripotency and enhances the efficiency of induced pluripotent stem cell (iPSC) generation (Yu et al., 2017). In particular, circBIRC6 was shown to bind to the RNA-induced silencing complex (RISC) and to function by inhibiting miR-34a- and miR-145-mediated suppression of the pluripotency factors NANOG, OCT4 (POU5F1) and SOX2, thus favoring their expression and maintaining the pluripotent state. Interestingly, circBIRC6 biogenesis was shown to be controlled by the alternative splicing regulator ESRP1, the expression of which in turn is controlled by NANOG and OCT4. Therefore, this is an interesting regulatory circuitry for the control of stemness, which involves the interplay of pluripotency factors, splicing factors, circRNAs and miRNAs (Yu et al., 2017; Fig. 4).

Mesenchymal stem cells (MSCs) have a circRNA expression profile that is very different to that of differentiated fibroblasts (Cherubini et al., 2019). In particular, circFOXPI is upregulated in MSCs compared with all in vitro-generated mesodermal derivatives and human biopsies of bone, cartilage and adipose tissue. Moreover, its downregulation reduces MSC growth, indicating circFOXPI as a marker of undifferentiated MSCs. In vivo, the knockdown of circFOXPI in MSCs injected into the rat femur of an atrophic non-union model – an animal model of nonhealed femur fractures – determines the lack of bone union development, demonstrating a role for circFOXPI in bone repair. Based on these findings, circFOXPI was suggested to regulate these different molecular pathways by acting as a ceRNA for multiple miRNAs (Cherubini et al., 2019).

Furthermore, circRNAs have been demonstrated to be abundant in male germ cells. The ratio of circular versus linear products increases during spermatogenesis for a large number of circRNAs. Interestingly, recent evidence such as the presence of large potential ORFs, m6A-modified start codons (associated with translation) and the association with polysomes during cell development suggested that a subset of these circRNAs might encode for proteins, and mass spectrometry analysis has indeed proved the coding ability of such molecules (Tang et al., 2020). Taking advantage of a sequence-based bioinformatic approach, a recent study provided a comprehensive dataset of circRNA/miRNA interactions in mouse germline stem cells (Li et al., 2019b). Although functional analyses of these interactions remain to be performed, these data unveil new potential circRNA/miRNA regulatory circuitries and has opened circRNA research up to studies of germ cell development and human reproduction.

**circRNAs in neurogenesis**

circRNAs have been shown to be abundant in neuronal tissues, to be upregulated during neuronal differentiation and to accumulate with age (Rybak-Wolf et al., 2015; Vene et al., 2015; You et al., 2015; Westholm et al., 2014; Yang et al., 2018). In cell culture models of neural differentiation, for instance, the expression of the majority of circRNAs was shown to be significantly upregulated and to vary during differentiation (Rybak-Wolf et al., 2015).

Very recently, the depletion of circSle45a4, a highly conserved circRNA, was shown to not only induce spontaneous neuronal differentiation in a human neuroblastoma cell line but also to produce a significant reduction in the basal progenitor pool in the developing mouse cortex and a decrease in cells in the cortical plate together with an increase in Cajal–Retzius cells, which are neurons involved in brain development (Sukenel et al., 2020). These results highlight the role of circSle45a4 in cortex formation and in the maintenance of the pool of neural progenitors both in vitro and in vivo.

Hundreds of circRNAs are highly and specifically expressed in the brain tissues of mouse, human, rat and pig. Notably, 80% of all efficiently expressed mouse neuronal circRNAs are also detected in the human brain, with very similar expression patterns seen across neuronal tissues (Rybak-Wolf et al., 2015). Moreover, approximately 20% of the splice sites involved in porcine circRNA production are functionally conserved between mouse and human (Vene et al., 2015). Deep sequencing of porcine brain samples has demonstrated that circRNAs are highly abundant and dynamically expressed in a spatiotemporal manner (Vene et al., 2015). For example, high levels of circRNA expression are observed in the cortex during early to mid-gestation, which is when neurogenesis occurs. Interestingly, the circRNAs expressed derive from genes related to axon guidance, Wnt signaling and the TGFβ signaling pathway, i.e. factors that strongly impact neuronal differentiation and migration (Yi et al., 2010; Salinas, 2012; Rosso and Inestrosa, 2013; Stipursky et al., 2014).
Beyond CDR1as, which we introduced above, a number of other circRNAs have been investigated in neuronal tissues. For example, circRIMS2 – a circRNA specifically expressed in the developing embryonic pig cortex – is also highly expressed in the human cortex, whereas it is almost exclusively located in the cerebellum of the murine brain (Rybak-Wolf et al., 2015). This indicates that organism-, tissue- and developmental stage-specific regulatory mechanisms are fundamental for the control of circRNA levels in the brain. A possible involvement of circRNAs in synaptogenesis has also been suggested. Indeed, it has been shown that circRNA expression dramatically changes at the beginning of this process in mice (You et al., 2015), and that circRNAs produced by synapse-related genes (Venø et al., 2015; You et al., 2015) are upregulated during the establishment of mature neural circuits (You et al., 2015). Furthermore, circRNAs are highly enriched in synaptoneurosomes (You et al., 2015) and their compartmentalization differs from that of their linear cognates (Venø et al., 2015). For example, circStau2a is mainly present in synapses, whereas its linear counterpart primarily localizes to the cytoplasm (Venø et al., 2015). Interestingly, one particular circRNA (circHomer1_a; encoded by the Homer1 gene) that is known to play a key role in postsynaptic density regulation was the most significantly upregulated circRNA after induction of synaptic trafficking in axons and dendrites, and the localization of proteins and mRNAs at synaptosomes, or they may be secreted at the synapse level and function in signal transmission. The intrinsic stability of circRNAs makes them ideal molecules for storing information and for making it available in response to different external stimuli.

circRNAs in osteogenesis

Bone development is a complex process that starts during embryonic development and is completed during postnatal life. Moreover, bone fractures can be healed thanks to the reaction of osteogenic mechanisms. A number of different transcriptional and post-transcriptional mechanisms modulate osteoblast differentiation, as well as bone size and shape (Berendsen and Olsen, 2015). Recently, circRNAs have been added to this list of modulators of bone development. The differential expression and possible molecular activities of circRNAs have been reported during osteoblast differentiation of adult stem cells such as bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs).

Regarding the role of circRNAs in BMSC osteogenesis, there is evidence for a functional circRNA in the context of bone tissue regeneration during fracture healing (Ouyang et al., 2019). In particular, the study focused on a pathological condition called nonunion, in which bone fractures cannot heal spontaneously. This work highlighted profound differences in circRNA expression patterns between controls and nonunion patients. Among the modulated circRNAs, hsa-circRNA_0074834, deriving from the circBIRC6 gene, was found to be upregulated during BMSC osteoblast differentiation but downregulated in the pathology. This circRNA was clearly shown to regulate osteogenesis positively by acting as a ceRNA for miR-942-5p, thus providing a good example of a ceRNA that functions during bone regeneration. Among the targets of miR-942-5p were ZEB1 and VEGF mRNAs, which are involved in the regulation of both osteogenesis and angiogenesis coupling, suggesting that this circRNA-miRNA-ceRNA network can control bone regeneration during fracture healing (Ouyang et al., 2019).

ADSCs are another type of mesenchymal cell that can give rise to osteoblasts. They are particularly useful in bone regenerative medicine because they can be more easily obtained (e.g. from adipose tissue with respect to bone marrow derivation) and have a higher proliferation and differentiation potential than BMSCs (Storti et al., 2019). Differential expression of circRNAs has been observed during human ADSC osteogenesis (Huang et al., 2019b). circPOMT1 and circMCM3AP are among the downregulated species. Their depletion favors ADSC-derived osteoblast differentiation and their expression trend negatively correlates with that of hsa-miR-6881-3p. In addition, both of these circRNAs...
were predicted to inhibit miRNA activity of the transcripts of SMAD6 and chorardin, two factors that regulate osteogenesis through the BMP signaling pathway (Huang et al., 2019b). The role of circPOMT1 and circMCM3AP in regulating activity of the same miRNA is interesting, as it points out the importance of cooperative circuits involving either more than one circRNA and the same miRNAs, or one circRNA sponging different miRNAs with the same targets. These cooperative circuits can corroborate the ceRNA activity of a circRNA, especially when it is not highly expressed or when it harbors a few binding sites for a specific miRNA.

Another example of cooperative circuit, in which two circRNAs sponge the same miRNA, is represented by circRFWD2 (RFWD2 is also known as COP1) and circINO80 (Huang et al., 2019a). They are upregulated upon the induction of NELL1, a protein factor that plays an important role in sustaining osteogenesis (Liu et al., 2012). circRFWD2 and circINO80 knockdown impairs NELL1-induced osteogenesis, suggesting that they are downstream modulators of its signaling. These two circRNAs sustain osteoblast differentiation by sponging hsa-miR-6817-5p, a negative regulator of this process (Huang et al., 2019a,b).

However, the pathways affected by the sponging of this miRNA have not yet been clarified; therefore, this circRNAs-miRNA interaction needs further investigation.

Besides ADSCs and BMSCs, an emerging model for bone development and regeneration is represented by periodontal ligament stem cells (PDLSCs), which have a crucial role in supporting osteoblast differentiation. PDLSCs are useful for studying the formation of periodontal and alveolar bone tissues, but also some nerves and blood vessels (Gu et al., 2017; Zheng et al., 2017). The differential expression of circRNAs has been analyzed in the context of PDLSC osteogenic differentiation, and some regulatory networks have been proposed (Gu et al., 2017; Zheng et al., 2017; Li et al., 2018b). One of them includes the long non-coding RNAs (lncRNAs) TCONS_00212979 and TCONS_00212984, as well as circBANP and circITCH. All four of these RNAs can bind miR-34a and miR-146a, thus upregulating some of their target mRNAs involved in the MAPK pathway (DUSP1, FAS, RAC1, PDGFRA, TGFBR2 and MYC) (Gu et al., 2017).

The well-studied CDR1as/miR-7 axis also plays a role in PDLCs. In fact, CDR1as can promote osteogenesis by avoiding miR-7-mediated repression of GDF5. The latter, in turn, sustains PDLC differentiation by promoting SMAD1/5/8 phosphorylation and MAPK pathway activation (Li et al., 2018b).

In addition to these studies focusing on circRNAs with a functional relevance to osteogenesis, there are others confirming the modulation of circRNA expression during osteoblast differentiation. For example, a microarray-based analysis of circRNA expression revealed that thousands of circRNAs are deregulated during osteoblast differentiation, the majority of which come from genes involved in osteogenesis (Zhang et al., 2019). In this work, a regulatory network involving circIGSF11 and miR199b-5p was hypothesized, based on the observations that circIGSF11 has one binding site for miR199b-5p, that there is an inverse correlation between these two molecules during osteogenesis, and that circRNA knockdown promotes upregulation of miR199b-5p (Zhang et al., 2019). However, this molecular circuit has not yet been further investigated, and the ceRNA activity of circIGSF11, as well as its effects on osteoblast differentiation, remain to be clarified. Indeed, circIGSF11 has only one binding site for miR-199b-5p, so it could exert limited sponging activity. Therefore, given the lack of strong evidence, the functional relevance of circIGSF11 and its hypothesized miRNA-sponge activity remain questionable.

Just as circRNAs can contribute to osteogenesis of human ADSCs, they also participate in the same process in mouse. From circRNA-miRNA co-expression network analysis, miR-338-3p emerged as an important node, showing an inverse correlation with mmu_circRNA_013422 and mmu_circRNA_22566. As miR-338-3p can potentially bind the two circRNAs and is known to target two key regulators of osteogenesis (Fgf2 and Runx2), the authors proposed that a mmu_circRNA_013422/22566-miR-338-3p - Fgf2/Runx2 circuit could act as a fine-tuning network during osteoblast differentiation (Long et al., 2018).

circRNAs in skeletal muscle development

Myogenesis is a highly regulated process, the success of which is ensured by a delicate equilibrium of transcription factors and non-coding RNA activities (Bentzinger et al., 2012; Zhao et al., 2019). In recent years, some circRNAs have been described to function alongside lncRNAs and miRNAs as novel regulators of skeletal muscle growth and differentiation.

Early studies of circRNAs in muscle differentiation identified 2100 and 1600 circRNAs that are expressed and modulated during human and murine myogenesis, respectively, with almost 600 of these being conserved between the two species (Legnini et al., 2017). Based on a large siRNA-based phenotypic screen, some of these circRNAs were shown to have a role in regulating different aspects of muscle development. For example, circQKI (as well as QKI mRNA) depletion was demonstrated to have a negative effect on myoblast differentiation, indicating that both the circRNA and its linear counterpart cooperate in this process. By contrast, although BNC2 mRNA depletion causes an increase in myotube formation, knockdown of its circular counterpart has no effect on differentiation. Interestingly, circBNC2 expression during myoblast differentiation increases at the expense of the corresponding mRNA, suggesting that circBNC2 could contrast the expression of the anti-differentiative BNC2 mRNA (Legnini et al., 2017).

A third interesting circRNA described to function in muscle development is circZNF609 (Legnini et al., 2017). It is expressed in human growing myoblasts, and its levels decrease upon differentiation. Its role in sustaining myoblast growth was confirmed by the evidence that its specific depletion strongly affects human primary myoblast proliferation (Legnini et al., 2017). Moreover, circZNF609 was recently discovered to be upregulated in a skeletal muscle-derived tumor (rhabdomyosarcoma), in which its knockdown induces an evident cell cycle slow-down at the G1-S transition (Rossi et al., 2019). Consistent with its role in supporting cell proliferation, circZNF609 was also found to be upregulated in Duchenne muscular dystrophy, which is characterized by a higher percentage of growing myoblasts at its early stages (Legnini et al., 2017). Another characteristic of circZNF609 is that it can be translated into two proteins. In fact, its sequence hosts two start codons (AUGs), shared with the ORF of ZNF609 mRNA. Upon circularization, the two AUGs are in-frame with a stop codon downstream of the back-splicing junction, giving rise to a circRNA-specific ORF that can actually be translated (Legnini et al., 2017). So far, the role of these circZNF609-encoded proteins in myoblast proliferation and differentiation is unknown, hence further studies are required.

More recently, circZNF609 has also been proposed to suppress myotube differentiation in the C2C12 murine muscle cell line via the sponging of miR-194-5p (Wang et al., 2019). It was observed that, upon circZNF609 knockdown, the expression of some myogenic markers (such as MYF5 and MYOG) increases,
whereas the levels of BCLAF1, a negative regulator of myogenic differentiation and a target of miR-194-5p, decrease (Wu et al., 2019). However, the presence of only one miRNA-194-5p binding site in circZNF609, the absence of its conservation in human, and the relatively low abundance of circZNF609 suggest that a role for direct ceRNA activity in this context needs to be further and more deeply investigated.

Besides functioning in human and mouse muscles, circRNAs have been found to regulate skeletal muscle development in other species. Because studying muscle biology is also relevant for the food industry and for meat production, some studies have focused on molecular processes regulating livestock myogenesis. These studies have revealed that there are circRNAs playing a role in bovine and goat myogenesis. For example, circFUT10 sustains bovine myogenic differentiation, inducing the exit of myoblasts from the cell cycle and their accumulation in G0/G1 phase, by sponging miR-133a (Li et al., 2018a). By contrast, circLM07 was suggested to sustain bovine myoblast proliferation. Indeed, its overexpression increases the proportion of cells in S phase, while inhibiting their differentiation (as shown by a decrease in MYOD and MYOG expression) and counteracting apoptosis (Wei et al., 2017). In goat mid-embryonic muscle tissue, expression of the well-known circRNA CDR1as can be transcriptionally induced by MYOD. In this system, CDR1as promotes goat satellite muscle stem cell differentiation by acting as a ceRNA for miR-7, its main miRNA partner, thereby releasing IGF1R mRNA from miR-7 targeting (Li et al., 2019a).

**circRNAs in other tissues**

There are also a number of other tissues in which the role of circRNAs has just started to be explored. Below, we highlight just two examples of differentiation processes in which circRNAs have been shown to be differentially expressed and to have a potential role.

- circRNAs recently emerged as possible new players in skin development and regeneration as well as in skin diseases such as psoriasis (Kristensen et al., 2018; Liu et al., 2019b). By analyzing RNA-seq data from epidermal stem cells and keratinocytes, circRNAs were found to be highly and differentially expressed along the differentiation process, with a preferential accumulation in keratinocytes with respect to expression in undifferentiated cells (Kristensen et al., 2018). Many of these circRNAs are also modulated independently of their linear counterparts. Interestingly, a significant number of circRNAs that are upregulated independently of their mRNAs are derived from genes related to epidermal growth and differentiation, and a large fraction of them also have a high number of AGO2- and miRNA-binding sites. One particular circRNA that emerged from this group is circZNF91, as it has 24 binding sites for miR-23b-3p, a miRNA involved in keratinocyte differentiation, as well as 23 binding sites for miR-766-3p, which is overexpressed in cutaneous squamous cell carcinoma and can target DNMT3B (Kristensen et al., 2018). Therefore, it was suggested that circZNF91 exerts its functions in epidermal differentiation by acting as a miRNA sponge. In this case, the abundance of binding sites for two different miRNAs makes circZNF91 a very interesting ceRNA candidate, which would be worth further investigation.

- circRNAs can also contribute to the development of adipose tissue. Recently, circH19 was shown to regulate the differentiation of human ADSCs into adipose cells (Zhu et al., 2020). circH19 is derived from the host gene of the lncRNA H19, which has already been characterized as a regulator of lipid metabolism. circH19 depletion was shown to promote adipogenesis in ADSCs by causing an increase in the expression of lipogenic transcription factors, such as PPARγ (Zhu et al., 2020). circH19 can also bind to the protein PTBP1, which is involved in the cleavage of SREBP1 (also known as SREBF1; which has a fundamental role in lipid metabolism and adipose cell differentiation) and the translocation of its N-terminal cleaved portion from the cytoplasm to the nucleus during adipogenesis. Indeed, it was observed that the knockdown of circH19 enhances the cleavage and translocation of SREBP1 to the nucleus. Therefore, circH19 could inhibit adipogenesis by binding to PTBP1 and counteracting its functions related to SREBP1 processing. The negative role of circH19 in adipogenesis could link it to metabolic syndrome, which is characterized by impaired ADSC differentiation. In fact, high levels of circH19 have been associated with this disorder; circulating circH19, but not the lncRNA, is highly expressed in the serum of patients with metabolic syndrome compared with controls (Zhu et al., 2020).

**Discussion**

The discovery of a large number and different functions of circRNAs has added further layers of gene expression regulation, increasing our knowledge on the transcriptional potential of eukaryotic cells. Moreover, this information has added a degree of complexity to the comprehension of the structure, function and evolution of eukaryotic genomes.

Despite the continuing growing interest in identifying and characterizing novel circRNA species, there are a few issues that should be considered when approaching circRNA studies. One of them is that even if computational approaches can help to identify bona fide circRNAs, they are not definitive even for very abundant circRNAs. Indeed, validation of the circularity of candidates by independent approaches, such as RNase R treatment to deplete linear molecules or specific RNase H assays to specifically cut the putative circular RNAs followed by RT-PCR or northern blot analysis, are strictly required.

Many aspects of circRNA function, origin and evolution are still awaiting clarification and new methodologies are required to improve these studies. In addition, methodologies to determine the protein and RNA composition of specific circRNA-containing complexes (circRNPs) is strongly needed. RNA pull-down techniques allowing purification of circRNA interactors could be achieved using specific complementary RNA or DNA probes followed by mass spectrometry and/or RNA sequencing. Biotinylated antisense probes have been utilized after crosslinking of cell extracts (Chu et al., 2015; McHugh et al., 2015). However, in order to prevent the co-purification of linear counterparts, either probes specific for the back-splicing junction need to be used or the cell lysate should be pre-treated with RNase R to degrade linear RNAs. The selection of the specific strategy strongly depends on the abundance of the circRNA under study as well as its relative expression level with respect to that of the linear isoform.

Similar attention has to be paid to setting up suitable imaging methodologies that allow the relative quantification and cellular localization of circRNAs versus their linear counterparts. In the last few years, several different enhanced fluorescence in situ hybridization methods have been utilized. Among them, the BaseScope Assay has resulted in a highly specific and sensitive strategy (Erben et al., 2018). It is based on the use of two Z-probes designed on the sequences flanking the back-splicing junction that, only when in proximity, can support the next steps of signal amplification (Xu et al., 2018; Harris et al., 2020; Nielsen et al., 2020). Thanks to the amplification step, this procedure enables single-molecule detection with a high signal/background ratio.
Similar to studies of lncRNAs, phylogenetic analyses coupled with appropriate bioinformatic tools could contribute to the extrapolation of different functional subdomains from the primary sequences of circRNAs. In particular, it would be useful to predict sequences involved in the recognition of other nucleic acids or those acting as scaffolding modules for protein interaction. If we consider that different types of circRNAs may derive from a single primary transcript, it becomes evident that many alternative functions can originate from a single genomic locus that might be subjected to evolutionary selection. The optimization of specific tools for circRNA sequence analysis and prediction of their molecular partners, accompanied by new wet techniques for their validation, will greatly help circRNA research and will hopefully shed light on other relevant roles for this class of transcripts. Recent advances in next-generation sequencing, such as the use of the Nanopore RNA sequencing technology (Oxford Nanopore Technologies, Oxford, UK), will also undoubtedly lead to further progress in the field. Indeed, exact circRNA exon composition cannot be defined by RNA-seq experiments producing short reads, thus limiting circRNA identification to the back-splicing junction. The Nanopore technology allows direct RNA-seq with very long reads to be performed, thereby providing the opportunity for full-length sequencing of circRNAs and therefore for defining their precise sequence (Rahimi et al., 2019 preprint).

Aided by the availability of several prediction tools, the function of a number of circRNAs has been linked to their ability to pair with miRNAs, suggesting that they could act as miRNA sponges by controlling miRNA-circRNA-mRNA networks in different cellular systems. Although in some cases the real proof of this activity has been rigorously provided, in other cases there is no reliable data and confirmations are often based on overexpression experiments. Indeed, one major problem with the sponge theory is the stochiometry of miRNA molecules versus miRNA-binding sites, as the abundance of miRNAs often happens to be lower than that of the corresponding target sequences. However, as suggested in some cases, specific subcellular compartments can be created where miRNAs and target sequences can be tethered together, thus overcoming the concentration issue.

Although many different molecular mechanisms are starting to be attributed to circRNAs in in vitro cellular systems, there is an urgent need for appropriate in vivo model systems that can be used to gain a deeper understanding of circRNA function in development and cell differentiation (see Box 1). However, owing to their intertwined expression with their linear counterpart, the design of suitable knock-out strategies that prevent circRNA formation without affecting the mRNA should not be overlooked. When intronic sequences responsible for circularization are known, such as the case of Alu sequences, the deletion of the reverse complementary sequence in the downstream flanking intron can effectively lead to circRNA depletion, as recently described in an in vivo mouse knockout model for cia-cGAS (Xia et al., 2018). One additional strategy is to precisely delete one of the introns flanking the exon(s) that undergo circularization; however, since a common feature of such introns is that they are very long, this approach can be challenging and side effects on the expression levels of the linear counterpart have to be checked. As a further strategy, when siRNAs highly specific for the back-splicing junction are available, it is possible to envisage their targeted expression in vivo as applied in Drosophila for the knockdown of the most abundant fly circRNA circMbl (Pamudurti et al., 2018 preprint).

Finally, understanding the regulation of circRNA expression, and its interdependence with its linear counterpart, is an important topic that has so far been poorly addressed. The main problems are related to understanding how the choice between back and linear splicing is controlled and to deciphering the factors that control circRNA subcellular localization. Moreover, it will be important to understand whether any functional interconnection between the circular and linear forms originating from the same genomic locus exists and how this contributes to the evolution and complexity of gene expression regulation.

In conclusion, circRNAs are much more abundant than previously thought and they exhibit clear cell type specificity. The regulation of this important class of RNAs, and the cis- and trans-acting factors that regulate their homeostasis in the cell, are important issues that need to be studied in depth and with appropriate technologies in the future.

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Competing interests

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References

Aktej, T., Avşar Ilık, I., Maticzka, D., Bhardwaj, V., Pessoa Rodrigues, C., Mittler, G., Manke, T., Backofen, R. and Akhtar, A. (2017). DmX suppresses RNA processing defects originating from the invasion of the human genome. Nature 544, 115-119. doi:10.1038/nature21715

Ashwal-Fluss, R., Meyer, M., Pamudurti, N. R., Ivanov, A., Bartok, O., Hanan, M., Evantal, N., Memczak, S., Rajewsky, N. and Kadener, S. (2014). CircRNA biogenesis competes with Pre-mRNA splicing. Mol. Cell 56, 55-66. doi:10.1016/j.molcel.2014.09.019

Barrett, S. P., Wang, P. L. and Salzman, J. (2015). Circular RNA biogenesis can proceed through an exon-containing lariat precursor. eLife 4, e07540. doi:10.7554/eLife.07540

Begum, S., Yiu, A., Stebbing, J. and Castellano, L. (2018). Novel tumour suppressive protein encoded by circular RNA, circ-SHPRT1, in glioblastomas. Oncogene 37, 4055-4057. doi:10.1038/s41388-018-0230-3

Bentzinger, C. F., Wang, Y. X. and Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. Cold Spring Harb. Perspect. Biol. 4, a003842. doi:10.1101/cshperspect.a003842

Berendsen, A. D. and Olsen, B. R. (2015). Bone development. Bone 80, 14-18. doi:10.1016/j.bone.2015.04.039

Capel, B., Swain, A., Nicolis, S., Hacker, A., Walter, M., Koopman, P., Goodfellow, P. and Lovell-Badge, R. (1993). Circular transcripts of the testis-determining gene Sry in adult mouse testis. Cell 73, 1019-1030. doi:10.1016/0092-8674(93)90279-y

Chen, Y. G., Kim, M. V., Chen, X., Cadena, C., Pulendran, B. et al. (2019b). N6-methyladenosine modification of circNSUN2 facilitates cytoplasmic export and stabilizes HMGA2 to promote colorectal liver metastasis. Nat. Commun. 10, 4695. doi:10.1038/s41467-019-12851-z

Chen, Y. G., Chen, R., Ahmad, S., Verma, R., Kasturi, S. P., Amaya, L., Broughton, J. P., Kim, J., Cadena, C., Pulendran, B. et al. (2019a). N6-methyladenosine modification controls circular RNA immunity, Mol. Cell 76, 96-109.e9. doi:10.1016/j.molcel.2019.07.016

Cherubini, A., Barillani, M., Rossi, R. L., Jalali, M. M. K., Rusconi, F., Buono, G., Ragni, E., Cantarella, G., Simpson, H. A. R. W., Pélault, B. et al. (2019). FOXP1 circular RNA sustains mesenchymal stem cell identity via microRNA inhibition. Nucleic Acids Res. 47, 5325-5340. doi:10.1093/nar/gkz199

Chu, C., Zhang, Q. C., Da Rocha, S. T., Flynn, R. A., Bhardwaj, M., Calabrese, J. M., Magnuson, T., Heard, E. and Chang, H. Y. (2015). Systematic discovery of Xist RNA binding proteins. Cell 161, 404-416. doi:10.1016/j.cell.2015.03.025

Cocco, P., Fajardo, R., Heleine, D. and Bailleul, B. (1993). Mis-splicing yields circular RNA molecules. FASEB J. 7, 155-160. doi:10.1096/fasebj.7.1.7678559
Li, Z., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X., Zhong, G., Yu, B., Hu, W., Kristensen, L. S., Okholm, T. L. H., Venø, M. T. and Kjems, J.

Dai, L. et al. (2015). Circular RNA SRD5A3 regulates osteoblast differentiation of periodontal ligament stem cells via the Wnt/β-catenin/SMAD and P38 MAPK signaling pathway. Stem Cell Res. Ther. 9. 232. doi:10.1186/s13238-019-01896-w

Li, H., Yang, Z., Xie, W., Song, C., Dong, D., Huang, Y., Lan, X., Plath, M., Lei, C., Ma, Y. et al. (2018a). CircFUT10 reduces proliferation and facilitates characterization of multiple myelomas by sponging miR-133a. J. Cell. Physiol. 233, 4843-4851. doi:10.1002/jcp.26230

Li, X., Zheng, Y., Zheng, Z., Huang, Y., Zhang, Y., Jia, L. and Li, W. (2018b). Circular RNA CDR1as regulates osteoblastic differentiation of periodontal ligament stem cells via the Wnt/β-catenin/SMAD and P38 MAPK signaling pathway. Stem Cell Res. Ther. 9. 232. doi:10.1186/s13238-019-01896-w

Li, X. and Tian, G. (2019b). Genome-wide identification and characterization of long noncoding and circular RNAs in germline stem cells. Sci. Data 6, 8. doi:10.1038/s41597-018-0261-1

Li, X. et al. (2015a). The output of protein-coding genes shifts to circular RNAs when the pre-mRNA processing machinery islimiting. Mol. Cell 68, 940-954. doi:10.1016/j.molcel.2017.03.002

Li, X. et al. (2017). Circular RNA circMbl functions as a ceRNA for miR-138-5p. Cell Res. 27, 96-107. doi:10.1038/cr.2016.112

Li, X., Liu, C.-X., Xue, W., Zhang, Y., Jiang, S., Yin, Q.-F., Wei, J., Yao, R.-W., Bu, J., Shi, H. et al. (2018). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443

Li, X. et al. (2017). Coordinated circRNA Biogenesis and function in psoriatic skin lesions. Nature 550, 232-236. doi:10.1038/nature14443
deregulation and affects brain function. Science 357, eaam8526. doi:10.1126/science.aam8526

Rahimi, K., Vene, M. T., Dupont, D. M. and Kjems, J. (2019). Nanopore sequencing of full-length circRNAs in human and mouse brains reveals circRNA-specific exon usage and intron retention. bioRxiv. doi:10.1101/567164

Rossi, F., Legnini, I., Migliorini, F., Colantoni, A., Santini, T., Morlando, M., Di Timoteo, G., Dattilo, D., Dominici, C. and Bozzoni, I. (2019). Circ-ZNF609 regulates G1-S progression in rhodobacterospora. Oncogene 38, 3843-3854. doi:10.1038/s41388-019-0699-4

Rosso, S. B. and Inestrosa, N. C. (2013). WNT signaling in neuronal maturation and synapogenesis. Front. Cell. Neurosci. 7, 103. doi:10.3389/fncel.2013.00103

Rybak-Wolf, A., Stottemeier, C., Glazär, P., Jens, M., Pino, N., Giusti, S., Hanan, M., Behm, M., Bartok, O., Ashwal-Fluss, R. et al. (2015). Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. Mol. Cell 58, 870-885. doi:10.1016/j.molcel.2015.03.027

Salinas, P. C. (2012). Wnt signaling in the vertebrate central nervous system: from axon guidance to synaptic function. Cold Spring Harb. Perspect. Biol. 4, a008003. doi:10.1101/cshperspect.a008003

Salzman, J., Gawad, C., Wang, P. L., Lacayo, N. and Brown, P. O. (2012). Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. PLoS ONE 7, e30733. doi:10.1371/journal.pone.0030733

Sanger, H. L., Klotz, G., Riesner, D., Gross, H. J. and Kleinschmidt, A. K. (2017). Circular RNA profiling reveals an abundant circLMO7 that regulates myoblast differentiation and affects brain function. Mol. Cell 80, 587-596. doi:10.1016/j.molcel.2017.09.015

Suenkel, C., Cavalli, D., Massalini, S., Calegari, F. and Rajewsky, N. (2019). Complementary sequence-mediated exon circularization. Nat. Biotechnol. 37, 134-147. doi:10.1038/s41587-019-0112-a

Senti, G., Venturini, S., Parrella, G., Gobbo, C. and Sanfilippo, P. (2018). A circRNA protects dormant hematopoietic stem cells from DNA sensor cGAS-mediated exhaustion. Immunity 48, 688-701.e7. doi:10.1016/j.immuni.2018.03.016

Xu, K., Chen, D., Wang, Z. M., Ji, Z., Zhou, J., Chen, N., Lv, L., Zheng, Y., Lu, H., Zhang, Y. et al. (2019). Annotation and functional clustering of circRNA expression in human rhesus macaque brain during aging. Cell Discov. 4, 48. doi:10.1038/s41421-018-0050-1

Yang, Y., Fan, X., Mao, M., Song, X., Wu, P., Zhang, Y., Jin, Y., Yang, L., Chen, L.-L., Wang, Y. et al. (2017). Extensive translation of circular RNAs driven by N6-methyladenosine. Cell Rep. 27, 626-641. doi:10.1016/j.celrep.2017.01.016

Yi, J.-J., Barnes, A. P., Hand, R., Poilieux, F. and Ehlers, M. D. (2010). TGF-β1 signaling specifies axons during brain development. Cell 142, 144-157. doi:10.1016/j.cell.2010.06.010

You, X., Vlatkovic, I., Babin, A., Will, T., Epstein, I., Tushev, G., Akbalik, G., Wang, M., Glock, C., Quedena, C. et al. (2015). Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. Nat. Neurosci. 18, 308-310. doi:10.1038/nn.4037

Yu, C.-Y., Li, T.-C., Wu, Y.-Y., Yeh, C.-H., Chiang, W., Chuang, C.-Y. and Kuo, H.-C. (2017). The circular RNA circBIRC8 participates in the molecular circuitry controlling human pluripotency. Nat. Commun. 8, 1149. doi:10.1038/ncomms14467

Zhao, Y., Chen, M., Lian, D., Li, Y., Li, Y., Wang, J., Deng, S., Yu, K. and Lian, Z. (2019). Non-coding RNA regulates the myogenesis of skeletal muscle satellite cells, injury repair and diseases. Cells 8, 988. doi:10.3390/cells08090988

Zheng, Q., Bao, C., Guo, W. L., Li, S., Chen, J., Chen, B., Luo, Y., Lyu, D., Li, Y., Shi, G. et al. (2016). Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. Nat. Commun. 7, 11215. doi:10.1038/ncomms11215

Zheng, Y., Li, X., Huang, Y., Jia, L. and Li, W. (2017). Circular RNA landscape of periodical limb stem cells during osteogenesis. J. Periodontol. 88, 906-919. doi:10.1902/jop.2017.170075

Zhong, Z., Huang, M., Lv, M., He, Y., Duan, C., Zhang, L. and Chen, J. (2017). Circular RNA MYLK as a competing endogenous RNA promotes bladder cancer progression through modulating VEGFA/VEGFR2 signaling pathway. Cancer Lett. 403, 305-317. doi:10.1016/j.canlet.2017.06.027

Zhou, C., Liu, H-S., Wang, F-W., Hu, T., Liang, Z-X., Lan, H., He, X-W., Zheng, X-B., Wu, X-J., Xie, D. et al. (2020). circCAMSAP1 promotes tumor growth in colorectal cancer via the miR-328-5p/EP302 axis. Mol. Ther. 28, 914-928. doi:10.1038/s41380-2019-0063-4

Zhu, Y., Gui, W., Lin, X. and Li, H. (2020). Knock-down of circular RNA H19 induces human adipose-derived stem cells adipogenic differentiation via a mechanism involving the poly(ADP-ribose) polymerase 1. Exp. Cell Res. 387, 111753. doi:10.1016/j.yexcr.2019.111753

Zimmerman, A. J., Hafez, A. K., Amoah, S. K., Rodriguez, B. A., Dell’Orco, M., Lozano, E., Hartley, B. J., Alural, B., Lalonde, J., Chander, P. et al. (2020). A psychiatric disease-related circRNA controls synaptic gene expression and cognition. Mol. Psychiatry. doi:10.1038/s41380-020-0653-4

Development (2020) 147, dev182725. doi:10.1242/dev.182725