Published in final edited form as:

Mech Ageing Dev. 2018 July ; 173: 71–79. doi:10.1016/j.mad.2018.05.001.

CircRNA accumulation: A new hallmark of aging?

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Abstract

Circular RNAs (circRNAs) are a newly appreciated class of RNAs found across phyla that are generated most commonly from back-splicing of protein-coding exons. Recent profiling of circRNAs genome-wide has shown that hundreds of circRNAs dramatically increase in expression during aging in the brains of multiple organisms. No other class of transcripts has been found to show such a strong correlation with aging as circRNAs—could they be playing a role in the aging process? Here, we discuss the different methods used to profile circRNAs and discuss current limitations of these approaches. We argue that age-related increases in global circRNA levels likely result from their high stability. The functions of circRNAs are only beginning to emerge, and it is an open question whether circRNA accumulation impacts the aging brain. We discuss experimental approaches that could illuminate whether age-accumulation of circRNAs are detrimental or protective to the aging brain.

Keywords

circRNA; RNA-Seq; aging; alternative splicing; nervous system

1. Introduction

Aging is characterized by the progressive deterioration of cells, tissues, and organs, ultimately leading to disease or death. Next-generation sequencing (NGS) technologies have enabled the molecular characterization of gene expression changes during aging in humans and other organisms. To date, most of the genome-wide gene expression characterization has been at the level of mRNA expression from protein-coding genes. Greater than 90% of the human genome is actively transcribed, yet only ~2% of transcribed RNAs will encode for protein (Consortium, 2012). RNA-Seq now enables characterization of not only the protein-coding transcriptome, but also characterization of alternatively spliced mRNA isoforms and non-coding transcripts. Indeed, many profiling studies have documented changes in small RNA, and long non-coding RNA expression during aging (reviewed in (de Magalhaes et al., 2009; Kato and Slack, 2013; Stegeman and Weake, 2017)). RNA-Seq based approaches have also led to the findings that RNA editing of microRNAs increases with age (Warnefors et al., 2014), and alternative splicing is progressively deregulated (Harries et al., 2011; Mazin et al., 2013; Stilling et al., 2014).

RNA-Seq studies have permitted the annotation and expression profiling of a newly appreciated, age-regulated class of RNAs called circular RNAs (circRNAs). CircRNAs are formed through a unique splicing mechanism termed back-splicing, where the downstream

splice donor site of a circularizing exon covalently joins with an upstream splice acceptor site (Figure 1). They are usually generated from protein-coding genes and have been found throughout eukaryotes (Memczak et al., 2013; Salzman et al., 2013; Salzman et al., 2012; Westholm et al., 2014). CircRNAs have long half-lives relative to mRNAs as they lack free ends and thus are not susceptible to degradation by exoribonucleases (Enuka et al., 2016; Jeck et al., 2013). CircRNAs are dynamically regulated during embryonic development (Dang et al., 2016; Szabo et al., 2015) and display expression differences among tissues, with a particular enrichment in brain regions (Rybak-Wolf et al., 2015; You et al., 2015). CircRNAs were originally found to increase with aging in an RNA-Seq analysis of *Drosophila melanogaster* (Westholm et al., 2014). Subsequent work has shown that this accumulation occurs in other animals as well (Cortes-Lopez et al., 2018; Gruner et al., 2016).

In this review, we focus on circRNAs in relation to aging, highlighting recent studies profiling their expression changes during aging. We review methods for circRNA detection, which may be relevant for the development of biomarkers to predict biological age and age-related diseases. We present methods to assess circRNA function while discussing limitations to current approaches and speculate on the mechanisms underlying circRNA accumulation in the aging brain.

2. Detecting circRNAs

Conventional RNA-Seq library preparation protocols select for polyadenylated RNAs to avoid sequencing of ribosomal RNAs, which constitute the majority of RNAs in the cell. CircRNAs lack polyA tails—thus, to detect them the RNA-Seq library must include non-polyadenylated transcripts. Today, such “total RNA” libraries are prepared using Ribo-depletion methods which are available in standard RNA-Seq library preparation kits. In 2012, circRNAs were serendipitously discovered on a global scale while attempting to examine splicing changes in Acute Lymphoblastic Leukemia by RNA-Seq (Salzman et al., 2012). Here, RNA-Seq reads aligned to “scrambled exon sequences”, where a downstream exon would appear to precede an upstream exon. Today circRNA detection by RNA-Seq continues to rely on split read alignment to these aforementioned, back-splice junction sites (Figure 2). However, these back-spliced reads constitute a very small proportion (<0.1%) of the reads generated from a typical total RNA-Seq experiment, which complicates analysis due to low read depth (Gruner et al., 2016). Hence, methods to enrich for circRNAs from total RNA populations have been developed.

RNase R, a 3′ to 5′ exoribonuclease, has been used to enrich for circRNAs prior to sequencing in several studies (Jeck et al., 2013; Panda et al., 2017; Zhang et al., 2014) (Figure 2). Devoid of free ends, circRNAs are generally resistant to RNase R treatment, while linear RNAs are not (Jeck et al., 2013) allowing for circRNA enrichment. One caveat to this approach is that linear RNAs with extensive secondary structures remain intact or fragmented after RNase R treatment, impeding quantitative analysis of circRNA abundance. Moreover, some circRNAs validated by independent detection methods are sensitive to RNase R treatment (Jeck et al., 2013). Despite these limitations, RNase R treated RNA-Seq

libraries have successfully enriched for back-splice junction reads in multiple studies (Jeck et al., 2013; Zhang et al., 2014).

There continues to be active development of circRNA enrichment strategies. For instance, a recently developed method called RPAD (RNase R treatment followed by polyadenylation and poly(A)+ RNA depletion) was developed to isolate a purer population of circRNAs prior to sequencing (Figure 2) (Panda et al., 2017). In this method, total RNA is treated with RNase R, followed by depletion of polyadenylated RNA using oligo d(T) beads. The resulting RNA population contains fragmented, RNase R-resistant linear RNAs and circRNAs. Fragmented RNAs are then artificially polyadenylated using polyA polymerase and removed by a second round of poly(A)+ capture. Finally, the RNA population is ribo-depleted, yielding an enriched population of circRNAs. Applying this method, hundreds of novel exon-derived circRNAs were identified in mammalian cell lines.

Other methods to profile circRNAs include custom microarrays (Chen et al., 2017a; Dou et al., 2016). Here, probes are designed to span the back-spliced junctions. These custom probes can be designed for many tissues and species as comprehensive circRNA annotations have been generated for human, and common model organisms including mouse, *Drosophila*, and *C. elegans* (Cooper et al., 2018). Although microarrays are an older technology, they have utility for circRNA differential expression analysis because of employing a targeted approach. In contrast, using total RNA-Seq for circRNA analysis requires very high sequencing depth for accurate quantification of differentially expressed circRNAs.

3. CircRNAs accumulate in the aging brain

3.1 Age-accumulation of circRNAs in *Drosophila*

Compelling evidence has amassed in various animals showing that circRNAs globally accumulate during aging. The first evidence for this was found from mining RNA-Seq libraries from *Drosophila melanogaster*. Examining more than 100 RNA-Seq libraries from various tissues and life-stages, Westholm and colleagues annotated >2500 circular RNAs. Over 90% of circRNAs annotated in these libraries were expressed in samples from dissected adult heads, providing evidence for a neural-tissue bias (Westholm et al., 2014). Profiling circRNAs in aging head samples (1, 4, and 20 days post-eclosion) showed that 262 circRNAs were significantly upregulated >2-fold in 20-day vs 1-day old samples (Westholm et al., 2014) (Table 1), thus uncovering a global age-increase in circRNAs during aging. Evidence was presented that suggested the observed increases were independent of general transcription of the circRNA hosting genes.

These findings have since been expanded to a study of aging *Drosophila* photoreceptor neurons (Hall et al., 2017). Here, Hall et al. sought to identify gene expression changes contributing to visual senescence in photoreceptor neurons at a comprehensive set of ages—10, 20, 25, 30, and 40 days post-eclosion. CircRNA profiling revealed an age-accumulation trend from 10-days to 40-days, extending the findings from Westholm et al, and providing evidence that global circRNA levels continue to increase from 20 days to 40 days of age in *Drosophila*. CircRNAs might thus have utility as aging biomarkers (*see 5.* CircRNAs as

potential biomarkers). Since nuclei of photoreceptor neurons were profiled in this study, this suggests that age-accumulation trends found from whole *Drosophila* heads (Westholm et al., 2014) might be largely attributed to accumulation in neurons.

Future work is needed to understand the cellular and subcellular expression patterns of circRNAs in aging animals. Fluorescence-activated cell sorting (FACS) of brain cell populations including, endothelial cells, neurons, and glia followed by circRNA profiling should elucidate cell-type specific differences in circRNA accumulation trends. Furthermore, it should be feasible in the future to profile circRNAs using emerging single-cell RNA-Seq technologies (Bacher and Kendziorski, 2016; Ofengeim et al., 2017). Finally, there is evidence that some circRNAs are enriched in subcellular compartments of neurons, including dendrites (You et al., 2015), axons (Shigeoka et al., 2016), and synapses (Rybak-Wolf et al., 2015). If accumulation in these regions during aging is pronounced, this could lead to clues as to how circRNAs might function during aging.

3.2 Age-accumulation of circRNAs in mouse brain tissues

Profiling of circRNAs in humans and mice uncovered that, like in *Drosophila*, neural tissues harbor a greater abundance of circRNAs compared to other tissues (Rybak-Wolf et al., 2015; You et al., 2015). To determine if age-accumulation of circRNAs also occurs in mammals, Gruner et al. profiled circRNAs in aging male C57Bl/6 mice (Gruner et al., 2016). Using total RNA-Seq, circRNAs were identified in the cortex, hippocampus, and heart of 1-month old and 22-month old mice. Using high sequencing depth and conservative thresholds for circRNA detection, >6,500 unique circRNAs were annotated. Comparing the old and young time points, a highly significant increase in the relative number of back-spliced reads was observed for hippocampus and cortex samples. This demonstrated a global bias for circRNA age-accumulation in the brain.

In addition to showing a genome-wide bias for increased circRNA levels in old versus young brains of mice, the authors also tested for differential expression of individual circRNAs. It was found that 258 and 250 circRNAs were upregulated >1.5-fold in the aging cortex and hippocampus, respectively. In contrast, only 40 and 53 circRNAs were significantly downregulated in cortex and hippocampus, respectively (Table 1). Similar to findings in fly, expression of the host genes of the circRNAs was not biased toward upregulation, meaning that the increase in circRNA expression was likely not due to increased transcription from the host genes. Due to the low number of back-spliced reads available to quantify circRNA expression changes, a limited number of individual circRNAs were differentially expressed after statistical analysis. The authors asserted that the quantification of ~250 age-accumulated circRNAs in either tissue was likely an underestimation. This might also explain why there was only some overlap between the individual circRNAs found to be age-accumulated in hippocampus versus cortex. Importantly, extensive validation of differentially expressed circRNAs was performed using qRT-PCR and Northern blots. As circRNA quantification methods and analysis tools are in their infancy, it is important for investigators to perform experimental validation of circRNA expression trends revealed by RNA-Seq.

Future work is required to generate a complete characterization of the circRNA transcriptome in the aging mouse. For instance, it will be important to profile changes between mature adults (5–8 months of age) in comparison to very old mice (> 28 months) of age, along with intermediate ages of both females and males. More accurate and specific methods to quantify circRNAs should be employed to obtain strong statistical evidence for age-accumulation trends of individual circRNAs. The targeted approaches for circRNA profiling mentioned above could be employed for these studies. Moreover, there is continual improvement in algorithms to detect differential expression of circRNAs from total RNA-Seq data. CircTest (Cheng et al., 2016) and Sailfish (Li et al., 2017a) are two algorithms that can determine circRNA differential expression trends that are independent of host gene expression.

Global circRNA age-accumulation appears to be specific to neural tissues. In contrast to the findings in brain tissues, global circRNA levels were statistically unchanged in the hearts of young versus old mice—instead, relatively equal numbers of circRNAs were found to increase or decrease with age (Gruner et al., 2016) (Table 1). This is in agreement with an independent study that profiled circRNAs in Rhesus macaque skeletal muscle and found no trend for circRNA increase during aging (Abdelmohsen et al., 2015). It is unknown why the circRNA age-accumulation trends appear to be restricted to brain tissues, although several likely responsible mechanisms are under current investigation (*see 4*. Mechanisms underlying increased circRNA levels during aging). It may be useful to profile specific cell types in different tissues during aging to strengthen the neural-specific age-accumulation hypothesis in mammals.

3.3 Age-accumulation of circRNAs in *C. elegans*

Very recently, circRNA profiling was performed in aging *C. elegans* (Cortes-Lopez et al., 2018), a model organism which has had incredible utility with respect to the study of aging (for review, see (Gems and Partridge, 2013)). Whole worms were profiled by total RNA-Seq at larval stage 4 (L4), day 1, day 7, and day 10 adults (Cortes-Lopez et al., 2018). Perhaps expectedly, circRNAs showed a global bias for age-accumulation. There were notable differences, however, from studies in fly and mice. In contrast to these previous studies, here whole worms were profiled, and not isolated neuronal tissues or neurons. Nonetheless, by far the strongest trends for circRNA age-accumulation observed to date were uncovered. Over 90% of circRNAs detected increased at least 1.5-fold between L4 and day 10. Using CircTest (Cheng et al., 2016), 194 circRNAs significantly increased between L4 and day 10 time-points, and this increase was independent of host gene expression levels. In contrast, 0 circRNAs decreased. Again, statistical limitations due to low read count likely led to a vast underreporting of the number age-accumulated circRNAs. Given the genetic tools and amenability to aging studies, *C. elegans* is uniquely poised as a model system to tackle what functions circRNAs play during aging.

Together, these studies in *C. elegans*, *Drosophila melanogaster* and mice (summarized in Table 1), demonstrate that age-accumulation of circRNAs is a phenomena conserved across phyla. Importantly, all these studies provide various levels of evidence that age-related circRNA increases were independent from host gene expression. This means that most

circRNA increases were not due to transcriptional upregulation of the host genes. Future work is required to determine whether circRNAs globally change during aging in humans. Such work will provide an indication of whether human circRNAs show parallel trends during aging compared to model systems. Moreover, this would permit identification of individual, conserved, age-accumulated circRNAs that could be studied using loss-of-function approaches in mouse (*see 6*- Probing circRNA function). We next turn to speculating on the possible mechanisms underlying age-related changes in circRNA levels.

4. Mechanisms underlying increased circRNA levels during aging

What mechanisms might account for the increased levels of circRNAs during aging? Current evidence suggests that the age-related increase trends for circRNAs are reflective of age-accumulation more so than specific age-related regulation. Still, multiple mechanisms might contribute to circRNA changes during aging, which we discuss below.

4.1 RNA stability-mediated age-accumulation of circRNAs

Due to their lack of free-ends, circRNAs are resistant to degradation from exoribonucleases (Jeck et al., 2013). This, coupled with the post-mitotic nature of neurons, implies that the increased levels of circRNAs reported in multiple animals could be a result of accumulation as opposed to specific gene regulation (Figure 3A). Following this logic, linear RNAs from the circRNA host genes would be synthesized in post-mitotic cells, and degraded, whereas the turnover of circRNAs in the same cells would be much slower. When cells divide or die, the stable circRNAs are lost, which would imply that post-mitotic cells would have more circRNAs than proliferative cells. Supporting this line of reasoning, circRNAs are less abundant in proliferative cells (Bachmayr-Heyda et al., 2015; Song et al., 2016) and are negatively correlated with increasing numbers of proliferative, glial subpopulations in cultured cells (Rybak-Wolf et al., 2015). The strong age-accumulation circRNA trends found in whole *C. elegans* samples also supports this explanation as the majority of cells in adult *C. elegans* are post-mitotic (Sulston and Horvitz, 1977).

Thus, there are multiple lines of evidence to support a hypothesis for stability-based age-accumulation of circRNAs. The direct monitoring of an individual circRNA molecule over the lifespan of a cell has not been performed to date. Could it be that a single circRNA might persist for days, or even months *in vivo*? Although the half-lives of individual circRNAs have been assessed using actinomycin D (Jeck et al., 2013) and metabolic labeling experiments in cultured cells (Zhang et al., 2016), we know nothing about the stability of circRNAs in intact neurons. Some circRNAs have recently been found to be translated. If such circRNAs are highly stable *in vivo*, could they provide messages for synthesizing aberrant proteins in neurons at accelerated rates as animal age?

4.2 Increased circRNA biogenesis due to age-related changes in alternative splicing

Aging is associated with global changes in splicing patterns in multiple organisms, including humans (Harries et al., 2011; Mazin et al., 2013; Rodriguez et al., 2016). Since circRNAs are products of alternative splicing, it is reasonable to assume age-related changes in back-splicing might contribute to the progressive age-accumulation of circRNAs (Figure 3B).

To date, a handful of splicing factors have been found to regulate subsets of circRNAs in both invertebrate and vertebrate systems (Conn et al., 2015; Errichelli et al., 2017; Kramer et al., 2015). Interestingly, the splicing factor FUS was shown to regulate circRNAs expressed in cultured mouse motor neurons (Errichelli et al., 2017). Whether the observed regulation is due to direct or indirect action of FUS remains to be investigated. Considering the role of FUS in ALS (Vance et al., 2009) and frontotemporal dementia (Deng et al., 2014), this may suggest a link between circRNAs and neurologic disease development. It is known that splicing factors compete for splice signals, which in-turn act to influence alternative splicing outcomes. Therefore, it is likely that many splicing factors contribute to circRNA biogenesis. Indeed, the *Drosophila* Laccase2 circRNA was found to be regulated by multiple hnRNP and SR proteins in a combinatorial approach (Kramer et al., 2015). Moving forward, it will be important to consider this interplay between multiple splicing factors when investigating circRNA biogenesis. Given the roles that splicing factors play in regulating circRNA biogenesis, it would be useful to correlate the abundance/activity of individual splicing factors with enhanced back-splicing events during aging.

4.3 Additional mechanisms underlying age-regulation of circRNAs

In addition to the contributions of inherent circRNA stability and splicing factor regulation of circRNA biogenesis during aging, other mechanisms could be at play (Figure 3B). For instance, RBPs surely affect stability of circRNAs like they do for other classes of RNAs. Although circRNAs are resistant to exoribonucleases, endoribonucleases likely facilitate their eventual turnover. What are the endoribonucleases that degrade circRNAs and does aging impact their activity? Are their catalytic activities diminished during aging? Individual circRNAs have been found to be localized to the nucleus (Li et al., 2015) and cytoplasm (Zheng et al., 2016), where RNA degradation kinetics are surely variable. Interestingly, circRNAs have been found in exosomes where they are enriched over their linear counterparts from the same host genes (Lasda and Parker, 2016; Preusser et al., 2018). This might provide a route for circRNA clearance independent of degradation pathways (Figure 3B).

5. CircRNAs as potential biomarkers

5.1 Biomarker of aging in experimental systems

The circRNA age-accumulation trends found in various animals suggest that they could serve as biomarkers of age. When profiling multiple aging time-points from *Drosophila* photoreceptor neurons, 34 circRNAs that significantly changed between 10 and 40 days were found to correlate highly with age (day 10, 20, 25, 30, 40), R^2=0.92 (Hall et al., 2017). Given that individual circRNAs can be accurately quantified using conventional RT-qPCR, it is plausible that detection of a handful of circRNAs might be highly accurate in predicting age. Future work is needed to determine whether circRNA levels are correlated with chronological age or with “biological” age. Modification of lifespan in model organisms by environmental or genetic means followed by precise quantification of circRNAs genome-wide at various ages should illuminate this question. Perhaps there are individual circRNAs that correlate linearly with chronological age in tissues such as the brain, whereas others are sensitive read-outs of biological aging which are correlated with stress or disease. Indeed,

higher temperature has been associated with enhanced circRNA levels in *Arabidopsis* (Pan et al., 2017) and *Drosophila* (Rybak-Wolf et al., 2015).

When profiling circRNAs for biomarker discovery, it is important that sequencing experiments are designed such that they will have enough statistical power to accurately measure changes between individual time-points. With most current methodologies, changes in the total number of circRNA reads can be compared with high statistical confidence between ages, but quantification of individual low expressed circRNAs can be problematic. New library cloning methods, custom microarrays or even high-throughput qPCR methods (*see* Section 2) could be applied in order to exploit the use of circRNAs as an aging biomarker.

The development of aging biomarkers for experimental model systems could be highly beneficial for aging research. For instance, tracking circRNA biomarkers reflective of biological age could facilitate screening for age-altering genes or compounds without the need for complete survival analysis in systems such as *Drosophila* and *C. elegans*.

5.2 Biomarker of aging and age-related neurodegeneration in humans

Given that age-accumulation of circRNAs appears to be a conserved phenomenon, it is plausible that circRNAs could serve as human aging biomarkers. However, this hinges on whether circRNA expression correlates with age in tissues accessible from living subjects, which has yet to be assessed. We do know that circRNAs can be detected in human saliva (Bahn et al., 2015), serum (Koh et al., 2014), and blood (Alhasan et al., 2016; Memczak et al., 2015). In saliva, circRNAs were found to be primarily derived from inflammatory and integrin-mediated signaling pathways, among others (Bahn et al., 2015). In blood samples, the average circular to linear RNA ratio was found to be much higher than in other tissues, suggesting that circRNAs might serve as easily detectable biomarkers in clinical blood samples (Memczak et al., 2015).

With recent development of what appears to be highly accurate DNA methylation biomarkers in humans from blood (Horvath, 2013), it is not clear if there will be a demand for circRNA-based biomarkers for human. However, there might be brain disease states for which circRNAs could serve as diagnostic biomarkers. The utility of circRNAs as biomarkers in humans for neurological disease will depend on whether circRNA changes in the brain are reflected in a tissue accessible from patients such as cerebrospinal fluid or blood. It will be important to determine if circRNA changes in blood are due to alterations in the blood-cell transcriptome or are due to circRNA release from diseased tissue. It is also unknown whether these circRNAs can originate from damaged brain tissue, or whether they share anything in common at the regulatory level with brain-expressed circRNAs.

A major risk factor for neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease is advanced age. Could circRNAs be deregulated in the brains of patients suffering from neurodegenerative diseases? Profiling for circRNAs in the cells, tissues or cerebrospinal fluid from patients suffering from the most devastating and common neurodegenerative diseases has not been performed to date. However, a recent re-analysis of an RNA-Seq dataset showed that several circRNAs are upregulated in multiple system

atrophy (MSA) samples (Chen et al., 2016). MSA is a sporadic neurodegenerative disease characterized by alpha-synuclein aggregation in oligodendrocytes, with a mean age-of-onset between 50 to 60 years old (Fanciulli and Wenning, 2015; Geser et al., 2006). Curiously, each upregulated circRNA confirmed in this work by RT-qPCR was enriched in cortical white matter, which is biased for glial subpopulations (Azevedo et al., 2009). Accordingly, it will be important to investigate splicing-changes in aging neurons and glia, which may provide clues to their age-accumulation.

6. Probing circRNA function in aging

6.1. CircRNA function in the nervous system

Functions of circRNAs are only beginning to emerge (Cortes-Lopez and Miura, 2016). Earlier work on the circRNAs CDR1as/ciRS-7 and Sry showed they can “sponge” microRNAs, preventing them from exerting their regulatory functions (Hansen et al., 2013; Memczak et al., 2013). The first circRNA knockout has been recently implemented in mice for the CDR1as circRNA (Piwecka et al., 2017). These animals have defects in neural function which provides the first evidence for a physiologically relevant circRNA by loss of function in mice (however, see (Barrett et al., 2017)). Interestingly, analysis of this knockout mouse showed that CDR1as enhances miR-7 activity (Piwecka et al., 2017), whereas previous work using overexpression of CDR1as showed it inhibited miR-7 activity (Hansen et al., 2013; Memczak et al., 2013) . These high-profile studies illustrate the need to assess the biological roles of circRNAs *in vivo* by loss of function approaches.

Moving beyond their impact on microRNA activity, emerging functions of circRNAs are broad—circRNAs can be translated (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017), participate in innate immune activation (Chen et al., 2017b; Li et al., 2017b), and localize to neuronal synapses (Rybak-Wolf et al., 2015; You et al., 2015). At the synapse, dozens of circRNAs were found to be biased toward upregulation after bicuculline-mediated neural activation, and this upregulation was largely independent from host mRNA expression changes (You et al., 2015). There is evidence that circRNAs can bind proteins and/or facilitate protein interactions (Du et al., 2017; Schneider et al., 2016). Perhaps circRNAs localized to synapses are trafficked there in circRNA-protein complexes. Functionally, it is unclear what role circRNAs have at neuronal synapses. One possibility is a contribution to local translation. In the mouse brain, polysome profiling failed to demonstrate circRNA enrichment (You et al., 2015); however, another study found some circRNAs to be associated with ribosomes at axon terminals, suggesting they might be locally translated or participate in regulating local translation (Shigeoka et al., 2016).

The age-accumulation of circRNAs in the brain warrants investigation for functional roles of circRNAs in aging. At this point, it is not clear whether the age-accumulation of circRNAs might be adaptive changes that are beneficial to the aging brain, or whether they are detrimental. Of course, it is possible that this accumulation does not impact aging neurons, and the low overall abundance of circRNAs in cells compared to mRNAs might support this view. With hundreds of circRNAs accumulating during aging, unique challenges present themselves to the dissection of circRNA age-related function. This includes the possibility

that there are collective functions of circRNAs as opposed, or in addition to, functions of individual circRNAs.

6.2. CircRNA loss of function approaches

The most informative approaches for determining the function of a gene product is creation of a genetic mutant or performing an siRNA based knockdown. However, as we detail below, such approaches that have been applied to the study of conventional gene products are highly problematic for the study of circRNAs.

Problems arise from the fact that the vast majority circRNAs are derived from protein-coding genes. How can one disrupt the circRNA generated from a gene, while not affecting the linear RNAs also generated? One approach is siRNA-based knockdown. This is challenging because an siRNA, typically ~20 nucleotides in length, must be designed to span the back-spliced junction of the circRNA. In this manner, the circRNA is targeted for knockdown, but not mRNAs bearing the same exons. This restriction of siRNA design to the back-spliced junction reduces the chances of finding an optimal circRNA-specific siRNA with minimal off-targeting events. Several studies have used this approach to knockdown circRNAs. Some of these studies performed extensive controls to evaluate unintended effects on host gene expression, but many have not (Du et al., 2016; Guarnerio et al., 2016; Jeck et al., 2013; Legnini et al., 2017). As the circRNA field matures, it will become increasingly important for studies to demonstrate specificity of knockdown before novel phenotypes are attributed to particular circRNAs.

Flanking introns of circularizing exons are important for circRNA formation (Ivanov et al., 2015). Flanking introns can base pair together forming reverse complementary matches (RCMs) that bring the 5′ splice donor site in closer proximity to the upstream 3′ splice acceptor site (Figure 1). Plasmid constructs that include such flanking introns have been used to drive overexpression of circRNAs (Kramer et al., 2015; Liang and Wilusz, 2014; Zhang et al., 2014). Likewise, it would seem plausible that deletion or mutation of one of the flanking introns to prevent base-pairing could prevent circRNA biogenesis (Figure 4A). If important intronic splice enhancers/inhibitors, the branch point, and the polypyrimidine tract are not disrupted by such deletion/mutations, then linear splicing should not be affected. Such an approach was recently employed in cultured cells. Knockdown of the *HIPK3* circRNA was accomplished in HEK-293T cells using CRISPR/Cas9 to delete a flanking intronic sequence (Zheng et al., 2016). Importantly, linear RNA expression from the circularizing locus was not perturbed. It will be exciting to determine whether such an approach is successful *in vivo.* If so, given the versatile, low cost, and continually improving CRISPR based tools (Wright et al., 2016), one could envision that the function of circRNAs in particular cellular processes could be tackled in large scale CRISPR-based screens. One limitation is the design of guide RNAs (gRNAs) for CRISPR genome editing. gRNAs require high GC content, and introns in most organisms are GC-poor. Thus, the more inefficient and resource-intensive homology-directed repair approach would have to be implemented in many cases.

6.3. Overexpressing circRNAs

CircRNA overexpression as a strategy has been used in many studies, most commonly by transient transfection . Often, complementary intronic regions have been employed to promote back-splicing of a subcloned exon (Kramer et al., 2015; Legnini et al., 2017; Li et al., 2015). However, these plasmid-based overexpression approaches often produce linear RNA by-products and concatemer circRNA transcripts, either of which might confound results. Moreover, recent work has shown that if the intronic regions used to promote the circRNA biogenesis are not endogenous to the host, then the innate immune response is triggered (Chen et al., 2017b). While these approaches should continue to uncover putative circRNA functions, it is possible that singular circRNAs which exhibit phenotypic consequences as a result of knockdown or overexpression are in the minority, potentially due to overlapping or redundant roles.

It would be interesting to probe the cooperative effects of an entire “pure” circRNA population with respect to disease progression or disruption of cellular homeostasis (Figure 4B). An attractive system to study this might involve the transfection of a highly enriched circRNA preparation into cultured cells. RPAD (Panda et al., 2017) could be used for such enrichment. For instance, to assess the role of an aged circRNA population, circRNAs may be extracted from aged tissues or FACS-sorted cell populations and transfected into cells. The cell populations with transfected circRNAs could then be monitored for age-related phenotypes, such as increased reactive oxygen species, age-associated disease development, or increased cellular senescence. This approach may provide evidence that circRNAs act as a class cooperatively to influence cellular function. A caveat to this approach for mammalian systems would be that innate immune response pathways would likely be triggered by an RNA preparation that contains highly structured RNAs, complicating the interpretation of cellular phenotypes.

7. Concluding Remarks

It is clear that increased circRNA levels in aging neural tissues is a universal phenomenon. Yet, due to their relatively low abundance, it is possible that the cooperative influence of many circRNAs acting in concert are needed to enact a function. Hence, single circRNA knockdown experiments might not reveal biological functions. Many questions remain to be answered in order to determine if circRNAs have utility as an aging biomarker, and whether circRNA accumulation has a functional impact on the aging nervous system. Given the recent findings that circRNA age-accumulation is common to metazoans and the development of new tools for manipulating circRNA expression, the time is ripe for investigation into the functions of circRNAs in aging. Enthusiasm for establishing circRNAs as a hallmark of aging is tempered by the unattractive possibility that circRNA age-accumulation might be a benign artifact. If circRNAs turn out to be detrimental or beneficial to the aging brain, then it will become lucrative to investigate the feasibility of therapeutic interventions to manipulate circRNAs.

Acknowledgements:

We thank Daphne Cooper and Hannah Gruner for critical reading of the manuscript.

Funding: This work was supported by NIH National Institute on Aging grant R15 AG052931 to P.M. and NIH National Institute of General Medical Sciences grant P20 GM103650. D.K. is supported by the National Science Foundation Graduate Research Fellowship Program.

Abbreviations:

circRNA	circular RNA
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate
NGS	Next-generation sequencing
RPAD	RNase R treatment followed by polyadenylation and poly(A)+ RNA depletion
RBP	RNA-binding protein
RCM	Reverse complementary matches

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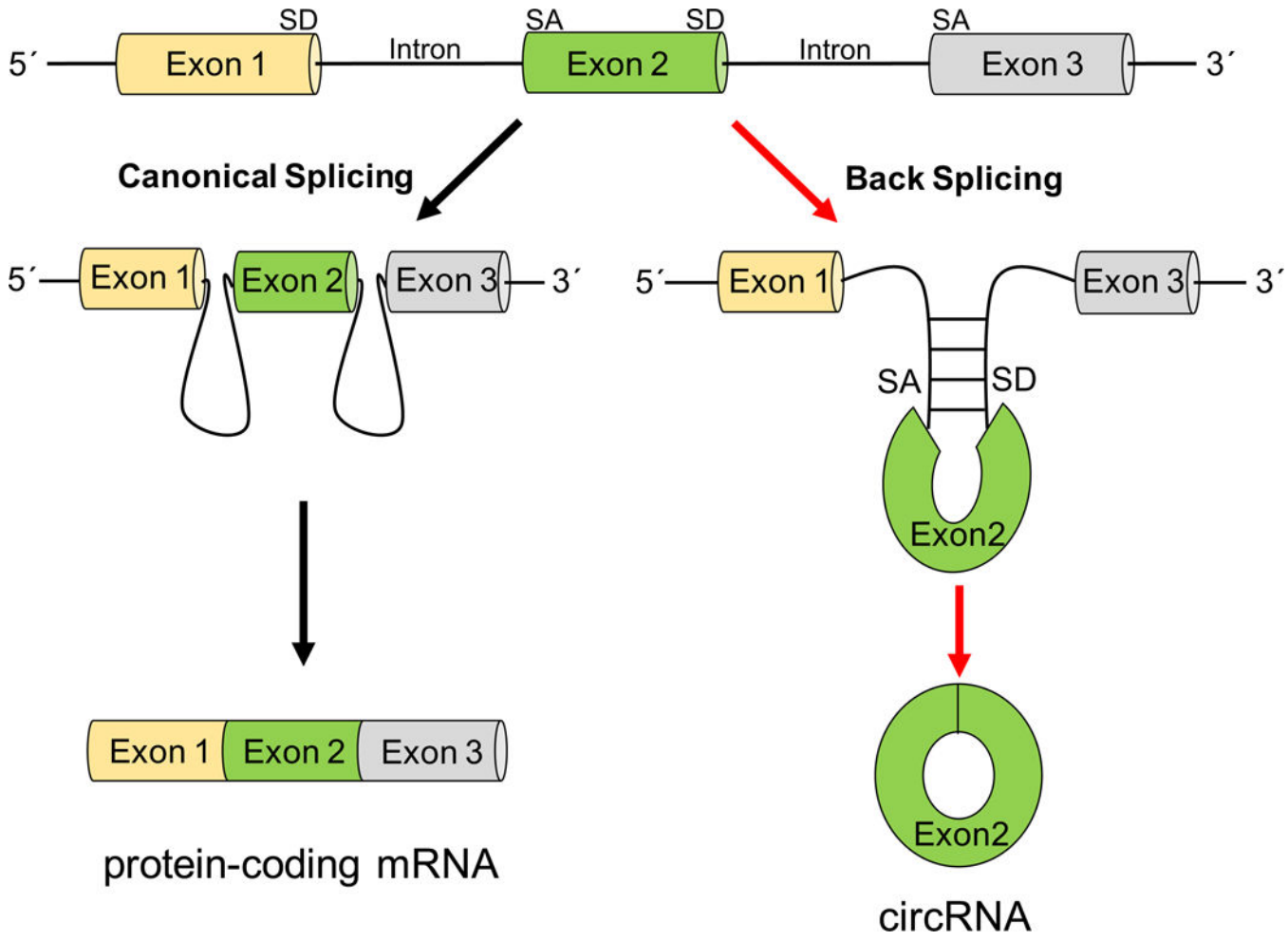

Figure 1: CircRNAs are formed by back-splicing.

In canonical splicing, (*left*) an upstream splice donor (SD) is spliced to a downstream splice acceptor (SA) site resulting in the production of a protein-coding mRNA. In back splicing, (*right*) complementary base pairing occurs between the flanking intronic sequences of circExon2, bringing the downstream SD and upstream SA into close proximity. This leads to the production of a circular molecule with no free ends.

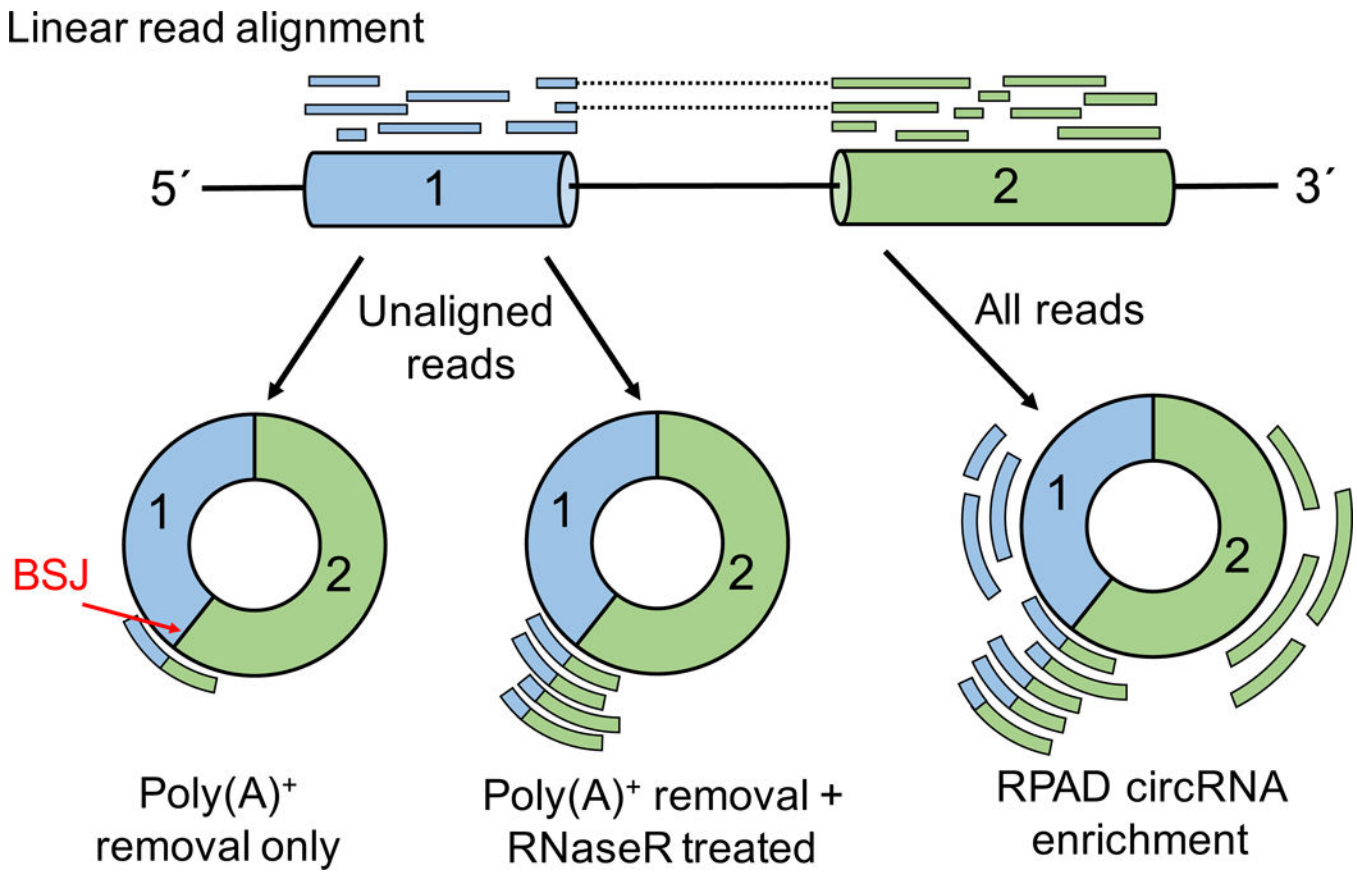

Figure 2: CircRNA detection strategies.

In a typical circRNA profiling strategy linear spliced reads are aligned to the genome. Unaligned reads (*left)* are then mapped to a custom scaffold consisting of only back-splice junction sequences (BSJ). Poly(A)$^+$ removal plus RNaseR treatment enriches the number of reads aligning to the BSJ in a RNA-Seq run. RPAD enrichment (*right*) allows for alignment to both back-splice junctions and exonic sequence resulting in greater read depth.

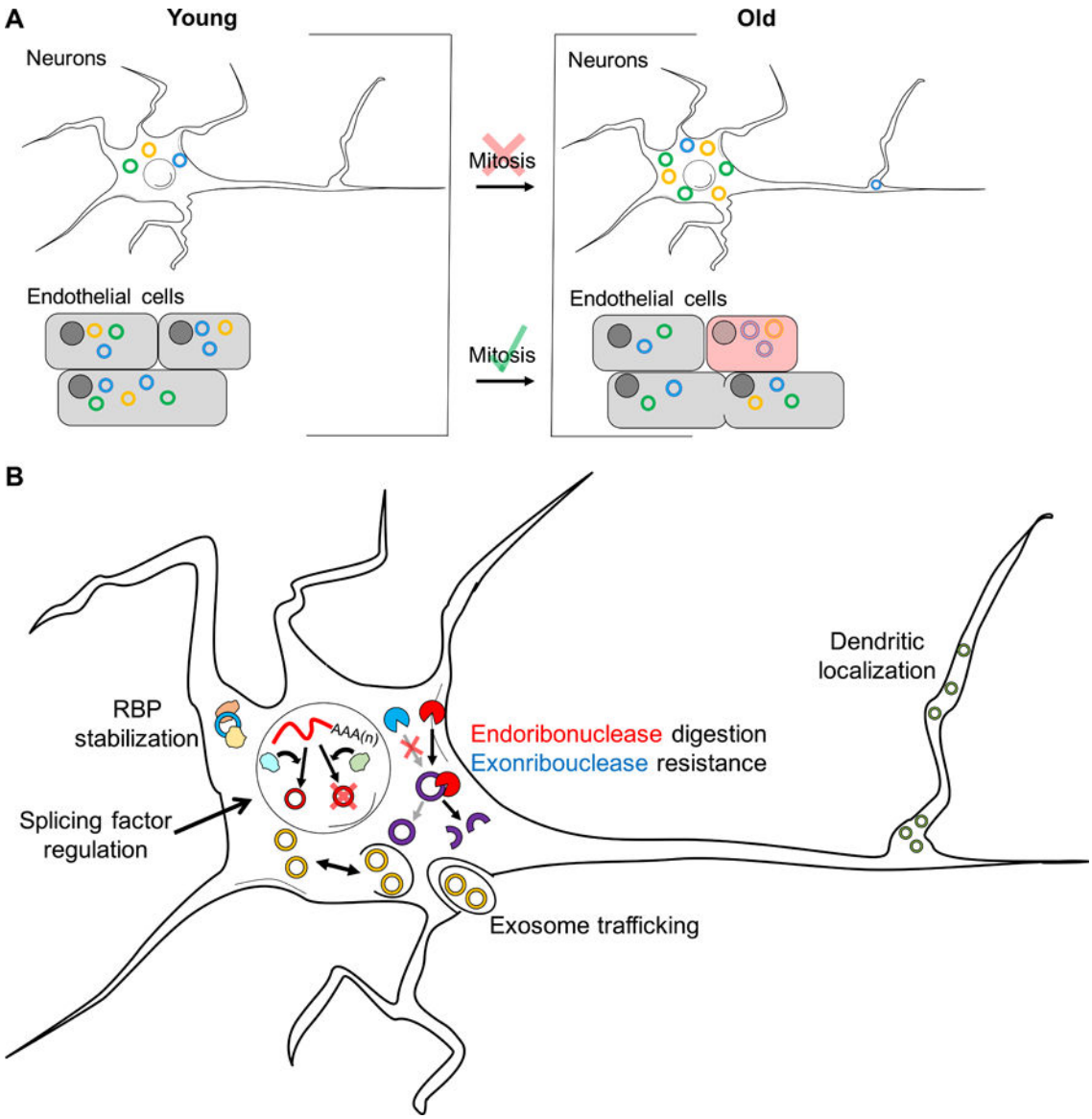

Figure 3. Potential mechanisms underlying circRNA accumulation during aging.

(A) CircRNAs accumulate in neurons (post-mitotic) but are diluted following mitosis and/or cell-death in proliferative cells (such as endothelial cells) during aging. (B) CircRNA accumulation in neurons may be attributed several cooperative actions in the cell including: stabilization by RNA binding proteins (RBPs), exoribonuclease resistance, endoribonuclease digestion, exosome trafficking, and splicing factor activation (light blue) or repression (green) that regulates circRNA biogenesis.

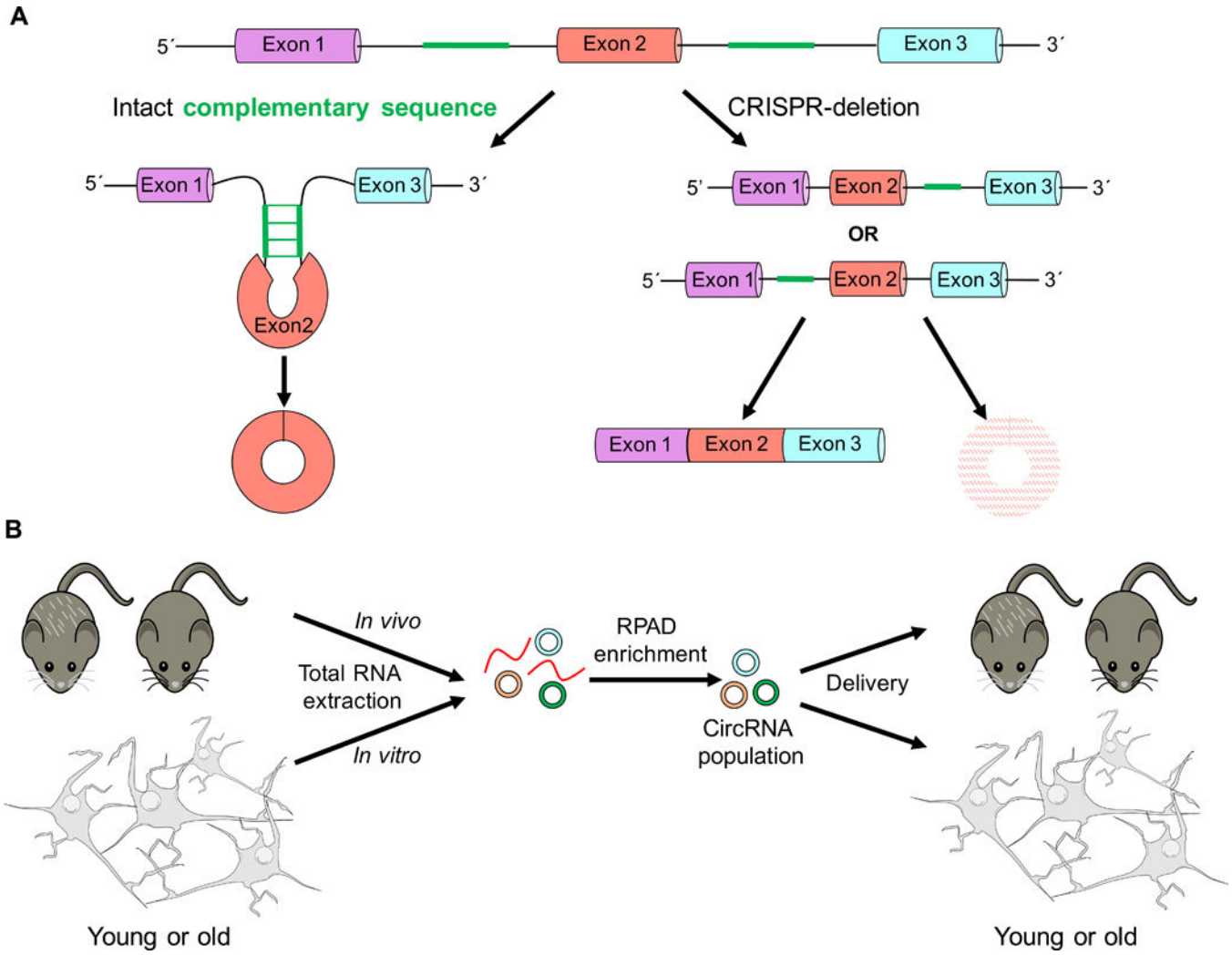

Figure 4. Methodologies to probe circRNA function.

(A) Reverse complementary matches (RCM) (*green bars*) facilitate back-splicing (*left*). CRISPR-mediated deletion of either the upstream or downstream complementary sequence (*right*) abrogates circRNA expression while leaving canonical splicing unperturbed. (B) Total RNA extraction from tissue or primary neurons of young or aged samples can be enriched for circRNAs using RPAD. Subsequently, the circRNA population can be transfected into a young or old host.

Table 1:
Studies profiling circRNAs during aging.

This table reports previous circRNA profiling studies that identified circRNA expression changes during aging. In *Drosophila,* circRNAs are increase during aging in adult head samples and photoreceptor neurons (numbers indicate circRNAs up/downregulated by at least a factor of 2). In mouse, circRNAs are biased toward upregulation (>1.5 fold-change cutoff) in the aging cortex and hippocampus. This trend is not observed in the aged mouse heart. In *C. elegans*, circRNAs are upregulated in whole-body samples during aging (>1.5 fold-change cutoff).

Host	Time-points profiled	Tissue profiled	# CircRNAs (up/down)	Refs
Drosophila	Adult day 1, 4, 20	Whole head	262/NA (20d vs 1d)	Westholm et al., 2014
Drosophila	Adult day, 10, 20, 25, 30, 40	Photoreceptor neurons	35/3 (40d vs 10d)	Hall et al., 2017
Mouse	Adult month 1, 22	Cortex	258/40	Gruner et al., 2016
Mouse	Adult month 1, 22	Hippocampus	250/53	Gruner et al., 2016
Mouse	Adult month 1, 22	Heart	68/57	Gruner et al., 2016
C. elegans	Larval stage 4 (L4) Adult day 1, 7, 10	Whole body	194/0 (10d vs L4)	Cortés-López et al., 2018