



# Molecular functions and specific roles of circRNAs in the cardiovascular system

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## ABSTRACT

As part of the superfamily of long noncoding RNAs, circular RNAs (circRNAs) are emerging as a new type of regulatory molecules that partake in gene expression control. Here, we review the current knowledge about circRNAs in cardiovascular disease. CircRNAs are not only associated with different types of cardiovascular disease, but they have also been identified as intracellular effector molecules for pathophysiological changes in cardiovascular tissues, and as cardiovascular biomarkers. This evidence is put in the context of the current understanding of general circRNA biogenesis and of known interactions of circRNAs with DNA, RNA, and proteins.

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## 1. Introduction

Today, nearly 28000 genes for long noncoding RNAs (lncRNAs) have been mapped. These lncRNA genes can be located in genomes separate from any other protein-coding gene, or overlapping in complex patterns with other genes [1]. lncRNAs have been shown to be expressed in a cell type- and state-specific mode during cell differentiation and during pathophysiological cell state changes including in diseases of the cardiovascular system [2]. Surprisingly, although genes are linear, and mRNAs are linear, and although typical lncRNA transcripts are linear as well, cells also express circular non-protein-coding RNAs, termed “circRNAs”. In a seminal study by Salzman et al. [3], advances in high-throughput sequencing and in annotating splice-junctions have allowed accumulating solid evidence that thousands of eukaryotic genes form circular RNAs as a physiologically normal process (see Ref. [4] for review). These circRNAs are not encoded as separate genetic units, like lncRNAs, but are produced by a variant form of splicing from the pre-mRNA of any transcribed gene *in cis*, may it be protein-coding or noncoding. To form circRNAs, the spliceosome covalently fuses an RNA's 5' end to its 3' end, which results in a circular

ribonucleic acid. Thus, circRNAs lack 5'Cap and poly(A) tail. These features are used to biochemically enrich for circRNAs. For example, one can negatively select against polyA-containing mRNA, or digest all linear RNAs in circRNA preparations by adding exonucleases like RNase R that attack open linear ends [5]. Irrespective of origin, and with only a tiny minority of exceptions, circRNAs are not translated into proteins and are thus falling into the class of noncoding RNAs. More specifically, with a median size of around 500 ribonucleotides, circRNAs are part of the larger superfamily of lncRNAs [3,6–9].

Two seminal findings showed that circRNAs do have important regulatory and developmental functions in animals [8,10]. This triggered a number of studies on circRNAs in basic and applied research, including studies relevant to cardiovascular research. Despite the large number of annotated circRNAs, so far only a handful of these has been approached by functional studies. This is due to technical challenges in detecting circRNAs, as well as problems in genetically manipulating circRNAs without affecting linear RNA expression from their host genes. By numbers, so far, 10 circRNAs have been firmly associated with cardiovascular pathophysiology by experimental evidence in cell culture systems that serve to model aspects of cardiovascular disease. Of these, only 3 circRNAs have been studied *in vivo*, that is in animal CVD disease models or by decisive evidence from genome-wide association studies (GWAS) in human patient cohorts. Another 27 circRNAs

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have been implicated as biomarkers for CVD but have not been functionally investigated. Given that many, if not most, circRNAs have been suggested to affect the expression of linear mRNA from their host genes, many more circRNAs can be expected to be linked to CVD in the future. Therefore, we will review here all the CVD-linked circRNAs, but start out by highlighting milestones in the investigation of general circRNA biogenesis and molecular function. In these introductory passages (chapters 2–4) we distill general concepts with relevance for cardiovascular disease, as then discussed later in the review of studies on specific CVD-linked circRNAs (chapters 5.1–5.3). Throughout, the main focus is the review of studies profiling circRNA expression in cardiovascular tissues, of studies exploring cellular functions of circRNAs in cardiovascular physiology and disease, and of studies translating these data to human pathophysiology. In the review, one major question will be how to prioritize circRNAs during the investigation of cardiovascular disease pathways, in times when expression profiles of thousands of circRNAs are accumulating through RNA high-throughput sequencing efforts in tissues, cells or blood samples. A second important question will be how to interpret genetic polymorphisms associated with CVD risk from GWAS, for example when risk single nucleotide polymorphisms (SNPs) are found to locate in CVD risk genes. Third, we will discuss how the spliceosome mediates circRNA biogenesis, and how alterations in splicing patterns are linked to the onset of cardiovascular disease.

## 2. Classes of circular RNAs

Three major classes of circular RNAs can be distinguished based on the type of covalent linkage underlying circularization, based on the molecular machinery circularizing 5' and 3' ends and based on the typical subcellular localization of the circularized RNA products. The large majority of circular RNAs in eukaryotes are produced by the spliceosome. Thereby, the major U2-containing spliceosome is the major machinery that produces circular RNAs. 3 groups of circular RNAs are produced by the U2-containing spliceosome: a. 3'-5'-linked circRNAs that only contain exonic sequences, b. 3'-5'-linked ElciRNAs, which contain both exons and introns, and c. ciRNAs, which contain only intronic sequences and have been

circularized by a 2'-5' covalent phosphodiester bond [11–13] (Table 1, Fig. 1). For the biogenesis of all these three groups of circular RNAs, in a reaction termed “backsplicing”, the spliceosome uses existing canonical splice signals [3,13] for cutting out and circularizing internal parts of pre-mRNA sequences *in cis*. In the following, we describe the classes of circular RNAs in more detail. The accompanying Box 1 contains a short list of vocabulary that can be used as an entry point into the field of circRNA biology.

### 2.1. Spliceosomal, exon-containing (3'-5')-linked circRNAs

The first and major class of circular RNAs is **3'-5'-linked circRNA** produced by backsplicing, a reaction whereby the spliceosome fuses a downstream splice donor to an upstream splice acceptor [6]. In the simplest case by this reaction a 5'→3'-linked circRNA is formed that consist of a single exon, where the end of the respective exon attacks its own start in the second transesterification reaction. circRNAs can, however also contain multiple exons, whereby intervening introns are spliced out in a subsequent step (Fig. 1). Exon-only circRNAs can exhibit a number of roles, ranging from regulating linear splicing, over binding proteins and modulating their activity, to binding and sequestering microRNAs, termed microRNA sponging. Since circRNAs are sequences of fused exons but do not display a 5'Cap and other upstream features necessary for ribosome entry to linear mRNAs, circRNAs are classically not translated. However, the small possibility exists that protein-coding potential is still exhibited, and this is a known function of a tiny subset of circRNAs. All these functions are described in detail in chapter 4.

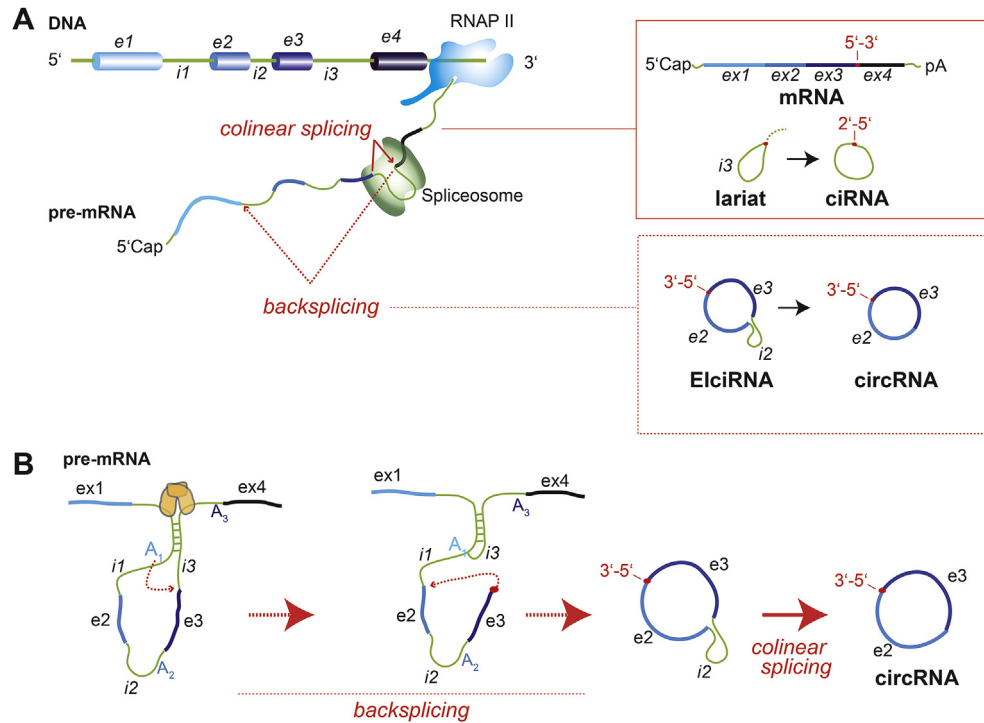
### 2.2. Spliceosomal, exon- and intron-containing (3'-5')-linked ElciRNAs

When one or several intervening intronic sequences are maintained during the biogenesis of a circRNA, these specialized forms are termed circular **exon-intron-containing circRNAs (ElciRNAs)** [14] (Fig. 1). In a typical cell, approximately hundred ElciRNAs exist. They are, thus, a minority compared to exon-only circRNAs. ElciRNAs are mostly nuclear and have been involved in stimulating RNAP

**Table 1**  
**Spliceosome-dependent circular RNAs.** Key features of circular RNAs produced by the spliceosome in eukaryotic cells. n (estimated number of relevant circRNAs expressed from a typical mammalian genome; counted per host gene), n<sup>total</sup> (total number of all predicted circRNA transcript isoforms) as deduced from mapping RNAseq reads during transcriptome profiling of different human organs/tissues/cell types.

Common features	<ul style="list-style-type: none"> <li>• Formed by the canonical cellular spliceosome (U2-containing and operating on canonical splice sites)</li> <li>• Formed from both coding and non-coding genes</li> <li>• Single-stranded RNA</li> <li>• No free termini/no 5' or 3' ends (circular)</li> <li>• No 5' Cap, no poly(A) tail</li> <li>• Stable because resistant to RNase R (3'-5' exoribonuclease)</li> <li>• Mostly non-protein coding (irrespective of origin)</li> <li>• Functioning mostly in control of gene regulation</li> </ul>
Exon-containing (3'-5')-linked circRNAs	<ul style="list-style-type: none"> <li>• Only-exon-containing, 3'→5'-linked, introns have been spliced out</li> <li>• Mostly cytoplasmic; few exceptional cases in the nucleus</li> <li>• n &gt; 10000 (per host gene)</li> <li>• n<sup>total</sup> = 140790 circRNA transcripts/isoforms in humans; listed in: <a href="http://www.circbase.org/cgi-bin/downloads.cgi">http://www.circbase.org/cgi-bin/downloads.cgi</a></li> </ul>
Exon- and intron-containing (3'-5')-linked ElciRNAs	<ul style="list-style-type: none"> <li>• Exon-and-intron-containing 3'→5'-linked circRNAs</li> <li>• Nuclear</li> <li>• n<sup>total</sup> = 111 in human cells (HeLa); listed in Ref. [14]</li> </ul>
Intron-only (2'-5')-linked ciRNA	<ul style="list-style-type: none"> <li>• Stabilized 2'→5'-linked excised introns, not containing exonic sequences</li> <li>• Nuclear</li> <li>• n &gt; 100</li> <li>• n<sup>total</sup> = 103 in human cells (HeLa, hESC) [17]</li> <li>• As a subgroup of ciRNAs, sisRNAs are stable intronic sequences (can be linear or circular, nuclear and cytoplasmic, n &gt; 9000)</li> </ul>

Abbreviations: circbase (curated database for circular RNAs [35]), HeLa (human cervical cancer cell line), hESCs (cultured human embryonic stem cell line).



**Fig. 1. Biogenesis of spliceosome-dependent circular RNAs.**

Overview of the three classes of spliceosome-dependent circular RNAs treated in this review: ciRNAs, ElciRNAs and circRNAs (A). Shown is the arrangement of exons (e1–4) and introns (i1–3) in 5′–3′ order in a gene in the genome, and the transcription into an exon- and intron-containing pre-mRNA, which is either collinearly spliced (top) or backspliced (bottom). Collinear splicing results in a major product (the exon-containing mRNA, where exons are arranged in the same 5′–3′ order as in encoded in the genome), and the byproducts (introns in the form of 2′–5′ branched lariats, of which only one case is shown). Lariats are usually rapidly degraded in the nucleus, but can become processed and parental molecules for ciRNAs. Backsplicing (bottom) results in a major product (3′–5′-linked ElciRNAs and circRNAs), and the byproduct (2′–5′ branched mRNA, not shown). Details of backsplicing are depicted in detail in (B). (B) Backsplicing resulting in 3′–5′-linked circRNA formation: Intramolecular backfolding between inverted intronic repeats in the pre-mRNA, assisted by dimerization of RNA-binding proteins with binding motifs on the pre-mRNA, brings canonical splice junction in such a three-dimensional context, that a downstream end of an exon (red dot) is spliced to the begin of an upstream exon. The two transesterification reactions are shown with small red dotted arrows (indicating the direction of the underlying nucleophilic attack). The result is a 3′–5′-linked ElciRNA that displays an intervening intron. This intron is further processed by conventional splicing to an exon-only 3′–5′-linked circRNA.

Introns (light green), exons (different shades of blue, indicating more 5′ or more 3′ location in a gene or mRNA), RNA-binding proteins serving as circRNA biogenesis regulators like Quaking or Muscleblind (orange), RNA polymerase III holocomplex (RNAP II), Spliceosome (dark green).

II-dependent initiation, likely via the intronic sequences they contain.

### 2.3. Spliceosomal, intron-only (2′–5′)-linked ciRNA

The third class of circular RNAs is **2′–5′-linked circular intronic RNA (ciRNA)**. ciRNAs are products of normal collinear splicing. ciRNAs essentially represent lariats, and are composed exclusively of intronic sequence. In the two transesterification steps of conventional collinear splicing, the adenosine at the intron branchpoint is linked to the 5′ of the excised intron, resulting in a lasso-like circular RNA, the 2′→5′-branched lariat. In contrast to normal lariats, which are rapidly degraded in the nucleus [15,16], ciRNAs do not get degraded [17]. Instead, their signature 3′ single-stranded extensions are nibbled off, and they resist further 2′→5′ debranching because of the presence of a 7 nucleotides (nts) long motif near the 5′ splice site and another 11 nts motif near the branchpoint [17]. As a result, ciRNAs are perfect RNA circle without branches that become stable and exhibit cellular functions (Fig. 1). Two independent experimental approaches basing on high-throughput sequencing have defined two different types of 2′–5′-linked circular intronic RNA: Firstly, several hundred circular intronic RNAs (ciRNAs) were identified by specifically screening for intronic sequences in poly(A)-negative and rRNA-depleted RNA pools of cultured human cell lines [17,18]. CiRNAs were found to be

localized in the nucleus of cells. Three ciRNAs have so far been functionally investigated to some extent, and they play a role in stimulating RNAP II-dependent transcriptional initiation at their host gene loci. Secondly, a family of several thousand stabilized intronic sequence RNAs (sisRNAs) were found as part of the nuclear RNA pool in the *Xenopus tropicalis* oocyte [19] and later also in other organisms and cells. sisRNAs are a more broadly defined class of RNAs compared to ciRNAs, as they contain both linear and circular intronic RNAs. sisRNAs are overall not well understood, their relevance in cardiovascular settings not studied, and they will, thus, not be treated in any detail in this review.

### 2.4. Non-spliceosomal circular RNAs

A small number of circular RNAs are not produced by the spliceosome. This class of circular RNAs is rather mixed and includes specialized cases of circularization that mostly happen in lower eukaryotes, for example, tRNA ligase-mediated circularization of tRNAs introns [20], or circularization by the ribozyme (self-splicing) activity of group I and group II introns encoded in mitochondrial and chloroplast genomes or in rRNA [21–23]. This class also contains circular RNAs that represent genomes of ssRNA viruses, like the hepatitis delta virus in mammals or virusoids in plants [24]. The relevance of the circular nature of all these types of RNAs is not specifically clear, and it can only be hypothesized that

**Box 1**

List of terminologies from circRNA biology used in this review.

Term/Reference	Definition
Splicing [214]	<ul style="list-style-type: none"> <li>Essential step in the maturation of mRNA: Enzymatic process (two consecutive transesterifications) that couples the removal of introns from pre-mRNA with the covalent 5'-3' linkage of exons. Splicing is executed by the spliceosome, a multiprotein-RNA complex that harbors five specialized RNAs (snRNAs U1, U2, U4, U5, U6) whereby RNA serves as the catalytic moiety.</li> </ul>
Splice sites [215]	<ul style="list-style-type: none"> <li>Recognition sites in pre-mRNA serving to define where the spliceosome executes splicing. A surprisingly minimal set of highly conserved features defines splicing (intronic dinucleotides GU as 5' motif/splice donor, and the intronic AG as 3' motif/splice acceptor). Additional nucleotides surrounding these sites enhance recognition, by allowing correct pairing with the snRNAs in the spliceosome. Additional exonic and intronic splicing enhancer or silencer sequences (within a few hundred nucleotides of the splice sites) define the strength of splice sites (their context-dependent usage).</li> </ul>
Splicing factors [216]	<ul style="list-style-type: none"> <li>Trans-acting regulatory RNA-binding proteins that act context-dependently. For example, a splicing enhancer binds motifs in the pre-mRNAs and promotes cross-exon interactions between two splice sites ("exon definition") but also interactions with spliceosomal proteins. Expressed and regulated cell-type specifically. Acting combinatorially.</li> </ul>
Branchpoint [217]	<ul style="list-style-type: none"> <li>Evolutionarily conserved adenine inside each intron, 20–40 nucleotides upstream of the 3' end of an intron. Contained in short pyrimidine-rich motif in an otherwise loosely conserved context. The free 2'OH of this adenine serves as a nucleophile to attack the free guanine at the 5' end of an intron (produced by the activity of the spliceosome) forming an intra-intronic 2–5' linked phosphodiester bond.</li> </ul>
Lariat [218,219]	<ul style="list-style-type: none"> <li>Lasso-like endproduct of splicing representing the spliced-out intron, which is intramolecularly branched via a 2'-5' bond between branchpoint adenosine and the intronic 5'G. Due to the single-stranded overhang and the absence of 5'Cap and polyA structures, the lariat is often rapidly degraded in the nucleus.</li> </ul>
Colinear splicing [220,221]	<ul style="list-style-type: none"> <li>Splice sites are recognized and used in the 5'-3' order in which they are encoded on the pre-mRNA, leading to the strict linkage of the 3' end of an upstream exon to the 5' end of a neighboring downstream exon. Not always is the immediate next neighboring exon is used, because alternative splicing can lead to skipping of exons and fusion to an alternative downstream exon (adhering to a colinear 5'-3' linkage).</li> </ul>
Circular RNAs [3]	<ul style="list-style-type: none"> <li>Single-stranded ring-like ribonucleic acids with continuous phosphodiester bonding in its backbone.</li> </ul>
Backsplicing [56]	<ul style="list-style-type: none"> <li>Linkage of the 3' end of a downstream exon to the 5' begin of an upstream exon (or to its own 5' begin) and thus working in the opposite direction compared to colinear splicing. Occurring often with a reduced frequency compared to colinear splicing. The consequence of backsplicing is the covalent circularization of the exon(s) involved (and consequently excision of exons and of any intervening introns from the pre-mRNA). The byproduct of the reaction is an internally shortened pre-mRNA, which now also contains an unspliced intron with internal 2'-5' branching (thought to become subject to degradation, see Fig. 1 for details).</li> </ul>
circRNAs [3]	<ul style="list-style-type: none"> <li>RNA that was covalently circularized by a 3'-5' phosphodiester linkage through the activity of backsplicing by the spliceosome. Either containing a single exon or containing multiple exons, whereby intervening introns had been splice out by conventional intron splicing after the backsplicing reaction (see Fig. 1).</li> </ul>
5'Cap [222]	<ul style="list-style-type: none"> <li>Here referred to as caps of RNAP II transcripts: Sum of a diverse array of cotranscriptional chemical modifications at the 5' (diphosphate) end of all nascent eukaryotic mRNAs (and, thus, not of internal 5' fragments). Serving to promote stability and resistance against 5'-3' exonucleases, to promote splicing, proper polyadenylation, and nuclear export. Often including a methylated guanine joined by a single 5' –5'-triphosphate linkage on top of the first nucleotide that is then further connected via conventional 5'-3' phosphodiester bonding. Also including several additional methylation events to the first few nucleotides, for example at 2'OH sites.</li> </ul>
3'polyA tail [223]	<ul style="list-style-type: none"> <li>Enzymatically induced cleavage near the 3' end of nascent eukaryotic pre-mRNAs, induced by nearby RNA sequence motif (polyadenylation signal) in the 3' untranslated regions; followed by posttranscriptional polyadenylation at the free 3' end by a specialized RNA polymerase. The polyadenosine 3' end is bound by polyA-binding protein (PABP), which interacts with eIF4G (eukaryotic initiation factor) at the 5'Cap, thereby stabilizing the mRNA in a loop (no covalent linkage). polyA tails influence transcription and translation.</li> </ul>
ElciRNAs [14]	<ul style="list-style-type: none"> <li>RNA that was covalently circularized by a 3'-5' phosphodiester linkage through the activity of backsplicing of the spliceosome. Introns are not spliced out from this circular RNA, resulting in an Exon-and-Intron-containing RNA ring.</li> </ul>
ciRNAs [17]	<ul style="list-style-type: none"> <li>circular RNA that represents an intronic lariat in principle. Produced by conventional colinear splicing activity by the spliceosome, and processed by digestion of the 3' single-stranded RNA overhang, resulting in a 2'-5'-linked perfect ring-shaped RNA.</li> </ul>

(continued)

Term/Reference	Definition
circRNA host gene [3]	<ul style="list-style-type: none"> <li>Gene that is encoded in the eukaryotic genome (protein-coding, or non-coding), which is transcribed to a pre-mRNA from which circRNA(s) derive (at least in specific cells in specific contexts). Experimental evidence for circRNA formation is the detection of the backsplice junction, and/or biochemical evidence for circularity (resistance to RNase R exonucleases, differential running behavior on 2D gels or in Northern Blots).</li> </ul>
Backsplice junction [4]	<ul style="list-style-type: none"> <li>Contiguous RNA sequence that can only be due to the fusion of non-contiguous 3' exonic sequence to an upstream 5' exonic sequence. It is not encoded as a contiguous stretch of DNA in the genome. Bioinformatically, it can, thus, not be mapped without penalties to the reference genome.</li> </ul>
ORF [114]	<ul style="list-style-type: none"> <li>Open reading frame of triplet-nucleotide codons that is decoded on mRNA by tRNAs and the ribosome during protein biosynthesis (translation). Since circRNAs contain exons, they contain ORFs (except potential frameshifts due to the noncontiguous linkage of exons by backsplicing). Yet, in the absence of 5'Cap and polyA tail (and the absence of internal ribosome entry sites in the vast majority of cases) translation cannot initiate.</li> </ul>
5' UTR	<ul style="list-style-type: none"> <li>Transcribed portion of a gene, prior to the first exon, which is not translated into protein in mRNAs. Important for regulating translational initiation using 5'Cap-dependent or –independent pathways. Can also contain regulatory sequences (microRNA binding sites regulating mRNA stability or translation).</li> </ul>
IRES	<ul style="list-style-type: none"> <li>Internal ribosome entry site. Usually, translation is initiated by recruitment of the ribosome and eIF5 (eukaryotic initiation factors) to the 5'Cap, followed by scanning for the Start codon in mRNA (AUG). Internal sequences in the 5' UTR of the mRNA can adopt secondary folds that can recruit the ribosome 5'Cap-independently, which is relevant for circRNAs that lack a 5'Cap.</li> </ul>
RNAP II [224]	<ul style="list-style-type: none"> <li>RNA polymerase II, 12 subunits multiprotein complex performing DNA-dependent RNA polymerization during transcription of mRNAs.</li> </ul>
Preinitiation complex [225]	<ul style="list-style-type: none"> <li>RNA polymerase II multiprotein complex competent to initiate transcription on double-stranded promoter DNA, containing a minimal set of RNAP II, TATA box binding protein, and general transcription factors TFIIB, TFIIF, TFIIE, and TFIIH.</li> </ul>
TFIIH [225]	<ul style="list-style-type: none"> <li>Only general transcription factor with enzymatic activity (helicases and kinase activity); Rate-limiting for transcription initiation by melting the promoter and thereby opening the two DNA strands for RNAP II in the absence of sufficient superhelical tension.</li> </ul>
P-TEFb [226]	<ul style="list-style-type: none"> <li>Positive transcription elongation factor-b (comprised of cyclin T1 and cyclin-dependent kinase9); Necessary for the release of paused Pol II by phosphorylating the carboxy-terminal domain of RNAP II at its Serine 2 residue, as well as negative elongation factors (causing their eviction from RNAP II). P-TEFb activity is required because RNAP II regularly pauses in promoter-proximal regions.</li> </ul>
RNAP II pausing [227]	<ul style="list-style-type: none"> <li>Pausing and elongation from paused states is the normal, regulated and regulative behavior of RNAP II. Control of elongation rate widely affects splicing, transcription termination and genome stability.</li> </ul>
R-loop	<ul style="list-style-type: none"> <li>Three-stranded nucleic acid structure. Arising from separation of coding and noncoding DNA strand in the genome and hybridization of one of these strands to single-stranded complementary RNA. Occurring physiologically with impacts on transcription, chromatin structure, and recombination.</li> </ul>
Pre-rRNA processing [104]	<ul style="list-style-type: none"> <li>Non-spliceosomal enzymatic processing of pre-ribosomal RNA (RNAP I product): involves excision of internal and external spacer elements (ITS/ETS) and the definition of individual rRNA molecules from a contiguous precursor (see Fig. 2).</li> </ul>

circularity promotes stability against nucleases and, as such, could hypothetically be of potential advantage for propagation on an evolutionary scale. Since these circular RNAs have not been studied in cardiovascular context, they will not further be treated in this review.

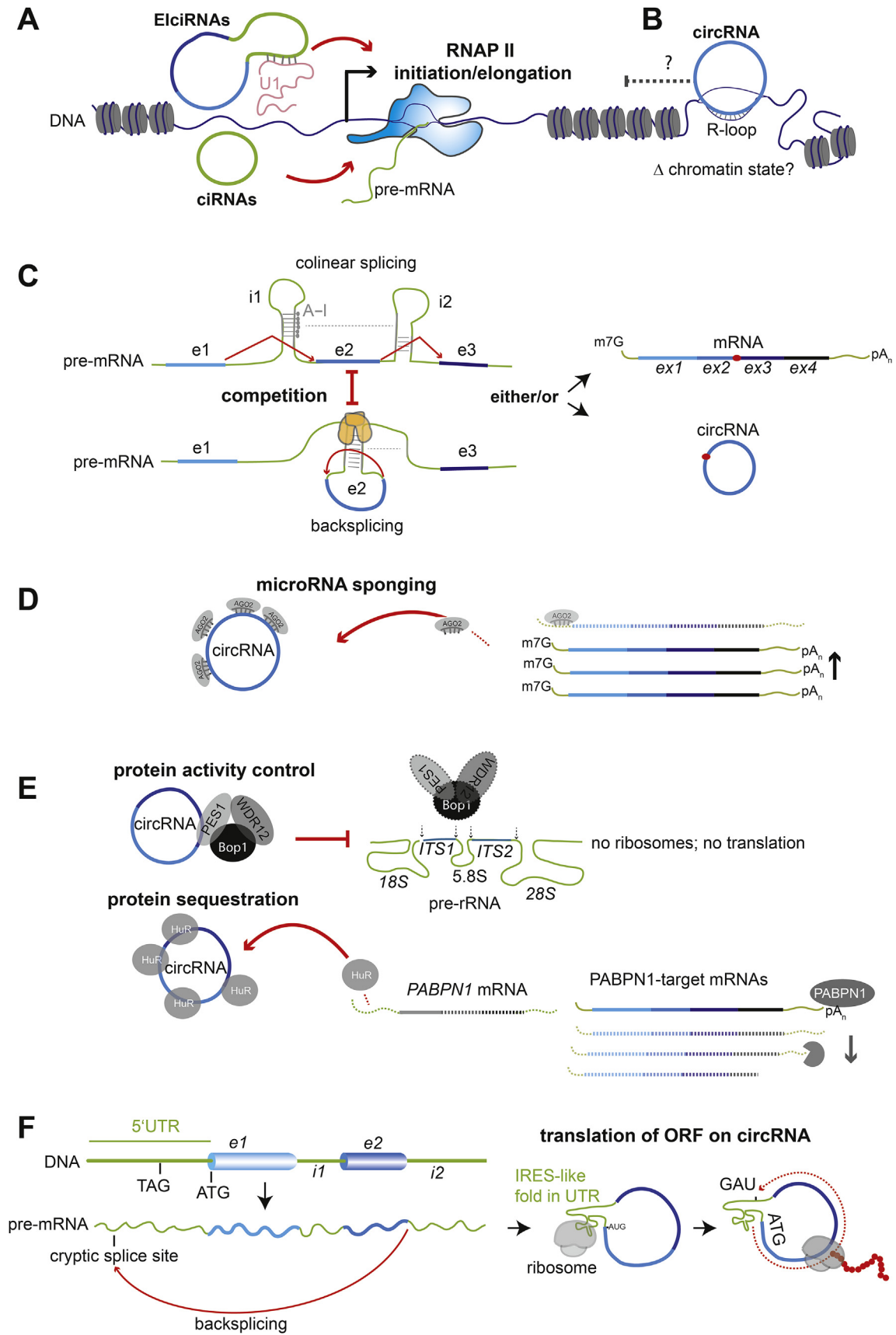
Pertaining to the spliceosomal circRNA, ElciRNAs, and ciRNAs, the formation of these circular RNAs seems to be a regulated process, as different cell types have been shown to exhibit differential circRNA expression profiles [9,11,25,26]. This is then reflected in differing circRNA expression profiles when comparing different tissues and organs [9,26–30]. Moreover, a single multi-exon gene can give rise to a number of different isoforms of circular RNA, depending on which splice donors and acceptors have been accessible in the pre-mRNA and how the biogenesis of a circular RNA proceeded [9,31,32] or depending on internal alternative splicing in circRNA-generating sequences [33]. Together, spliceosomal RNA circularization greatly enhances the complexity of transcriptomes.

## 2.5. Tools and databases to study circular RNAs

Researchers in the circRNA field are beginning to realize that both, the bioinformatics mapping of circRNAs, as well as the experimental validation of circularity and the quantification of circRNAs, are far less trivial than anticipated and prone to variation.

### 2.5.1. Bioinformatic tools and databases

Studies using different circRNA profiling algorithms come to circRNA datasets that differ by up to 40%. Thus, when exploring existing circRNA databases that become ever more specialized (like circBase [35], CircNet [36], starBase [37] or CSCD [38]), or when choosing from existing circRNA detection tools (circRNA\_finder [12], find\_circ [8], CIRCexplorer [32], CIRI [39], DCC [40], KNIFE [11], MapSplice [41], NCLScan [42], PTESFinder [43], Segemehl [44], Uroborus [45]) care has to be taken that no gold standards exist yet in assessing the accuracy of bioinformatics algorithms to map circRNAs.



**Fig. 2. General molecular functions of circular RNAs.**

Proposed functions of circular RNAs in the cell nucleus (A–C) and the cytoplasm (D–F). Functions are not arranged by the prominence where circular RNAs are expected to be most important but instead are ordered by how gene expression proceeds, starting from transcription and ranging over co-/post-transcriptional processing to cytoplasmic regulation and

### 2.5.2. Biochemical circRNA detection and quantification

No gold standard exists, either, for the decision which biochemical approach is taken for RNA preparation during circRNA analysis, or for molecular techniques used to quantify circRNAs. Specifically, no benchmarks for enzymatic procedures have yet been developed that could be followed when enriching circRNAs in RNA preparations. In fact, potential artefacts in the enrichment steps for circRNAs and the depletion steps of linear RNAs in biochemical preparations may account for high rates of false-positive circRNAs (up to 30%). For example, RNase R is employed to deplete non-circular RNAs, but may lead to decay also of some circRNAs [6], and is also not always completely efficient [34]. Excellent recent reviews and comparative studies summarize the current understanding of the underlying problems in biochemical circRNA prediction, detection, and quantification [4,11,46–48]. Consequently, it is advised to use a number of complementary methods instead of a single method, for example for circRNA quantification, or for the experimental validation of circularity of candidate circRNAs (e.g. applying also other enzymes equivalent to RNase R treatment, and quantifying circRNAs not only by RT-PCR, which can cause artefacts due to template switching, but also by Northern blotting or 2D gels [34]).

### 2.5.3. Functional genetic analysis of circRNAs

Not last, care must be taken when making functional arguments based on genetic activation or inactivation of circRNAs, for example by overexpression or knockdown approaches in cells, or by mutation analysis or genomic knockouts *in vivo*. In a commonly used cellular approach, ectopic overexpression of circRNAs is accomplished by transfecting into cells mini-gene constructs on plasmids that carry inverted repeats in the introns that flank the exons to be circularized. Such mini-gene constructs have been experimentally shown to promote artificial spliceosomal exon circularization [34], but have also been found to give rise to RNA concatemers, off-target effects [34], and unphysiologically high levels of circRNAs. The latter is especially problematic when dose-dependent functions like microRNA sponging are implicated as prime effector mechanism by such a study [49]. Finally, an in-depth analysis of circRNA function may ultimately involve genome-engineering of the endogenous host locus, but this can be complicated, as it is not often clear how to avoid affecting linear RNA expression from the host locus [50].

## 3. Molecular mechanism of circRNA biogenesis

Colinear splicing and alternative splicing are regulated processes and under the influence of a number of factors. This includes a range of sequence motifs and contexts, splicing factors, chromatin-dependent processes, and RNAP II-dependent parameters. Similarly, the regulation of circRNA formation is expected to be equally complex, but only part of the determining factors have so far been detected. Conceptually, two different modes of circRNA biogenesis are distinguished: a. cotranscriptional circRNA

formation from within the linear pre-mRNA, and b. post-transcriptional backsplicing from within an already excised exon-containing lariat, which is not connected anymore with the linear pre-mRNA molecule [51,52]. This distinction is relevant because cotranscriptional circRNA biogenesis is able to negatively inflict the fate of the linear mRNA molecule directly *in cis* [53] (Fig. 1B), while posttranscriptional circRNA biogenesis cannot (see Ref. [34] for review). As a consequence, circRNA formation from a gene with disease relevance may impact disease parameters.

### 3.1. RNA sequence motifs promoting RNA circularization

A number of bioinformatics, biochemical and genetic experiments have explored which factors promoted RNA circularization from pre-mRNA. Bioinformatic approaches involve the analysis of the sequence space in an around circularizing exons, biochemical approaches involve the isolation of circular RNA species, their quantification and the identification of circRNA-binding proteins and factors, and genetic experiments involve the use of mutation screens or reverse genetic overexpression or knockdown of circRNA-regulatory candidates or of circRNAs themselves. Especially genetic screens combining mutation analysis with circRNA biogenesis reporter assays, sometimes also in different model systems such as yeast, *Drosophila* or mouse, have been very successfully in identifying circRNA-regulating factors [52,54,55]. From these combinatorial approaches, some higher-order parameters for circRNA biogenesis became apparent, such as a sufficient length of the exon(s), an above-average length of flanking introns, the relative positioning of a circularization event in the gene body, and the absence of secondary RNA structures that might disfavor circularization [3,52,56]. However, also RNA sequence content *per se* appears to be important for circRNA biogenesis. RNA sequence content determines circRNA biogenesis in three different ways:

#### 3.1.1. Splice donor and splice acceptor sequence motifs in RNA

High-throughput sequencing and genetic experiments have determined that the vast majority of circular RNAs in cells were formed by the cellular spliceosome. Three-quarters of all circularization events employ canonical splice donors (GU) and acceptors (AG) and, thus, the same sequence signals that are also used during conventional linear splicing by the U2-containing spliceosome [11,13,53,57]. In the rest of cases, existing cryptic splice sites are used, or the U12-containing minor spliceosome is involved, but these features, as such, do not make the difference to colinear splicing [13,52,58]. Rather, what determines the frequency of RNA circularization is whether upstream splice acceptor sites are still available for backsplicing (and not already used up by colinear splicing). In fact, colinear splicing occurs rapidly, within seconds, after the pre-mRNA is synthesized and exits from the RNAP II complex [59] (Fig. 1A). Splice sites are then used colinearly, one by one, as the pre-mRNA emerges from the exit-channel of the RNAP II and becomes accessible to the spliceosome [53,60]. The spliceosome fuses splice sites with each other, first come first serve. A

finally to translation. (A) EicRNAs and ciRNAs binding to host locus and stimulating RNAP II initiation and/or elongation. (B) circRNA binding to DNA sequence encoding the circRNA-generating exon in the host locus and forming an R-loop at this site. This is proposed to impair RNAP II progression, a function so far not studied as a transcription-regulating process, but rather as a splicing regulating process. (C) Model for competition between colinear splicing (top) and co-transcriptional backsplicing (bottom): competition is due to backfolding between different intronic repeat motifs (grey segments). Selective adenosine deamination to inosine by ADAR enzymes within double-stranded RNA patches in the backfolded regions can promote linear splicing (top). RNA-binding protein like Quaking or Muscleblind (orange) bind and homodimerize, and thereby can promote backsplicing by favoring backfolding of introns flanking the circularization event (bottom). (D) Sequestration of Argonaute 2-bound microRNAs on the circRNA (left) reduces the cellular pool of active microRNAs and, thereby, increases the abundance of mRNAs that would otherwise be degraded by these microRNAs (right). (E) Known examples of circRNA:protein interactions: *circANRIL* binding PES1 protein of the PeBoW complex. This interaction blocks PeBoW activity in ribosomal RNA (rRNA) maturation (Top). The second example (bottom): Sequestration of HuR proteins by *circPABPN1* reduces the free levels of the *PABPN1* mRNA-stabilizing HuR and leads to reduced levels of PABPN1 protein, leading to destabilization of PABPN1-dependent mRNAs. (F) An exceptional case of protein translation from an open reading frame (ORFs) encoded on a circRNA: Sequences in the genes 5'UTR that serve as unconventional internal ribosome entry site (IRES) for translation beginning with the ATG start codon. Translation proceeds until an in-frame stop codon (UAG) located, for example, in the 5' UTR.

head-to-tail backsplice reaction is, thus, disfavored compared to linear splicing [51] and for many genes, the amount of circRNA produced is an order of magnitude smaller than mRNA expression [6,7,9]. Less than 0.1% of genes express more circRNA than linear mRNA [9].

### 3.1.2. Reverse-complementary RNA repeat motifs in introns flanking a circularization event

Backsplicing has been found to be especially favored when the downstream splice site is brought in close 3-dimensional proximity of the upstream splice site because of base-pairing between sequence-complementary inverted repeats in the two introns flanking the circularization event in the pre-mRNA [6,13,32,60–63] (Fig. 1B). A number of different types of repeat motifs have been identified in introns, some of which are oriented in reverse-complementary orientation to each other. Alu repeats are one example. In humans, Alu elements are a major type of repeats associated with circRNA formation and exist in more than 1 million copies in our genomes [60]. Alu elements derived from the 7SL noncoding RNA and are primate-specific retrotransposons that insert in gene-rich genomic regions but are losing their mobility as they accumulate mutations on an evolutionary timescale. As a consequence of their insertion preference, they are prevalent in expressed RNAs, and also in noncoding parts, such that 66% of Alu elements reside in introns [64,65]. It has been suggested that the likelihood and strength of repeat-mediated backfolding of introns contained within pre-mRNA can determine the frequency of backsplicing [32,51,54,63,66]. This applies to both cotranscriptional as well as to posttranscriptional backsplicing. Genome engineering approaches have documented that deleting one such repeat on one side of the circularization event with CRISPR/Cas9 was sufficient to abolish circularization, corroborating the repeat-induced backfolding model of biogenesis [51,66].

### 3.1.3. Binding motifs for RNA-binding proteins and splicing factors in RNA

circRNA formation is suggested to be additionally supported by proteins that bind to introns or intron-exon-junctions and stabilize backfolding. A number of circRNA biogenesis-regulating proteins have been identified in genetic screens using synthetic backsplice reporters. Some of these were known from before to be regulators of normal alternative mRNA splicing. Common to these proteins is their capacity to bind RNA, and some additionally homodimerize to assist RNA backfolding and appropriately position backsplice junctions next to each other (Fig. 1B). Examples are the evolutionarily conserved proteins Quaking (QKI) [29] and Muscleblind (MBNL) [53], which bind to introns flanking a circularization event and promote circRNA biogenesis. These two proteins have, however, also been previously described as regulators of developmentally programmed alternative linear mRNA splicing in mouse and in *Drosophila melanogaster*, respectively [67,68]. Beyond that they also influence translation, decay, and localization of sets of mRNAs, making the selective analysis of circRNA regulation very complex [69,70]. Another circRNA regulator is Fused-in-sarcoma (FUS) [71], a well-known RNA- and DNA-binding protein that pleiotropically regulates also transcription start and transcript site control, mRNA length control and alternative linear mRNA splicing. Similar to QKI and MBNL, FUS was found to bind to the exon-intron junctions that formed the RNA circle, consistent with stabilizing a microenvironment that favored backsplicing. Dozens of other RNA-binding proteins have since been implicated in RNA circularization and stabilization by measuring circRNA abundance during RNAi-based screens in *Drosophila* cells [54] or in human cells in culture [55]. Among the hits, serine-arginine-rich SR proteins and hnRNPs turned up, which are well-known mRNA splicing regulators, but

also factors with still unclear function. It should also be mentioned that not all of the identified circRNA regulator candidates were stimulators, as some also repressed circularization by still unknown mechanisms [54,55]. Important for the aetiology of cardiovascular diseases, some of these circRNA-regulating proteins have been studied *in vivo*, and have been clearly implicated in both development of the cardiovascular system, as well as in pathophysiological cellular changes, yet without any evidence whether these functions were due to regulating linear mRNA splicing, or circRNA biogenesis. This will be discussed in chapter 5.3 below.

Most recent models for circRNA biogenesis base on the observation of an inversely correlated link between the speed and efficiency of transcription-coupled mRNA processing, and the efficiency of circRNA formation [72]. CircRNA biogenesis has been found to be favored when levels of splicing factors and core spliceosomal factors dropped and when linear splicing slowed down. This may have to do with the fact that in such conditions, spliceosomal “cross-exon” interactions [73] are only slowly converted to linear splicing-competent “cross-intron” interactions [73], which would then allow the alternative pathways of circRNA biogenesis to prevail [72].

## 4. Molecular functions of circular RNAs

Similar to lncRNAs, circular RNAs have been proposed to be functional because their biogenesis itself has functional consequences, or because the RNA exerts functions as a molecular entity. In the following chapters 4.1–4.6, we will describe the already known roles of circRNA biogenesis and of circular RNAs themselves (Fig. 2). We will highlight relevant aspects that also play a role in how circRNAs affect cardiovascular pathophysiology.

### 4.1. Circular RNAs and the regulation of transcription

A number of different circular RNAs have been found to regulate the transcription of their cognate host gene, either by affecting initiation or elongation of RNA polymerase II. These will be reviewed in this paragraph. In general terms, how circular RNAs affect transcription is different from how many linear noncoding RNAs control transcription: One of the best understood and prototypical functions of linear lncRNAs is their ability to tether chromatin-regulatory complexes to specific gene loci, and, concurrently, to scaffold bound complexes. Both functions are used in regulating transcription of genes but have so far not been described in circular RNAs. A second paradigm is that a linear lncRNA can interact with multiple proteins at once. Through their modular buildup, lncRNAs can bind, for example, several different transcription-regulating enzymes (see Ref. [74] for review). Conceptually, circular RNAs, at least in published reports, have so far not been found to function as scaffolds of chromatin regulatory protein complexes, either. This may, in part, have to do with the fact that circular RNAs have rather recently been identified.

#### 4.1.1. 3'-5'-linked circRNAs and the regulation of RNAP II?

Exon-only circRNAs have so far not been directly linked to the regulation of transcriptional initiation at gene promoters. Although the cytoplasmic abundance prevails for circRNAs, as experimentally observed in individual cases as well as biochemically by fractionation [3,6,8,10,63,66,75], some mature circRNAs do also exist in the nucleus. Examples are *circ-SEP3* [76], *circ-Amot1* [77], circRNAs from the *CAMSAP1*, *GLIS3*, or *c-01*, *c-87* and *c-88* [71], or circRNA from the *HIPK3* gene, which has cardiovascular relevance [78]. The specific role of *circHIPK3* in CVD will be summarized in detail in chapter 5.2. For some of these nuclear circRNAs, a nuclear function may exist. The only relevant evidence for a nuclear function of a



circRNA comes, however, from a study in *Arabidopsis thaliana*, a model plant. A circRNA was found that stemmed from a transcription factor gene that regulated floral development. The circRNA was shown to bind to its parental DNA, from which it had been transcribed [76]. *In vitro*, it could be documented, that during binding, the investigated circRNA locally opened the DNA helix and hybridized to one DNA strand. At this site, the second DNA strand is locally displaced (Fig. 2B). Such a configuration is called an R-loop (see Ref. [79] for review). The investigated plant circRNA formed an R-loop even with slightly higher efficiency than a linearized RNA with same sequence [76]. Functionally, the circRNA:DNA R-loop was shown to impair transcriptional elongation at this site, and possibly as a consequence stimulated skipping of the exon from which the very circRNA derived [76]. Thus, this circRNA established a feed-forward loop in stimulating its own production by hybridizing to its locus of origin (Fig. 2B). How often circRNAs form R-loops on a genome-wide scale is unknown, but if this mechanism was more far-spread the implications could be very broad and be relevant for every cell type of the cardiovascular system. R-loops will be described in more detail in the following chapter on the role of circRNAs in splicing (4.2).

Also in mammalian systems, an indirect link has been established between circRNAs and RNAP II progression: It has been suggested that the process of circRNA biogenesis (backsplicing) was influenced by the speed of RNAP II transcription: A faster progression of RNAP II was suggested to give circRNA-forming splice sites more chance to engage with each other, decreasing the frequency by which linear splicing could use them up [51,53]. Together, at least in some cases, it may be possible that circRNA biogenesis and RNAP II progression mutually impair each other, even though 3'-5'-linked circRNAs are not known to themselves bind RNAP II or transcription factors or affect their activity. Overall, circRNAs exist in rather low copy numbers, which makes the biochemical pulldown and functional analysis of circular RNA-binding complexes more challenging. By numbers, 90% of circRNAs are present with only 1–10 molecules per cell, which is at 10-fold lower levels than their cognate host gene [7]. Only 2% of circRNAs are present at levels >50% of their cognate linear mRNA [7]. Yet, circRNAs would not need to exist in high copy numbers, if the very process of circRNA biogenesis was the essential determinant in affecting the expression of a circRNA host locus.

Specific classes of different circular RNAs have been linked to RNAP II-dependent transcription more directly, namely ElciRNAs and ciRNAs, as described in the following:

#### 4.1.2. ElciRNAs stimulate transcription initiation at gene promoters

**ElciRNAs** are a separate class of approximately hundred circular RNAs that also stimulate transcription. They have been found to co-immunoprecipitate with RNAP II at their parent locus [14]. The understanding of ElciRNA function is a bit more advanced compared to ciRNAs. ElciRNAs stimulate transcription by interacting with the small nuclear U1 that is otherwise well known for its classical and conserved role in the spliceosome [14] (Fig. 2A). U1 has, however also life on its own at promoters, where it functions independently of the other snRNAs and spliceosomal proteins [80]. There, U1 binds the general transcription factor TFIID to stimulate transcriptional initiation [81], as well as the transcription elongation factor P-TEFb to stimulate elongation of RNAP II [82]. U1 can also determine the directionality of transcription at promoters and can prevent premature polyadenylation and cleavage of linear mRNAs [83]. Which of these functions ElciRNAs associates with is still unknown, but a number of different RNAP II-dependent processes might be under their influence.

ElciRNAs have not yet been specifically studied in cardiovascular context, but are expressed also in cardiovascular tissues, as

determined by RNA-seq analyses, for example of the heart. 5–18% of circular RNAs in hearts do contain introns [84–86], a ratio that fits with the overall initial estimation that up to 20% of circular RNAs expressed from mammalian genomes contain introns [7]. Of those, of course, only a fraction belongs to the class of ElciRNAs/ciRNAs, but this fraction is also predicted to be significantly large (e.g. 30% of all intronic RNAs >200 nts are circular [17]). Also, intron-containing circRNAs have been reported to map to a gene with cardiovascular relevance, such as to *MYH7*, *Slc8a1*, *Ryr2*, or *Camk2d* [84–86]. Yet, any function of such intron-containing RNAs in cardiovascular cell types remains to be established.

#### 4.1.3. ciRNAs stimulate transcription elongation by RNAP II

Around 100 **ciRNAs** were found in cultured cell lines [17,18]. Of these, three representatives were functionally tested in more detail. In biochemical purifications ciRNAs from the *ANKRD52*, *MCM5*, and *SIRT7* genes segregated with the nuclear insoluble fraction, which represents chromatinized DNA and strongly associated molecules. These ciRNAs stimulated the transcriptional elongation of RNAP II on the gene from which they derived. Thereby, the ciRNAs were activating only when expressed from within the locus, and not when provided from plasmids *in trans*. The actual molecular mechanism remains to be dissected [17]. Interestingly, a number of nuclear ciRNA foci were observed by immunostaining. This indicated that ciRNAs regulate also other gene loci, but direct evidence for *trans*-regulation is still missing, as well as which sequence determinants would guide ciRNAs to other loci [17]. Overall ciRNAs are expected not to lead to an all or nothing decision in transcription, but to a certain degree of modulation of transcriptional activity. ciRNAs have not yet been specifically studied in cells of the cardiovascular system, but given their generality of function, similar to ElciRNAs, ciRNAs can be expected to be found and to be functional in hearts, vessels and blood cells. For example, if it holds true *in vivo* that *circSIRT7* regulates linear *SIRT7* mRNA transcription [17], *circSIRT7* may very well be of cardiovascular relevance because linear *SIRT7* is known from before to be important for survival and stress resistance in cardiomyocytes [87], especially also in the infarcted heart [88]. This remain to be determined, and the specific list of ciRNAs and or ElciRNAs in cardiovascular cell types to be derived.

#### 4.2. 3'-5'-linked circRNA:DNA interaction and the regulation of splicing

Cotranscriptional RNA circularization has been found to anti-correlate with the abundance of linear mRNA levels produced from the very circRNA-hosting gene (Fig. 2C). Insight from these experiments constitutes a very generalizable model for circRNAs function that affects all classes of genes, and thus also different traits and disease, including cardiovascular disease. The underlying mechanism, how circRNA competes with linear mRNA expression, is not yet fully understood and will be explained in detail in the following:

A number of independent studies have documented that there is competition between circRNA biogenesis and linear mRNA formation. mRNA formation usually is dominant in this competition, but circRNAs formation can have measurable negative effects on linear mRNA abundance of a given locus [51,53]. In other cases, when assessing this competition in higher temporal resolution in single cells, linear and backsplicing were suggested to be completely mutually exclusive [89]. A first possible explanation for competition between mRNA formation and circRNA formation concerns the accessibility of splice sites as a result of competition between different flanking introns to pair with each other in the pre-mRNA [32] (Fig. 2C). A second explanation follows the concept of “kinetic

coupling” defined earlier in studies of alternative splicing regulation (see Ref. [90] for a recent review): It has been suggested that the rate of backsplicing was influenced by the speed of RNAP II transcription: A faster progression of RNAP II was suggested to give circRNA-forming splice sites more chance to engage with each other, decreasing the frequency by which linear splicing could use them up [51,53]. A third explanation is that, once backsplicing has indeed occurred co-transcriptionally, the linear mRNA may hypothetically be less stable, because carrying an internal 2'-5'-linked covalent branch [52,56]. In fact, in 55% of cases, the linear mRNAs that have likely undergone circRNA formation (because lacking circRNA-generating exons) cannot be detected anymore in cells [6]. In a fourth possible explanation, backsplicing can also change the downstream splicing pattern and render linear host pre-mRNA less stable after producing a circRNA: in one case, knockdown of a circRNA led to the abnormal retention of a downstream intron in the host mRNA, and this intron contained a premature stop codon [17,91]. Whether this observation related to nonsense-mediated mRNA decay has, however, not been decisively explored yet.

Together, though the frequency of circRNA formation is on average an order of magnitude smaller than linear mRNA formation, circRNA biogenesis is thought to be potent enough to compete with concurrent mRNA formation.

#### 4.3. circRNA:RNA interaction and the regulation of mRNA stability

A major topic in many of currently published papers on circRNAs is their role in sequestering microRNAs. The ease to predict potential microRNA seed sites on noncoding RNAs has led to a flurry of papers implicating microRNA sponging as effector mechanism, and this is occurring also in papers on circRNAs with cardiovascular relevance. The function of microRNA sponging is, however, seen more and more controversially and will be discussed in some more detail here (Fig. 2D).

##### 4.3.1. *CDR1as* as bona-fide microRNA sponge

The idea that certain RNA transcripts can serve as decoys or sponges for microRNAs to regulated sequence homologous target mRNAs is not new. It originally emerged from tools of synthetic biology, which used antisense sequences to sequester microRNAs [92]. Later, endogenous microRNA-sponging lncRNAs were found, including those transcribed from noncoding pseudogenes [93,94]. As a consequence of sequestering microRNAs, microRNA-sponging lncRNAs were shown to promote the expression of their cognate protein-coding genes. By coincidence, the first published report on a physiologically relevant circRNA had picked one of the very few circRNAs that do indeed contain multiple binding sites for microRNAs, and which are expressed at substantially high levels, namely the circular *antisense to the cerebellar degeneration-related protein 1 transcript (CDR1as)* [8,10]. *CDR1as* displays 74 microRNA seed regions for the miR-7 microRNA. Since *CDR1as* lacks full sequence complementarity with this microRNA, *CDR1as* degradation is thought to be avoided [8,10]. Indeed, *CDR1as* does form stabilized microRNA:AGO2 endonuclease complexes, as would be expected for a microRNA sponge [10]. That sponging was indeed a biological function for *CDR1as* was concluded from genetic experiments: The depletion of *CDR1as* caused the upregulation of miR-7 targeted mRNAs, and the overexpression of *CDR1as* phenocopied a miR-7 loss of function [8]. This did not only apply in cells *in vitro* but also in situations *in vivo*. A mouse knockout of the *CDR1as* locus has recently been reported [95]: Since the *CDR1as* locus had been thought to produce only a circRNA and no linear RNA, the straight knockout of the exon encompassing the circRNA was used to explore the function of the *CDR1as* circRNA as microRNA sponge *in vivo*. The authors showed that *CDR1as* mutant mice upregulated

miR-7 targeted mRNAs in neurons. Neurons are the cell type most strongly expressing this circRNA in the brain, and the deregulation of excitatory synaptic transmission and abnormal sensorimotoric gating in the *CDR1as* mutant mice could, at least phenotypically and in part, be ascribed to deregulation of miR-7 targets [95]. The picture is likely more complex, because a recent reinvestigation of the *CDR1as* loci in human and mouse revealed that the *CDR1as* circRNA (also known as *ciRS-7*) is not a stand-alone circRNA, but embedded in a previously unannotated linear long noncoding RNA (ASINC/LINC00632), from which it is produced by splicing [50]. In the reported *CDR1as* circRNA mouse knockout the levels of this linear lncRNA were increased. The lncRNA transcripts from the locus were located in the nucleus and thus were unlikely to participate in the cytoplasmic microRNA sponging typical of the circular *ciRS-7*. Together, this suggests that, normally, the circRNA impaired the expression or stability of the cognate linear host lncRNA. Consequently the question is raised whether all neurological phenotypes were solely due to circRNA misregulation [50]. On a more general level, the recent study by Salzman et al. also raises the possibility that the transcriptional start sites of other circRNAs in the genome may still be misannotated, which is a problem in studying how circRNAs crosstalk with transcription factor-based gene regulatory mechanisms. Despite the undisputed evidence for a function of transcripts from the *CDR1as* locus in the central nervous system, *CDR1as* has also been studied in cardiovascular context, and one report suggested that it promoted myocardial infarction size (see in detail in chapter 5.2).

##### 4.3.2. microRNA sponging is not a common function for circRNAs

MicroRNA sponging is, however, not considered a very common function for many circRNAs on a genome-wide scale for several reasons [9]: Few other circRNAs contain microRNA binding sites in numbers >10, and equally critically, circRNAs are expressed mostly at relatively low levels, certainly overall lower than many linear mRNAs and microRNAs [7]. MicroRNA sponging can also not be a specific function of circRNAs, as circularized exons were found to associate with Ago2 protein complexes not more frequently than exons of the same sequence content inside the linear mRNAs [7,26]. Also, only very few circRNAs and microRNAs are present in cells at such stoichiometric levels – relative for example to the number of their seed-targets in mRNAs – that they possibly can participate in a sponge effect [49,96–100]. For a sponge effect to occur, calculations suggest that competing endogenous RNA abundance has to be close to the target mRNA abundance [101]. While a number of microRNAs have been described as important regulators of tissue-specific gene expression networks underlying cell proliferation, cell-cell communication or cell type specification in development and disease [102], such as in cardiovascular disease (see Ref. [103] for a recent review), microRNA sponging is thought to be a rather exceptional mechanism for circRNAs [9,49].

#### 4.4. circRNA:protein interaction

CircRNAs do not only bind other nucleic acids but can also interact with proteins (Fig. 2E). Three different examples for how circRNAs interact with protein complexes are reviewed in the following, one of which (*circANRIL*) has direct functional relevance for cardiovascular disease.

##### 4.4.1. *circANRIL* regulates the PeBoW protein complex

In a first example, *circANRIL* was found to bind to members of the evolutionarily conserved PeBoW complex (*Pes1*, *Bop1*, and *WDR12*-containing). The PeBoW complex is essential in organisms ranging from yeasts to animals and plants. It is required in the nucleolus for pre-60S ribosome maturation and it is thought to

stabilize RNases that excise the internal transcribed spacer 1 (ITS1) from pre-rRNAs [104,105]. *circANRIL* impaired rRNA maturation, which led to impaired cellular translation capacity (Fig. 2E, top panel) [106]. *circANRIL* stems from the long noncoding RNA *ANRIL* [106,107]. *ANRIL* is the acronym for *antisense noncoding RNA in the INK4 locus* because the *ANRIL* locus is located in antisense to the *INK4* tumor suppressor locus on chromosome 9p21. *ANRIL* is considered a central effector of cardiovascular risk, as will be described separately in chapters 5.1 and 5.2 on circRNAs in cardiovascular disease.

#### 4.4.2. *circPABPN1* as decoy for the protein HuR

In a second example, *circPABPN1* was suggested to sequester HuR, a central RNA-binding protein that is known to stabilize specific linear mRNAs (Fig. 2E, bottom panel) [108]. The mRNA encoding *polyadenylate-binding nuclear protein 1 (PABPN1)* was found as one of the most important targets of HuR [108]. Among other functions, PABPN1 protein defines the site of polyadenylation and polyA tail length in a number of mRNAs and augments their stability and thus translation. The notion emerged that *circPABPN1* competed with its parental linear mRNA in binding to HuR, such that the *circPABPN1* served as a decoy for HuR, and impaired *PABPN1* translation and, secondarily, the translation of PABPN1-dependent mRNAs [108]. Together, the activity of RNA binding proteins may be fine-tuned by the relative abundance of circular and linear RNAs emerging from a locus, but a generalization of this theme remains to be determined.

#### 4.4.3. *circRNAs* associate with hundreds of different RNA-binding proteins

Thirdly, genetic screens have identified over 100 RNA binding proteins (RBPs) that have the potential to regulate circRNA biogenesis [55]. Of these, some have been tested for their capacity to physically bind to circRNAs. For example, the RBPs NF90/NF110 (NFAR1/2) represent protein isoforms expressed from the *interleukin enhancer binding factor 3 (ILF3)* gene. These RBPs contain double-strand RNA-binding motifs that were found to interact with backfolded intronic RNA during circRNA biogenesis [55]. Coincidentally, for still unknown molecular reasons, NF90/NF110 also interacted with mature circRNAs, and a quantitative preference was observed in circRNA-binding over binding the cognate linear RNA sequences [55]. The functional implications of circRNAs binding to NF90/NF110 are not so clear. NF90/NF110 proteins are usually required for an efficient host immune active in defending against RNA viruses [109,110]. Correlative evidence suggests that circRNA biogenesis decreases during viral infection at a time when NF90/NF110 is released from circRNAs and starts to bind the invading viral genomic RNA instead [55]. Currently, it is thought that structured RNA motifs in some circRNAs are similar to viral RNA motifs and may, thus, be effective in binding NF90/NF110. This circRNA:protein binding is thought to serve as kind of reservoir and to provide a sufficient amount of ready-and-go NF90/NF110 for fast antiviral surveillance [55]. The causality in this hypothetical model remains to be demonstrated. Lastly and as evident from independent co-immunoprecipitation experiments, ElciRNAs and ciRNAs interact somehow with the initiating or elongating forms of RNAP II in the nucleus [14,17]. Details of the nature of the underlying presumptive circular RNA:protein interactions have not been reported, and these circular RNAs may very well also interact with histones or chromatin proteins or DNA or RNA at the locus (Fig. 2A).

Together, it is likely that many other circRNAs functionally interact with proteins, and reported cases will likely increase as the biochemical purification of circRNA-bound complexes becomes routine.

#### 4.5. Exons encoded on circRNAs are not generally translated into proteins

Linear lncRNAs do not contain open reading frames (ORFs) that are longer than 300 nucleotides (encoding for more than 100 amino acids), which is the central definition of the non-coding character of lncRNAs [111,112]. The vast majority of lncRNAs has been experimentally found not to be translated into proteins [113]. In reality, there is, however, a grey zone in distinguishing non-coding from coding transcripts: In fact, small ORFs are present just by chance in any sufficiently long RNA transcript, including the best characterized long lncRNAs [114], and experimental evidence demonstrates that more and more micropeptides are translated from such small ORFs in regions that have been previously annotated as noncoding.

##### 4.5.1. *circRNAs* do not productively associate with ribosomes

At first glance, the question whether circRNAs are translated or not seems clear: Many circRNAs derive from protein-coding genes and constitute fusions of exonic sequences. Thus, circRNAs do contain open reading frames per definition. The vast majority of endogenous circRNAs is, however, not translated because circRNAs do not contain 5'Caps which could serve as entry points for ribosomes for translation. Several ribosome footprinting (RFP) studies agree on that the vast majority of circRNAs are not associated with active polyribosomes and are thus not translated [6,7,26]. This is likely a consequence of circularization from internal portions of genes and, consequently, the absence of a 5' Cap, of linear ends, and of the Kozak sequence for ribosome entry and translation initiation in linear 5' capped mRNAs (see Ref. [115] for review). Advanced biochemical conditions in RFP experiments have, however, succeeded to demonstrate that some hundred circRNAs could be potentially translated [58], and a few dozens of those had the potential to encode a polypeptide [58] (Fig. 2F). So far, translation of only two circRNA molecules, *circMbl* [58] and *circZNF609*, have been documented with confidence [75]. Paramount for their translation was that RNA circularization led to the inclusion of the endogenous start codon, as well as of specialized 5' untranslated regions (UTRs) that folded into specific secondary RNA structures with a potential to serve as internal ribosome entry site (IRES) [58,75].

For the cardiovascular field, especially the study on the translation of *circZNF609* is of potential relevance because knockdown of *circZNF609* revealed a role of this circRNA in myoblast proliferation *in vitro* [75]. This suggests a role of this circRNA in muscle biology, with implications also for heart function. As explained in chapter 4.6.2 the role of protein derived from *circZNF609* is much less clear.

##### 4.5.2. Exceptional translation of protein fragments from selected circRNAs

Whether circRNA-encoded polypeptides from the *Mbl* or *ZNF609* genes exhibited any function is still unknown. Current experiments have succeeded in knocking down the relevant circRNAs by siRNAs targeting the circRNA-specific backsplice sequence and, thus, avoiding to affect the linear host mRNA [75]. However, the knockdown depleted the circRNA, and, thus, it remained unclear whether the observed proliferative defects were due to a function of the circRNA itself, or due to the circRNA-derived polypeptide [75]. In this specific case, the circRNA-derived 251 amino acid long ZNF609\* protein is a truncation of the native ZNF609, and as such, may theoretically behave as a dominant-negative version of this transcriptional regulator (Fig. 2F). More experiments are needed to test this hypothesis in muscle cells and in pathophysiologically relevant settings.

#### 4.5.3. Micropeptides translated from circRNAs?

Small ORF-encoded peptides have recently been found to be much more common than previously thought. Specifically, this relates to the observation that there are rather many instances where functional protein translation occurs outside of annotated protein-coding open reading frames [116,117]. Translation has been observed to occur also on RNAs that have been thought to be purely noncoding. Micropeptides from small ORFs are well-known to have especially important functions in cardiac physiology: Several of such micropeptides have been found to be translated from linear long noncoding RNAs and to regulate heart function. This includes the sarcolamban family of ORFs which repress the  $\text{Ca}^{2+}$  pump SERCA to terminate muscle contraction [118,119]. Also, another small ORF is important, namely DWORF, which displaces SERCA inhibitors and enhances contraction [120] and shows reduced expression in mouse models of dilated cardiomyopathy and ischemic failing human hearts [120]. At the moment, all known small peptides stem from linear RNAs and not from circRNAs. Also, circRNA formation from the relevant linear lncRNAs has not been specifically addressed, nor whether any of the few circRNAs that are associating with productively translating ribosomes do encode peptides that have the potential to regulate SERCA or function in other aspects of muscle biology. Thus, while the view emerges that translation of small ORFs from circRNAs is not impossible in theory [58,75], current evidence does not yet support the view that many such cases with relevance for cardiovascular physiology exist. More focused analyses are required to address this problem.

## 5. circRNAs in cardiovascular disease

So far, only a single circRNA knockout has been described in an animal model system, and this knockout affected neurological function [95]. Evidence from loss-of-function or transgenic overexpression of specific circRNAs in cardiovascular development *in vivo* does not yet exist. Those circRNAs that will be described in this chapter have been associated with cardiovascular disease by three conceptually different approaches:

First, circRNA expression profiling has been performed in cardiovascular tissue as well as in blood. Some of these studies compared circRNAs in control and diseased cardiovascular states. For biomarker studies, RNA preparations of whole blood, or of specific blood cells were analyzed. Such studies ended in speculations whether a specific circRNA had the potential for biomarker development, or could serve as candidate effector of cardiovascular pathophysiology.

Secondly, some studies have functionally implicated circRNAs as effectors of cardiovascular disease. None of these circRNAs have been selected based on measuring differential abundance alone. Evidence was collected from multiple pieces of evidence. For example, such studies analyzed circRNAs together with host mRNA expression investigated the competition between circRNAs and parental RNA, or they explored GWAS datasets with a focus on circRNAs that stemmed from disease-linked loci (e.g. *circANRIL*). Other cases followed the rationale to focus on a highly abundant or enriched circRNAs that carried seed regions for well-known microRNAs (e.g. *HRCR*).

Thirdly, in yet another type of approach, some studies focused on specific RNA-binding proteins that regulated splicing of mRNAs and that had, separately, been linked to cardiovascular pathophysiology in mouse knockout or transgenic models. Since some of these RNA-binding proteins and splicing regulators (e.g. QKI, MBNL, RBM20) have recently also been found in genetic screens to serve as regulators of circRNA biogenesis, the question arose whether cardiovascular functions were due to circRNA regulation. We will review reports how these three factors related to cardiovascular

pathophysiology, although current evidence supports the view that these roles are due to regulation of linear mRNA splicing.

Considering these three major routes, we will summarize the current state of knowledge about circRNAs in cardiovascular pathophysiology in the three chapters 5.1–5.3: a. evidence determined by differential circRNA expression profiling in cardiovascular tissue and blood, b. evidence determined by functional tests of candidate disease effectors, and c. evidence determined by deducing functionality from studies of circRNA-regulating proteins. Evidence from circRNA expression profiling studies will be summarized in Table 2, and *in vivo*, functional evidence from candidate approaches in Table 3. A summary of the suggested cellular functions of circRNAs and circRNA regulators, as determined from experiments with cells cultured *in vitro*, is presented in Fig. 3. To enable a quick overview of key relevant papers, we have highlighted the major publications in Box 2.

### 5.1. circRNAs associated with cardiovascular disease by RNA expression profiling

#### 5.1.1. CircRNA expression profiling in cardiovascular tissues

A number of studies have reported on profiling heart-specific sets of circRNAs, either in physiological or pathological conditions. For example, myocardial circRNAs were delineated by profiling hearts samples from different species: samples from healthy mouse and rat were compared to mouse hearts after pressure overload and to human samples, including RNA from hearts of heart failure patients [84]. Likewise, both human and mouse heart-specific circRNA sets were determined in a recent comprehensive approach that aimed to delineate cardiac circRNAs expressed in healthy or in pathological conditions [85]. The pathologies included ischaemic and non-ischaemic dilated cardiomyopathy and hypertrophic cardiomyopathy in humans, and material was derived from left ventricle myocardium in these cases. In mouse, RNA was analyzed from cardiomyocytes that were isolated from hearts after pressure-overload-induced hypertrophy as a consequence of transverse-aortic constriction (TAC) [85]. Independent papers focused on cataloging heart-specific circRNAs, from fetal to adult stages: For example, circRNAs profiles were established in young and maturing adult mouse hearts [121] or mapped based on existing publically available datasets [122]. Yet another circRNA profile was established by profiling expression in mouse ventricular tissue after permanent ligation of the left anterior descending coronary artery [123].

In another set of approach, circRNAs profiles were determined while profiling differentiation of human embryonic stem cell (hESC) to cardiomyocytes over 3 weeks in culture conditions [85]. In this study, among all the identified circRNAs, 1664 were heart-specific as compared to expression in other organs. Some of these stemmed from genes that had been independently functionally studied in the heart (*Titin*, *RYR2* gene, *HIPK3*). A circRNA from the *SLC8A1* gene, which encodes a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, was the most abundant circRNA in human hearts and the second most abundant in mouse cardiomyocytes. Conceptually similar experiments led to the definition of circRNA species expressed when induced pluripotent stem cell (iPSC)-derived cardiomyocytes matured upon beta-adrenergic stimulation in culture [86]. The latter condition is used to reflect acute sympathetic stimulation of heart contractility or when chronic, hypertrophic proliferation and changes in apoptosis (see Ref. [124] for review). Top candidates from this diverse range of circRNA profiling studies may serve as entry points for future tests whether these circRNAs influenced cardiovascular diseases also in functional terms.

In another very recent approach, a team of researchers sequenced multiple human tissues of a single person in parallel, in

**Table 2**  
circRNA as biomarkers for cardiovascular disease.

Name	Disease	Regulation (in Disease)	Function	human GWAS/Mouse models	Role <i>in vivo</i>	Ref.
<i>circNPPA</i> <i>circCORIN</i> <i>circRYR2</i> <i>circMYH6</i> <i>circQKI</i> <i>circSLC8A1</i> <i>circTitin</i> (h.s.)	Heart defects (multiple)	n.a. (expressed in diseased tissue)	n.a.	Right atrium, vena cava, heart tissues	n.a. (but produced from disease-linked mRNAs): <i>NPPA</i> : heart failure [228] <i>CORIN</i> : heart failure [229] <i>RYR2</i> : atrial fibrillation [230] <i>MYH6</i> : hypertrophic cardiomyopathies [231] <i>QKI</i> : CAD [193] <i>SLC8A1/NCX</i> : heart failure and arrhythmia, hypertension [232] <i>TITIN</i> : DCM [233]	[234] [85] [84] [195]
<i>circANRIL</i> (h.s.)	CAD	Down	Protective	Blood T-cells, PBMCs, carotid endarterectomy tissue	n.a. (Human GWAS: Associated with CAD risk SNPs (chr9p21); Generally antiproliferative, proapoptotic; Anticorrelation with linear <i>ANRIL</i> isoforms)	[107] [106] [148,149,235]
<i>hsa_circ_0124644</i> <i>hsa_circ_0082081</i> <i>hsa_circ_0113854</i> <i>hsa_circ_0098964</i> <i>hsa_circRNA5974-1</i> (h.s.)	CAD	Up	n.a.	PBMCs	n.a.	[137]
<i>hsa_circ_0089378</i> <i>hsa_circ_0083357</i> <i>hsa_circ_0082824</i> <i>hsa_circ_0068942</i> <i>hsa_circ_0057576</i> <i>hsa_circ_0054537</i> <i>hsa_circ_0051172</i> <i>hsa_circ_0032970</i> <i>hsa_circ_0006323</i> (h.s.)	CAD	Up	n.a.	Blood plasma	n.a.	[138]
<i>circHIPK3</i> orthologs: <i>hsa_circ_0000284</i> <i>mmu_circ_0001052</i> (h.s., m.m.) <sup>#</sup>	Diabetic retinopathy (TIID)	Up	n.a.	Blood plasma, aqueous humor of eyes	n.a.	[139]
<i>Hsa_circ_0054633</i> (h.s.) <sup>#</sup>	Prediabetic state (TIID)	Up	n.a.	Peripheral whole blood	n.a.	[140]
<i>hsa-circ-0005870</i> (h.s.) <sup>#</sup>	Hypertension	Down	n.a.	Blood plasma	n.a.	[141]
<i>MICRA</i> (h.s.) <sup>#</sup>	MI: LVD	Down	n.a.	Peripheral whole blood	n.a. (predictor for LVD)	[142]
<i>circR-284:mir-221 ratio</i> (h.s.)	Stroke (acute phase)	Up	n.a.	Blood serum	n.a. (circRNA Up/microRNA Down)	[144]

<sup>#</sup> (marking circRNAs associated with CAD risk factors), DCM (dilated cardiomyopathy), MI (myocardial infarction), LVD (Left ventricular dysfunction), LAD (permanent ligation of the left anterior descending), PBMCs (blood mononuclear cells), ECs (vascular endothelial cells), h.s. (*homo sapiens*), m.m. (*mus musculus*).

**Table 3**  
**circRNAs and circRNA regulators in cardiovascular disease in vivo.** List of circRNAs implicated as regulators of cardiovascular disease entities by *in vivo* evidence. Levels of expression and proposed functions *in vivo* are indicated. Asterisks (\*) mark RNA-binding proteins that have been functionally studied and linked to CVD, and which have independently been found to regulate also circRNA biogenesis - It is not known whether their role as circRNA regulators has any impact on their role in CVD.

Name (Species)	Disease	Regulation (in Disease)	Function	Mouse Models	Role <i>in vivo</i>	Ref.
<i>HRCR</i> (m.m.)	Heart injury	Down	Protective	Cardiomyocytes?	ISO-induced heart injury mouse model: vein-injected <i>HRCR</i> -expressing construct rescued from cardiac hypertrophy	[170]
<i>MFCR</i> (m.m.)	Heart injury	Up	Deleterious	Cardiomyocytes?	I/R-induced heart injury mouse model: intracoronary injection of <i>MFCR</i> siRNA rescued from heart dysfunction	[171]
<i>Cdr1as</i> (m.m.)	MI	Up	Deleterious	Heart	LAD ligation-induced MI in mouse: intracardially injected <i>CDR1as</i> -expressing construct prior to MI increased infarction size	[143]
MBNL* (h.s. m.m. d.r.)	DM	Down	Protective	Cardiac muscle	<ul style="list-style-type: none"> <li>• Mouse double KO (<i>Mbnl1</i><sup>-/-</sup>; <i>Mbnl2</i><sup>+/-</sup>): DM-like phenotypes (atrial dilatation left ventricular hypertrophy, arrhythmias)</li> <li>• Zebrafish <i>Mbnl2</i> mutant: DM-like heart defects</li> <li>• Expression correlation in human: CUG repeat-expressing RNA inhibits MBNL</li> </ul>	[236] [237] [238]
MBNL* (h.s., m.m.)	MI	Down	Protective	Cardiac myofibroblasts?	<ul style="list-style-type: none"> <li>• Left coronary artery ligation-induced MI mouse model/<i>Mbnl1</i> KO: Mbnl was required for preserving ventricular wall integrity after MI</li> <li>• Fibroblast-specific human <i>MBNL1</i> expression caused heart fibrosis</li> </ul>	[180]
QKI* (h.s. m.m.)	CAD	Up	Deleterious	VSMCs and macrophages?	<ul style="list-style-type: none"> <li>• Vascular femoral-cuff-injury mouse model: hypomorphic <i>Qki</i> mutant showed reduced neointima and foam cell formation</li> <li>• Expression correlation in human and mouse vessel intima</li> </ul>	[190] [193]
QKI* (h.s. m.m. d.r.)	MI	Down	Protective	Cardiomyocytes?	<ul style="list-style-type: none"> <li>• I/R-induced MI in leptin-mutant diabetic mouse model: intramyocardial injected <i>QKI</i> resolved heart injury</li> <li>• Loss-of-function in zebrafish: <i>Qki</i> promoted myofibril formation</li> <li>• Human GWAS: SNPs near <i>QKI</i> associated with MI</li> </ul>	[191,192] [187]
RBM20* (h.s. m.m. d.r., r.n.)	DCM	Down	Protective	Heart	<ul style="list-style-type: none"> <li>• Mutation in rat: DCM-like heart phenotype and altered <i>Titin</i> splicing</li> <li>• Mouse KO: DCM-like heart phenotype and altered <i>circ-Titin</i> isoforms</li> <li>• Mutations human: associating with familial DCM</li> </ul>	[239] [194] [195]

Abbreviations: CAD (coronary artery disease), ISO (isoproterenol), I/R (ischemia/reperfusion), T-cell (T-lymphocyte), PBMC (peripheral blood mononuclear cell), VSMC (vascular smooth muscle cell), GWAS (genome-wide association study), MI (myocardial infarction), LAD (left anterior descending artery), DCM (dilated cardiomyopathy), ER (endoplasmic reticulum), CM (congenital myopathy), DM (myotonic dystrophy), KO (knockout), h.s. (*homo sapiens*), m.m. (*mus musculus*), d.r. (*danio rerio*), r.n. (*rattus norvegicus*).

order to focus on potentially disease-relevant circRNAs [125]. Among the identified candidates, those tissue-specific circRNAs were identified, for example, that mapped to genes with known physiological roles, as well as to genes where mutations had previously been associated with tissue-specific diseases. Related to

cardiovascular disease, an atrial circRNA was identified in the *NPPA* gene, which encodes the natriuretic peptide and had been associated with diabetes. Other circRNAs identified were from *CORIN*, which is a peptidase that activates NPPA. With a similar rationale, circRNAs were highlighted in the ryanodine receptor gene *RYR2*,

A circRNAs		B RNA-binding proteins	
Blood monocytes			proadhesive; promigrative, pro-differentiation (PBMC → MΦ) po-inflammatory/lipid uptake [193]
Macrophages	<i>circANRIL</i> proapoptotic [106]	HuR	production of angiogenic factors [208, 209]
Endothelial cells	<i>hsa_circ_0003575</i> proapoptotic, anti-angiogenic antimigrative under oxLDL [138]	QKI	pro-differentiation (iPSC → EC) proangiogenic (matrigel) [202]
	<i>hsa_circHIPK3</i> prosurvival, pro-proliferative, promigrative, tube formation [139]	HuR	proinflammatory; proadhesive [205] proangiogenic elastolysis [206] stabilization of VEGF [207, 208]
	<i>cZNF292</i> proproliferative, proangiogenic sprouting [166]		
	<i>hsa_circ_0010729</i> proapoptotic under hypoxia [167]		
VSMCs	<i>circANRIL</i> proapoptotic and antiproliferative [106]	QKI	dedifferentiation (anticontractile; proproliferative, promigrative) [190]
	<i>hsa_circWDR77</i> proproliferative, promigrative [168]		proproliferative [203]
	<i>hsa_circ_000595</i> proapoptotic upon HIF-1 activation [169]	HuR	promigrative [204] VSMC-mediated vasoconstriction [210]
Cardiomyocytes	<i>mm_CDR1as</i> proapoptotic [143]	QKI	antiapoptotic in injured CMs [191, 200]
	<i>mm_HRCR</i> antihypertrophic (antiapoptotic?) [170]	RBM20	Titin AS → increased sarcomer compliance/elasticity [194]
	<i>mm_MFACR</i> mitochondrial fission, proapoptotic [171]	MBNL	pro-differentiative [197] contraction-relaxation [198]
Cardiac fibroblasts		MBNL	fibroblast/EC → myofibroblast transformation (injury healing) [180]

**Fig. 3. Cellular roles of circRNAs in cardiovascular cell types *in vitro*.**

Blood cells and cells of the cardiovascular system that are known to contribute to cardiovascular diseases are depicted on the left. Evidence for cellular roles of (A) circRNAs and of (B) RNA-binding proteins that have recently also been identified as circRNA biogenesis regulators. Evidence stems from *in vitro* experiments in cultured cells. Note that it is unknown whether the cardiovascular roles of the indicated RNA-binding proteins are related in any way to their function as circRNA biogenesis regulators.

which is linked to atrial fibrillation, in myosin heavy chain *MYH6* which associates with hypertrophic cardiomyopathy, and in the solute carrier *SLC8A1* which controls  $Ca^{2+}$  levels and associates with cardiomyocyte dysfunction, heartbeat and regulation of hypertension [125] (Table 2). Follow-up studies on these circRNAs will reveal whether these circRNAs may be biomarkers or even effectors of cardiovascular disease endpoints.

#### 5.1.2. Expression profiling establishes cell-free circRNAs as potential CVD risk biomarkers in the blood

Apart from tissue-centered expression profiling, the minimal-invasive molecular analysis of peripheral blood is gaining diagnostic importance. In the field of cardiometabolic diseases, classically, protein serum markers like cardiac troponins T/I and creatine kinases (CK, CK-MB) are profiled in blood samples. More recently, also microRNA profiles in blood have been shown to be potentially useful. For example, *miR-1*, *miR-133*, *miR-208*, and *miR-499*, as well as circulating lncRNAs, such as *LIPCAR*, *MYHEART* or *UCA1*, can be informative for monitoring myocardial infarction and heart failure, and a number of other small and long linear noncoding RNAs are emerging as possible biomarkers also of coronary artery disease (see Ref. [126] for review). Studies are currently performed that assess whether also cell-free circRNAs may serve as meaningful molecular targets in the analysis of blood. Exploratory studies have documented that circRNAs are not an exception from all other nucleic acids, and can be found in the cell-free transcriptome of biofluids like blood [74,152–154] or saliva [127]. Thereby, circRNAs are expected to be particularly interesting during blood profiling because of their increased stability against cellular exonucleases compared to nucleic acids with linear ends [6,35,125,128–133]. In blood, a

major degradation pathway is the action of RNase A-like endonucleases and RNase A also degrades circular RNAs very efficiently [155]. Mechanisms exist that protect linear RNAs and circRNAs in blood. For example, RNAs (and other cellular components) are encapsulated in phospholipid-membrane-bound vesicles. These can be exosomes or the slightly larger microvesicles, or other fragments of cells like ER fragments [73,74,153,154,157,158]. RNAs may also be protected by coating with diverse RNA binding proteins, high-density and low-density lipoproteins or Argonaute proteins [134–136], which abolishes recognition by RNase A. Together, circRNA degradation routes are still an open field, but circRNAs stability, whatever the underlying mechanism, may indeed turn out to be an advantage when screening for disease-initiating that occurred in distant tissues or at some time point in the past. Subsequent studies have probed whether circRNAs can serve as biomarkers for cardiovascular disease. For example, specific *circANRIL* isoforms in whole blood-derived T-cells or in blood mononuclear cells (PBMCs) were found to anti-correlate with atherosclerosis risk genotypes and disease phenotype severity [106,107]. Conversely, levels of linear noncoding *ANRIL* RNA correlated with atherosclerosis (Table 2). This suggested that measuring both circular and linear *ANRIL* could be a robust indicator of atherosclerosis. *ANRIL* may also be an example, where one could think of gaining robust information by combining information from RNA and DNA sequencing of blood cells since specific predisposing atherosclerosis risk SNPs in the *ANRIL* locus have been suggested to affect the splicing pattern of *ANRIL* pre-mRNA and the decision between linear and circular RNA formation [107]. This is to our knowledge the only known case where such advanced insight is available and GWAS data have been firmly linked to circRNA biogenesis.

**Box 2**

## Reading highlights

**Historical detection of circRNAs**

- Cell-type specific features of circular RNA expression [9]. Building on their seminal paper that established the field of circRNA biology in 2012, Salzman and colleagues report on the genome-wide scale of circRNA expression by giving quantitative answers to numbers, relative abundance, cell-type specific expression, dynamic expression in development, circRNA isoforms and evolutionary conservation. First statistical methods for circRNA detection are presented which influenced the field.
- **Detecting and characterizing circular RNAs** [56]. Insightful study of the general properties of circRNAs on a genomic level. Inspiring models for how circRNAs can originate both, cotranscriptionally by backsplicing, and posttranscriptionally from backsplicing inside lariats that contain skipped exons.
- **Early history of circular RNAs, children of splicing** [240]. Comprehensive overview how exon circularization was detected early on in the analysis of splicing, how circRNAs were long overlooked as singular peculiar cases in few genes, and how recent bioinformatics approaches using RNA sequencing developed the notion that circRNA expression is a phenomenon with genome-wide relevance.

**Benchmarking circRNA detection and quantification**

- **Detecting circular RNAs: bioinformatic and experimental challenges** [4]. Comprehensive and insightful overview of the wide range of possible sources of error, bias and artefacts in the bioinformatics analysis of circRNA expression by RNAseq experiments, as well as in the experimental validation of RNA circularity and in the wet-lab quantification methods for circRNAs.

**CircRNA biogenesis**

- **Complementary sequence-mediated exon circularization** [32]. Formalization of the concept that circRNAs circularize because of intramolecular backfolding of reverse-complementary inverted repeats, such as of the Alu repeats that exist in >1 million copies in the human genome. This is a competitive process between different available inverted repeats, with implications for alternative circRNA formation, and representing an avenue for bioinformatics detection of novel circRNAs.
- **Combinatorial control of *Drosophila* circular RNA expression by intronic repeats, hnRNPs, and SR proteins** [54]. Genetic screen revealing that circRNA biogenesis is not only driven by RNA sequence determinants in flanking introns, but by a number of different proteins, including different hnRNPs and SR splicing factors. One can, thus, expect that circRNA biogenesis is governed by proteins serving as enhancers and silencers, and that circRNA biogenesis may use many of the linear splicing regulators in ways that are not yet fully understood.

**Molecular functions of circular RNAs**

- circRNA biogenesis competes with pre-mRNA splicing [53]. Cotranscriptional circRNA biogenesis is able to negatively impact the efficiency/frequency of coinciding linear mRNA formation and/or influence linear alternative splicing from the host gene.
- Circular RNAs are a large class of animal RNAs with regulatory potency [8] and Natural RNA circles function as efficient microRNA sponges [10]. Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function [95] and ciRS-7 exonic sequence is embedded in a long non-coding RNA locus [50]. Suite of papers on the *in vivo* analysis of a circRNA, representative of high quality research and controls necessary for studying circRNAs: *CDR1as* is a circRNA that serves as a microRNA sponge. First circRNA to be studied *in vivo*. First circRNA to be knocked out *in vivo* in mice. Reinvestigation of the host locus revealed that the circRNA knockout had unintentional effects on expression of unannotated linear long noncoding RNAs from the same locus.
- **Endogenous microRNA sponges: evidence and controversy** [49] and **Cell-type specific features of circular RNA expression**. [9] microRNA sponging is not expected to be a common function of circRNAs.
- **Circ-ZNF609 Is a Circular RNA that Can Be Translated** [241]. CircRNAs are generally not serving as messages for protein translation, but in a small minority of cases can be translated to proteins, or at least to protein fragments, therefore.

**CircRNA expression profiling in cardiovascular tissue**

- **Characterization of circular RNAs in human, mouse and rat hearts** [84]. Resource paper for circRNAs as potential future biomarkers in heart tissue analysis. The study profiled by RNAseq the expression of circRNAs specific to hearts in humans, mouse, and rat, in normal and myocardial infarction conditions.

**CircRNAs as biomarkers in the blood**

- **A map of human circular RNAs in clinically relevant tissues** [125]. Useful resource paper for circRNAs as future disease biomarkers in blood. This study addresses inter-individual variability in tissue-specific circRNA expression, by profiling 20 disease-relevant tissues from a single human individual, including CVD-relevant tissues and cells, and mapped circRNAs with tissue-specific enrichment to known disease-associated host genes.

**CircRNAs as effectors of cardiovascular disease**

- **A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223** [170]. *In vivo* analysis of a circRNA in the context of heart failure in the mouse. Systemic overexpression of the circRNA *HRCR* reduced hypertrophy in failing rodent hearts.
- **Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk** [107]. First demonstration of a circular RNA expressed from the human *ANRIL* locus. Careful annotation of *circANRIL* isoforms and implication of CVD risk SNPs in modulating splice site strength in *ANRIL*, with potential influence on *circANRIL* formation.
- **Circular non-coding RNA *ANRIL* modulates ribosomal RNA maturation and atherosclerosis in humans** [106]. Functional study of a circular RNA (*circANRIL*) stemming from the strongest known human cardiovascular risk locus (9p21): While the expression of the linear host RNA from 9p21 (the noncoding RNA *ANRIL*) associates with atherosclerosis (proatherogenic), the expression of the *circANRIL* RNA anticorrelates with disease (protective). *circANRIL* binds and inhibits protein complex that mediates ribosomal RNA processing, and thereby inhibits ribosome maturation and translation, and consequently, curbs the pathological cellular overproliferation in nascent atherosclerotic plaques.



In another case, circRNA profiling in PBMCs from venous blood has delivered several candidate circRNAs, like *circ\_0124644*, *circ\_0082081*, *circ\_0113854*, *circ\_0098964* and *circRNA5974-1*, which may also be useful as biomarkers of coronary artery disease (CAD) [137]. Dozens of other circRNAs were found in another study to be differentially expressed in plasma samples from (CAD) patients and control individuals. Based on existing microRNA profiling data, and using predictions of putative sponge effects to find anticorrelating circRNA:miRNA pairs, modest upregulation of *hsa\_circ\_0089378*, *hsa\_circ\_0083357*, *hsa\_circ\_0082824*, *hsa\_circ\_0068942*, *hsa\_circ\_0057576*, *hsa\_circ\_0054537*, *hsa\_circ\_0051172*, *hsa\_circ\_0032970*, and *hsa\_circ\_0006323* was suggested to be indicative of CAD [138] (Table 2).

A set of separate studies investigated whether circRNAs could serve as biomarkers for cardiometabolic risk factors, such as dyslipidemia, diabetes or hypertension (Table 2): No circRNAs have yet been found to indicate dyslipidemia in blood. With respect to CAD risk factors, *circHIPK3* has been shown to be upregulated in diseased retinal vascular tissue in diabetic patients. Compared to controls, *circHIPK3* is more abundant also in blood plasma of patients as well as in the aqueous humor (fluid) of the eye [139]. This finding also shows that circRNAs will likely be found also in other biofluids. *Hsa\_circ\_0054633* was identified in a separate study as a potential biomarker in whole blood for the onset of the pre-diabetic phase in type II diabetes [140]. Another example of a circRNA for a CAD risk factor is *hsa-circ-0005870*, which was found to be less abundant in blood plasma of individuals with hypertension [141] (Table 2).

Relevant to myocardial infarction, *myocardial infarction associated circular RNA (MICRA)* in preparations of peripheral blood has been proposed to be useful as a predictor of left ventricular dysfunction [142]. Also, the well-described *Cdr1as* circRNA was shown to be upregulated in whole blood from acute myocardial infarction patients [143] (Table 2).

Finally, *circR-284* was recently shown to be elevated to some extent in blood serum of patients with CAD and suffering from a recent cerebrovascular stroke, as compared to CAD patients having not yet experienced plaque rupture leading to stroke [144].

Summarizing, a few dozen human circRNAs have been suggested to potentially serve as blood biomarkers for state or stage of cardiovascular diseases. Since specific cells of the blood mechanistically contribute to CAD onset and progression, it is not excluded that some of the reported circRNAs in this list may also have functional pathophysiological relevance. For example, specific monocyte and macrophage subtypes, diverse T-lymphocyte subtypes, and diverse antigen-presenting cell types shape, indirectly or in the lesion itself, when and how atherosclerotic plaques form and develop (see Ref. [145] for review). On the other hand, a number of circRNAs have been shown to be differentially regulated in tissues of mouse models of myocardial fibrosis, cardiac hypertrophy, and myocardial infarction or in cultured models of vascular cell types. It is, however, currently unclear whether circRNAs orthologous to these mouse candidates are also differentially abundant in blood samples or in human patients. Also, more work is needed to independently consolidate the initial biomarker candidates and to rigorously assess biomarker performance in larger, independent, and appropriately control-matched cohorts. This relates not only to the normal range of circRNA biomarker levels in healthy individuals and the quantification of false-positive- and false-negative classification rates (see Ref. [146] for review) but also to benchmarking biochemical and bioinformatics approaches used to sequence and identify circRNAs [4].

## 5.2. Candidate approaches to delineate circRNAs as potential effectors of cardiovascular disease

Only a few circRNAs have been functionally studied so far. Therefore the number of circRNAs that serve as candidate effectors of cardiovascular disease is rather small. Relevant studies concurrently analyzed circRNA regulation during cardiovascular injury in rodent models *in vivo* mimicked pathological processes in physiological relevant cell types in culture, and eventually correlated circRNA expression with known disease risk genotypes in human association studies. From the existing evidence, it becomes clear that no single overarching effector mechanism is apparent. In the few cases where circRNAs were tested in the context of cardiovascular disease *in vivo*, such as by classical transgenic expression in mice, or from injection of virus-encoded expression cassettes into the circulation or into the heart, the induced expression was system-wide (not cell-type specific) and constituted ectopic overexpression (not from the endogenous locus). This is important to realize when interpreting the results of these studies. In the following, we will first report on circRNAs that have been studied with connotation to human cardiovascular disease, and then circRNAs that have emerged in animal disease models.

In humans, the strongest known genetic risk determinant for atherosclerosis resides on a locus on chromosome 9p21, where independent studies have discovered tightly-clustering SNPs that associate with myocardial infarction [147], coronary artery disease (CAD) [148,149] and aneurysms [150]. These SNPs are independent of the traditional risk factors like dyslipidemia, hypertension, type II diabetes (TIID), or obesity. While independent from association with TIID, a TIID-linked haplotype block lies adjacent to the CAD and myocardial infarction-linked haplotype blocks on 9p21 [151–153]. Regarding CAD/MI, the relevant SNPs correlate with atherosclerosis plaque development and the risk for MI is a consequence of increased atherosclerosis burden [154]. The major effector of this locus is not a protein-coding gene but a long non-coding RNA, human *ANRIL*, whose expression levels associate with more severe atherosclerosis phenotypes [149,155–160]. Mechanistically, on the one hand, human linear *ANRIL* has been implicated in the chromatin-based silencing of the *INK4* locus: *ANRIL* was shown to bind both PRC1 and PRC2 polycomb complexes, which are one of the most well-studied repressive histone modifying complexes in multicellular organisms [161,162]. *INK4* silencing by *ANRIL in cis*, thus, may contribute to cell cycle progression by promoting G0-G1 cell cycle entry. This may hypothetically be relevant for *ANRIL*'s described roles in activating atherosclerosis [163]. On the other hand, *ANRIL* can also interact with transcriptional coactivators to alter the transcription of target genes *in trans* and, thereby, affect cell adhesion, apoptosis and inflammation in ways that stimulate atherosclerosis [164,165].

In any case, the human *ANRIL* locus has recently also been shown to yield a number of circular RNA isoforms [106,107] making the picture more complex. Specifically, *circANRIL* expression was found to correlate with the occurrence of cardiovascular risk alleles [106,107]: Specifically, atherosclerosis disease severity correlated with reduced *circANRIL* levels in a number of cell types, such as PBMCs, blood T-lymphocytes and cells of the vascular wall [106,107] (Table 2). This evidence and subsequent results from functional testing suggested that *circANRIL* protects from CAD. Indeed, when expressed in cultured cells, including vascular cell types, *circANRIL* was found to reduce cell proliferation and to increase the sensitivity to pro-apoptotic signals, functions that are conceptually able to protect from atherosclerotic progression in the vascular wall in specific contexts [106] (Table 3, Fig. 3). For such a net atheroprotective function, *circANRIL* was suggested to bind to and inhibit the PES1 protein, a member of the PeBoW complex,

which is known to be essential for pre-rRNA processing and consequently for the formation of functional ribosomes. As a consequence of increased *circANRIL* levels, rRNA maturation was impaired, the capacity of cells to translate proteins dropped, and this results in nucleolar stress, p53 activation in cultured cells and antigrowth conditions [106].

A number of studies investigated the role of the first described circRNA, *CDR1as* circRNA, in diverse tissues, cells, and contexts. *CDR1as* circRNA was found to be induced in the blood of acute myocardial infarction patients. Separately, *CDR1as* circRNA was also described to be upregulated in a mouse model for myocardial infarction, both in infarcted tissue and in circulating blood. The model was put forward that *CDR1as* circRNA might not only serve as a biomarker for disease, but that *CDR1as* circRNA also might be a stimulator of infarction size. The authors suggested that for this function *CDR1-as* circRNA served as a sponge for *miR-7a* and inhibited the antiapoptotic effects of this microRNA in cardiomyocytes [143]. This latter interpretation has, however, to be met with caution, as the implication of both *CDR1as* and *miR-7*, and their genetic interaction, was made based on overexpression of both factors, and functional experiments were not performed *in vivo*. Moreover, *CDR1as* and *miR-7* are known to be highly expressed in brain tissues, their expression is low or absent in non-neuronal tissues [8] and the mouse knockout shows a specific neurological deficit [95]. Thus, further experiments have to substantiate the proposed model.

Also, the gene encoding the zinc finger *ZNF292* expresses circRNAs (*cZNF292*) [166]. *cZNF292* was identified by RNA high-throughput sequencing of cultured human umbilical vein endothelial cells (HUVECs). Hypoxic culture conditions were found to affect the abundance of selected circRNAs, and among those, *cZNF292* became one of the most abundant circRNAs [166]. siRNA-mediated inhibition of a skipped exon that was included in *cZNF292* allowed to deplete this circRNA in HUVECs. This intervention inhibited the typical proangiogenic phenotypes that can be tested in cultured endothelial cells such as sprouting or tube formation in Matrigel. The mechanism of action of *cZNF292* is unknown, but the authors excluded the possibility that a microRNA sponge effect was involved [166] (Fig. 3).

*Hsa\_circ\_0010729* is another human circRNA that was found to be induced by hypoxic conditions in HUVEC cells [167]. It was implicated as an antisurvival factor in HUVEC cells under hypoxic conditions. The effector mechanism for *hsa\_circ\_0010729* is not clear [167]. *Hsa\_circ\_0003575* is yet another circRNA that is differentially regulated in stressed HUVECs. It is upregulated upon stimulation with oxidized LDL, a classical *in vitro* model for studying effects of cardiovascular pathology [138]. siRNA-mediated knockdown showed that *hsa\_circ\_0003575* was antiproliferative and antiangiogenic in HUVEC cells (Fig. 2).

Another cell type relevant for cardiovascular diseases is the vascular smooth muscle cell (VSMC). For example, several dozens of circRNAs were found to be differentially regulated in human VSMCs cultured *in vitro* under high glucose administration, a culture system used to mimic diabetic conditions [168]. Among the differentially expressed circRNAs, *circWDR77* was found to be induced. Based on siRNA-mediated knockdown, it was concluded that *circWDR77* contributed to cell survival, S-phase cell cycle entry and migrative behavior [168]. Since *miR-124* bound *circWDR77*, and since *miR-124* knockdown rescued the cellular defects of *circWDR77* depletion, this circRNA was suggested to act as a sponge for *miR-124* [168]. As a consequence, one possibility is, that FGF2, a well-known promigrative and pro-proliferative factor for VSMCs, and targeted by *miR-124*, is one of the potential effectors of *circWDR77* in this pathway [168]. A similar study explored circRNA profiles in cultured VSMCs under hypoxic conditions as well as in

aortic tissue from patients with aortic aneurysms [169]. *hsa-circ-000595* was found to be induced in these cases, and siRNA-mediated knockdown experiments suggested that this circRNA slightly contributed to apoptosis under chemically-induced HIF-1 activation (hypoxic-like conditions) in cell culture [169] (Fig. 3).

In yet another mouse model of ischemia/reperfusion, the *MFACR* circRNA was found to be upregulated [171]. Nuclear-encoded mitochondrial protein MTP18 is known to be important for cellular viability by facilitating mitochondrial fission during the complex homeostatic balance of fusion and fission that is necessary for mitochondrial function. Especially the unbalanced overshooting of mitochondrial fission in many different cell types, including cardiomyocytes, has been previously implicated in cardiometabolic syndrome. In the injury model, mouse circRNA *MFACR* promoted MTP18 protein levels and mitochondrial fission. In the injury model *in vivo*, based on systemic manipulation of *MFACR* levels, the authors showed that this circRNA stimulated MTP18, mitochondrial fission, apoptosis and heart dysfunction [171]. The authors suggested that *miR-652-3p* was impaired downstream of *MFACR* function and repressed MTP18 in a deleterious pathway (Fig. 3).

In another set of experiments, some circRNAs were mouse *circ\_000203*, as well as mouse *circRNA\_010567*, were hypothesized to function as potential promoters of cardiac fibrosis by two separate studies that used the same experimental setup [172,173]: In these reports, *circ\_000203* and the transcript of its linear host gene *Myosin 9a*, as well as *circ\_010567* were found to be induced in the myocardium of *db/db* mutant mice, which serve as a model for type II diabetes. Further, *in vivo* evidence was not reported, and insight is limited to experiments in cardiomyocytes stimulated with angiotensin II. Under these conditions, overexpression of *circ\_010567* induced the expression of three fibrosis marker genes that were concurrently repressed by *miR-26b* [172]. The authors of this study discussed the possibility that *circ\_000203* served as a sponge for *miR-26b*. In the second report, *circ\_010567* knockdown in cultured cardiac fibroblasts repressed the same three fibrosis marker genes under conditions of angiotensin II exposure. Since *miR-141* was repressed by angiotensin II, and *circ\_010567* inhibition partially rescued the downregulation of *miR-141*, the authors hypothesized that *circ\_010567* might stimulate fibrosis as an upstream inhibitor of *miR-141* transcription [173]. The mechanism underlying the proposed negative regulation is unknown.

Finally, and with respect to CAD risk factors, *circHIPK3* was identified as an abundantly expressed circRNA induced in diabetic conditions. *circHIPK3* was expressed both, in mice, as well as in diabetic human retinas and in cultured human retinal vascular endothelial cells (HRVECs) under glucose stimulation, which served as a relevant stressor *in vitro* [139]. *circHIPK3* was found to be required for optimal growth of HRVECs in baseline and stressed conditions and for expression of selected *miR-30* targets. The possibility was discussed that *circHIPK3* served as a sponge for *miR-30* targets not only *in vitro* but also in the retinal vasculature. Systemic depletion of *circHIPK3* by expression of shRNAs targeting the backsplice junction from injected adenoviral constructs, the phenotype of retinopathy in the diabetic mouse model was partially ameliorated. Conversely, *miR-30* downregulation in a similar experiment increased retinopathy. Since an epistatic relationship was found in double-knockdown experiments for *circHIPK3* and *miR-30*, and since *miR-30* was pulled down by *circHIPK3* in Ago2 complexes, the possibility exists that *circHIPK3* is indeed a physiologically relevant sponge of *miR-30* [139]. A similar pro-proliferative cellular function for *circHIPK3* was previously also suggested in a separate study, that showed that *circHIPK3* more generally stimulated proliferation of a number of different cell types by sponging growth-suppressive microRNAs like *miR-124* [66]. Together, *circHIPK3* may exert a pro-proliferative role in

different tissues, possibly by sponging different growth-restrictive microRNAs (Fig. 3).

### 5.3. Some circRNA-regulatory proteins have an independently reported function as regulators of cardiovascular physiology

Some upstream regulators of circRNA biogenesis or stability have been studied already before the re-discovery of circRNAs in the context of cardiovascular disease. For example, more than 700 RBPs are known to be encoded in the genome, and more than a hundred of these have been shown to specifically affect circRNA biogenesis [174]. Despite their expected pleiotropic roles as RNA-binding proteins, some tissue- and lineage-specific RBPs and splicing regulators have been successfully connected to rather specific sets of linear target mRNAs (see Refs. [175,176] for review). Still, the large number of targets makes it then difficult to associate a single mRNA or a single splicing event with a phenotype, let alone to associate a cardiovascular function with the so far independent role in circRNA regulation. Therefore it is only a curiosity, that rather specific cardiovascular functions have been reported for three RBPs that have recently (but independently) also been revealed as circRNA biogenesis regulators:

#### 5.3.1. The Muscleblind family of RNA-binding proteins

The first of these RBPs is the Muscleblind protein family (mouse *Mbnl1-3*, human *MBNL1-3*). *Mbnl/MBNL1-3* proteins are RNA binding proteins, splicing regulators [177,178], alternative polyadenylation regulators [70] and circRNA regulators [53]. Muscleblind proteins are not specifically expressed in cardiovascular tissues, but compared to other organs they are relatively highly expressed in hearts of both mice and man. *Mbnl1* knockout studies have shown that Muscleblind it is required for normal heart valve development [179] and for fibroblast-to-myofibroblast differentiation [180] (Fig. 3). Besides a number of other phenotypes, and with relevance to disease, *Mbnl1* knockout mice also develop myotonic dystrophy 1 (DM)-related cardiac defects including myocardial fiber death, fibrosis, cardiac hypertrophy and conduction problems [180] (Table 3). These heart problems are the second most frequent cause of death in DM patients [181]. Cell differentiation defects in *Mbnl1* mutants have been ascribed to a failure of cells in transitioning from a naïve and undifferentiated splicing pattern to a differentiation-determining splicing pattern [177,178]. In fact, *Mbnl* regulates mRNA splicing in the maturing heart [182]. The question that has not yet been addressed is whether *Mbnl*-dependent regulation in the heart is due to circRNA regulation or primarily to regulation of linear splicing. The implication of *Mbnl1* in DM is better understood in this regard: Critical to the onset of DM is that specific RNAs acquire CUG repeat extensions, which causes these mutant RNAs to sequester *Mbnl* and to impair *Mbnl*-dependent linear mRNA splicing [183,184]. In the cardiovascular system, it might be the mis-splicing of surprisingly few important cardiovascular regulatory mRNAs, such as of *SCN5A*, that trigger disease. *SCN5A* mRNA splicing has been found to contribute to a significant part of the cardiac-conduction delay and heart arrhythmia in *Mbnl1* mutants [185]. Yet, also in a disease context, any involvement of circRNA biogenesis is speculative. To the contrary, as a test circRNA, *circMbnl1* levels were quantified in DM patients and in transgenic CUG-repeat-expressing mice [183] that serve as a mouse model for *Mbnl1* -protein inhibition in DM, but no changes were found in the diseased state [186]. This may speak against an implication of circRNAs in DM and be explained by the known upregulation of *Mbnl1* transcription upon *Mbnl1* protein loss, which would effectively counteract decreases in *circMbnl1* by increasing *Mbnl1* transcription.

#### 5.3.2. The RNA-binding protein Quaking

The second relevant RBP is the global linear splicing regulator Quaking (QKI). Apart from its major role in nervous system function, it is also required for muscle fibril formation [187], visceral endoderm differentiation, and vessel formation in embryogenesis [188,189]. QKI also partakes in the onset of atherosclerosis, where it regulates neointimal vascular smooth muscle cell (VSMC) proliferation in adult human coronary lesions [190]. QKI may also participate in the resolution of heart injury [191] (Table 3). Others studies showed that QKI is involved in endothelial layer formation. Not last SNPs in QKI have recently been associated with risk for myocardial infarction and coronary heart disease [192] (Table 3). It is still not fully clear in which cell types QKI would function in disease, but Qki is known to promote monocyte adhesion, and to stimulate differentiation of monocytes to inflammatory macrophages and foam cells in atherosclerotic plaques [193] (Fig. 3). Together, QKI has a surprisingly large number of functions in the cardiovascular system, but as for *Mbnl*, whether any of these is due to circRNA regulation is unknown.

#### 5.3.3. The RNA-binding protein RBM20

A third example is *Rbm20*, a splicing regulator implicated in hereditary dilated cardiomyopathy, one of the leading causes of heart failure [194] (Table 3). *Rbm20* has been found to be responsible for alternative splicing of at least 30 genes, some of which are known to regulate cardiomyocytes, sarcomere organization or biomechanical muscle function, or are otherwise implicated in heart failure or cardiomyopathy [194] (Fig. 3). Among these is *Titin*, a gene encoding a sarcomeric filament protein that is inherently linked to force generation in muscle cells and hence to myocardial function. The *Titin* gene is known to be massively alternatively spliced and, thus, became the focus of interest with respect to circRNA biology. Indeed, both mouse and human *Titin* express up to 80 different circular RNA isoforms in the heart [195]. Moreover, the splicing factor *Rbm20* has been found to be involved in the biogenesis of *circTitin*, because it potentially binds to introns flanking the circularization events. An *Rbm20* mutation was generated in the mouse, and it was found that these knockout mice experienced early onset dilated cardiomyopathy [195]. In the absence of *Rbm20*, the formation of a specific subset of circRNAs from the *Titin* gene was found to be defective in heart tissue [195]. Together, this raised the possibility that RBM20-dependent changes in circRNA biogenesis of *Titin* contribute to disease onset [195,196], hypothetically related to the fact that *circTitin* formation occurs in exons encompassing the region determining linear *Titin*'s biomechanical rigidity [195]. This possibility has, however, so far not been tested and the implication of RBM20-dependent circRNAs in CVD is solely correlative at this point.

Complicating the analysis, in addition to the already described functions in regulating linear mRNA expression, Muscleblind [178,197–199], Quaking [200–202], or also other RBPs like HuR [203–210], have specific pleiotropic and context-dependent functions in cardiovascular cell types. Therefore, whether and to what extent the regulation of circRNAs, as opposed to the regulation of alternative linear splicing, contributes to the known *Mbnl*- or *Qki*- or *Rbm20*-dependent processes in cardiovascular physiology and disease will be difficult to be teased apart.

## 6. Outlook

During the past years, it has become clear that thousands of genes express circular RNAs and that these have gene regulatory potential. CircRNA formation is a regulated process, and deregulation of circRNA formation can contribute to cellular and organismal defects. CircRNAs have not only been involved in cardiovascular

disease but also in neurological functions (see Ref. [211] for review) and in cancer-related processes such as overproliferation, transformation, metastasis, and chemotherapeutic drug resistance (see Ref. [212] for review). So far it has not been reported whether circRNAs can serve as successful targets or therapeutic agents in preclinical settings. Approaches focusing on RNA as a therapeutic molecule, are, however, gaining importance. What makes them interesting candidates is their intracellular stability. So far, approaches centering on RNA transgenics pertain to linear RNAs, such as mRNAs or interfering RNAs: technologies for their delivery into cells of living tissues are improving, whereby chemical modifications are used to increase their stability [213]. In the future, also circRNAs might be administered for therapeutic purposes *in vivo*, their endogenous stability may be superior, and additional covalent modification of their backbone may increase their application even further. Also, one could imagine that antisense oligonucleotides could be transfected into tissues *in vivo*, which bind to splice-regulatory sequences and could be designed to affect backsplicing at a specific locus. Thereby one might be in the position to either increase or decrease the frequency of endogenous circRNA biogenesis from a specific locus. In this context, and what is maybe best shown in the example of *ANRIL*, it is likely the defined expression levels of both, linear and circular RNA, that determines the function of a gene. Finally, even if not all circRNAs were always functional, circRNAs may still be used as biomarkers for defining cell or disease states.

## Disclosures

None. There are no competing interests.

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