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Cirscan: a shiny application to identify differentially active sponge mechanisms and visualize circRNA-miRNA-mRNA networks.

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Abstract *Non-coding RNAs and their biological functions are still incompletely understood, while representing a large part of the human transcriptome and having an important role in cancer. Among these, circular RNAs (circRNAs) have recently been discovered for their microRNA (miRNA) sponge function, which allows them to modulate the expression of miRNA target genes: they take on the role of competitive endogenous RNAs (ceRNAs). Their closed-loop structure makes them highly stable and their miRNA binding capacity seems much more powerful than that of any other ceRNAs, leading to their super-sponge naming. Today, few ceRNA prediction computational tools have been published and most of them do not consider ce-circRNAs. Moreover, the few studies focusing on circRNA-miRNA-mRNA networks have not developed a tool that automates the search for ceRNAs from user's transcriptomic data. In this study, we present an interactive Shiny web application, called Cirscan for CIRcular RNA Sponge CANDidates. Cirscan automatically infers circRNA-miRNA-mRNA networks from human multi-level transcript expression data (including circRNAs, mRNAs, and optionally miRNAs in the case of cancer study) from two biological conditions (e.g. tumor versus normal conditions) in order to identify potential sponge mechanisms, active in a specific condition. Cirscan calculates a global sponge score for each miRNA-circRNA interaction, integrating multiple criteria based on interaction reliability and on RNA expression level using the TOPSIS method. The ranking of this score provides the most relevant circRNA candidates being miRNA sponges. Finally, the user is able to visualize the top ranked sponge mechanisms as graphs, where nodes correspond to the different types of RNAs and edges to the miRNA-target interactions. In order to help the user in the biological interpretation of the visualized network, an enrichment analysis of the biological functions is performed. We applied Cirscan on a public multi-level microarray transcript expression dataset from colorectal cancer (CRC) and retrieved previously described sponge mechanisms as well as novel circRNA sponge candidates.*

Keywords circular RNAs, sponge mechanism, transcriptomic data, regulation network, cancer

1. Introduction

Non-coding RNAs (ncRNAs), genome sequences not translated into proteins, and their biological functions are still incompletely understood, because they were characterized as junk transcriptional products. Recently, it has been demonstrated that ncRNAs represent a large part of the human transcriptome and have important functional roles, particularly in cancers [1]. Advances in sequencing technologies have led to the identification of different types of ncRNAs in the cell, such as long non-coding RNAs and microRNAs (miRNAs) [2]. In this study, we are interested in circular RNAs (circRNAs), recently discovered and known for their miRNA sponge function [3,4]. CircRNAs can indirectly regulate the expression of genes involved in cell plasticity by sequestering miRNA(s) and can

play a role of a competitor, called competitive endogenous RNA (ceRNA) [5–7]. One of the most well-known ce-circRNA is ciRS-7 (CDR1as), mainly expressed in the brain, which acts as a regulator of the miRNA-7 with more than 70 binding sites and leads to increased expression levels of miRNA-7 targets [8]. Thanks to their closed-loop structure formed by reverse-splicing, circRNAs are very stable and their binding capacity to bind to miRNAs seems to be stronger than that of any other ceRNA, leading to their super-sponge naming [9]. These interactions between coding and non-coding RNAs can be represented as circRNA-miRNA-mRNA interaction networks.

Today, few computational tools have been developed for the identification of ceRNA but are not adapted to the search for ce-circRNA [10,11]. Moreover, the studies focusing on circRNA-miRNA-mRNA networks have not developed a tool that automates the search for ce-circRNA from its own transcriptomic data [12–18]. Recently, two circRNA functional annotation databases predicting sponge mechanisms in several tissues have been developed [19,20], but it is not possible to query one's own dataset and to identify sponge mechanisms specific to a biological condition. Here, we present an interactive Shiny web application, called Cirscan for CIRcular RNA Sponge CANDidates. The aim of this tool is to automatically infer circRNA-miRNA-mRNA networks from human multi-level transcript expression data (including circRNAs, mRNAs, and optionally miRNAs in the case of cancer study) from two biological conditions (e.g. tumor versus normal conditions) and to identify sponge mechanisms, active in a specific condition. From the user's expression data and an in-house database of highly reliable prediction interactions, Cirscan is able to construct an interaction score matrix including multiple criteria based on interaction reliability (e.g. affinity of the interaction, enrichment of binding sites for a given miRNA) and on RNA expression (e.g. minimum level of expression, co-expression between circRNA and mRNA targets for a given miRNA). To optimize the search for effective sponge mechanism, a global sponge score is calculated using the different criteria for each miRNA-circRNA predicted interaction. The ranking of this global sponge score provides the most relevant circRNA candidates being miRNA sponges. Finally, the user is able to visualize the top ranked sponge mechanisms as graphs, where nodes correspond to the different types of RNAs and edges to the miRNA-target interactions. In order to help the user in the biological interpretation of the results and to better understand the functional impact of the selected sponge mechanism, an enrichment analysis of the biological functions is performed by Cirscan for each visualized network.

We applied our tool on a public multi-level transcript expression dataset from two conditions: colorectal cancer samples and normal adjacent samples [21]. We showed that Cirscan was able to retrieve previously described sponge mechanisms as well as novel circRNA sponge candidates [22].

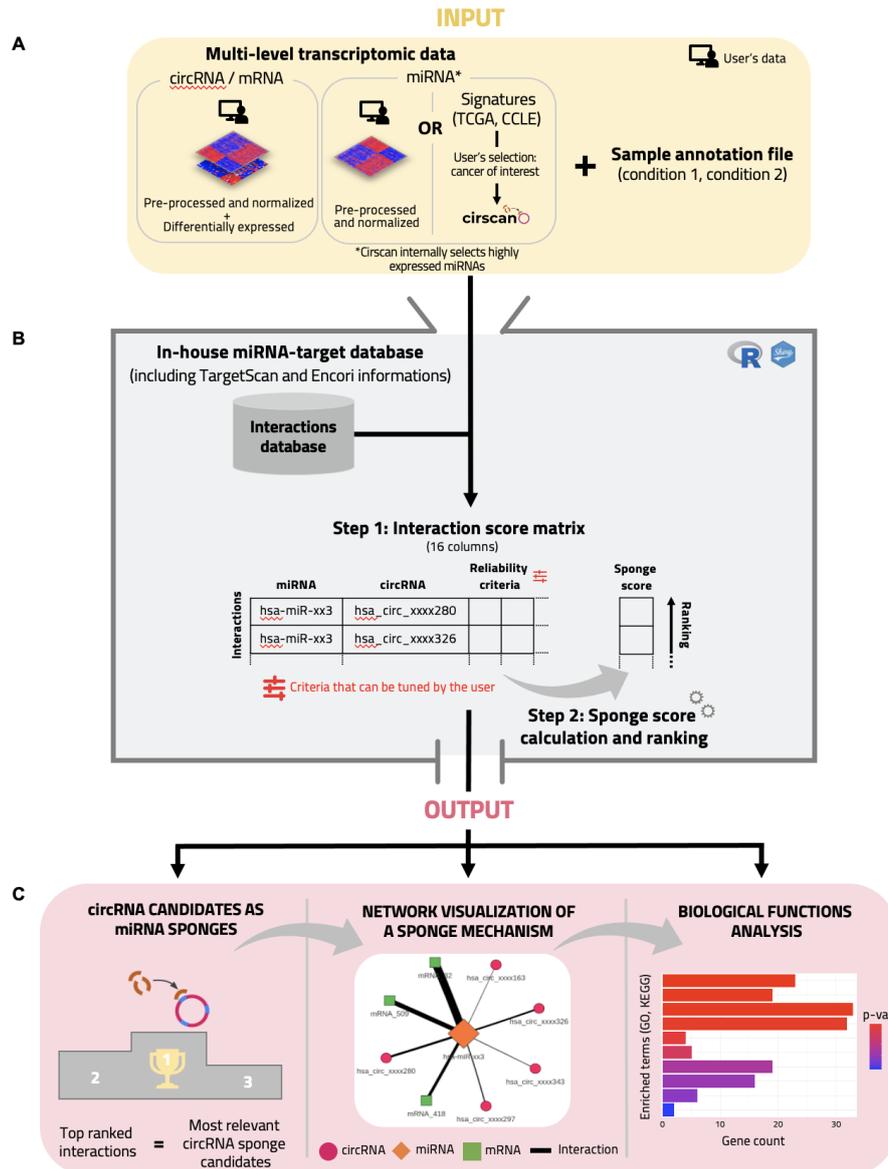
In summary, Cirscan provides a user-friendly tool to identify and visualize circRNA-miRNA-mRNA networks with potential sponge mechanisms from user's human multi-level transcript expression data, and may provide potential novel biomarkers for the development of RNA targeted therapies [23].

Cirscan shiny app is freely available on Gitlab at the following link: https://gitlab.com/geobioinfo/cirscan_Rshiny.

2. Software description

2.1. Overview of the tool

Cirscan infers circRNA-miRNA-mRNA interactions from coding and non-coding transcriptomic data (Fig. 1A) and identifies circRNA candidates that can act as miRNA sponges (ce-circRNA). The tool comprises the following main steps: 1) Construction of an interaction score matrix including different criteria: criteria of interaction reliability and criteria of effectiveness of a sponge mechanism as defined in the following sections (Fig. 1B. Step 1). 2) Calculation of a global sponge score for each circRNA-miRNA interaction by integrating the different criteria using the TOPSIS method [24] [see more details in the section 2.4.3] (Fig. 1B. Step 2). Cirscan outputs the top ranked circRNA sponge candidates based on the ranked global sponge scores. It also enables the visualization of the identified sponge mechanisms as networks and biological enrichment analysis of the mRNAs of the networks of interest (Fig. 1C). A video tutorial and an example dataset are available to guide the user in the different steps on the application.



2.2. Effective sponge mechanisms prerequisites

Prerequisites based on both sequence and expression information have been put forward for the identification of circRNAs acting as miRNA sponges: 1) A miRNA must be sufficiently expressed whatever the biological conditions to have a functional impact on its targets and must target at least one circRNA and one mRNA, with a strong affinity. Importantly we do not assume a differential expression between both conditions due to a sponge mechanism: we assume that a sponge mechanism involving a circRNA only affects the bioavailability of the miRNA but does not affect the miRNA expression. 2) The mRNAs and circRNAs that are targets of the miRNA must be co-expressed, i.e differentially expressed between the two conditions in the same direction. Indeed, it is expected that the increased presence of a circRNA that can sponge a miRNA will induce an over-expression of the mRNA targets of the miRNA, as the miRNA can no longer degrade them.

2.3. Input files

Cirscan requires two types of CSV files from the user that are depicted in Figure 1A. First, multi-level transcript expression data are required as input files, including circRNA, mRNA and optionally miRNA expression matrices. If the user does not have miRNA expression data, miRNA signatures for different types of cancer (respectively 32 and 20 from the TCGA consortium (<https://www.cancer.gov/tcga>) and CCLE database [25] (www.broadinstitute.org/ccle)) are internally available in Cirscan. There is no constraint concerning the technology used to obtain the expression matrices (array or sequencing), which in return requires the user to properly normalize his data beforehand. Each expression matrix needs to be normalized (e.g. RMA for expression array, TPM for RNA-seq data) and log-transformed, and a minimum of 3 samples per condition is required to ensure reliability of the downstream analysis. Based on the sponge mechanism prerequisites, circRNA and mRNA expression matrices should be restricted to the differentially expressed circRNAs and mRNAs prior to the use of Cirscan. As mentioned above, this restriction is not relevant for miRNAs and no filter is required. Cirscan will automatically keep miRNAs that are sufficiently expressed considering the following assumptions: (1) the sum of the expression of a given miRNA in all samples must be non-zero, (2) for a given miRNA, its expression must be higher than a defined cutoff in more than 90% of the samples, the cutoff being the quartile Q1 of the overall miRNA expression distribution. This selection was also made for the establishment of the miRNA signatures from the TCGA and CCLE datasets.

CircRNA identifiers must correspond to the human circRNAs referenced in circBase (“hsa_circ_XXXXXXX”) [26], human miRNAs from miRBase (“hsa-miR-xxx” or “hsa-let-xxx”) [27], and mRNAs must be referenced as gene symbols. Furthermore, the user must provide another CSV file corresponding to the sample annotation file. This file must contain two columns: “samples” with the sample names referenced in the RNA expression matrices and “conditions” with the corresponding condition it refers to (“condition_1”, “condition_2”), knowing that the first condition refers to the control condition. The file format required by Cirscan is detailed in the Home panel of the Shiny application and in the example dataset provided in the application.

2.4. Identification of sponge mechanisms

2.4.1. In-house miRNA-target interaction databases

A miRNA-target interaction is established between a region of the miRNA, called “seed sequence” and a complementarity sequence called “miRNA response element” (MRE) on the target (circRNA or mRNA). An in-house database of interactions between miRNAs and putative mRNA or circRNA targets was constructed using the TargetScan interaction prediction tool and information from experimentally validated interaction database (ENCORI [28]) (Fig. 1B).

TargetScan was used to predict miRNA-circRNA and miRNA-mRNA interactions, and to obtain an affinity score for each interaction, by taking into account the specificity of the RNA type considered (mRNA or circRNA). For miRNA-circRNA interactions, we used the context+ score available in the TargetScan v6 [29]. The context+ score is the sum of the contribution of six features (site-type, 3' pairing, local AU, position, target site abundance, seed-pairing stability). The feature 3' pairing was removed here because it is not relevant for circRNA as the pairing is not expected to be restricted to that region and can appear on the whole circRNA sequence. The lower the context+ score, the stronger the affinity between the miRNA and its target. To reduce the number of false positive predictions, we defined a cutoff on the context+ score, based on its distribution on experimentally validated interactions given by the ENCORI database. We defined as a cutoff the 95th percentile of the context+ score distribution restricted to the interactions supported by at least two CLIP-seq and degradome-seq experiments. For miRNA-mRNA interactions, we calculated the context++ score provided by TargetScan v8 [30]. Compared to the context+ score, the context++ score includes additional criteria specifically relevant for miRNA-mRNA interactions (e.g. 3' UTR length, ORF length, probability of conserved targeting between species), which are expected to reduce false positive predictions.

MiRNA-circRNA and miRNA-mRNA interactions were restricted to specific MRE binding sites: 7mer-m8 (exact match to positions 2-8 of the mature miRNA), 7mer-1a (exact match to positions 2-7 of the mature miRNA followed by an “A”), and 8mer-1a (exact match to positions 2-8 of the mature miRNA followed by an “A”).

2.4.2. Criteria of interaction reliability and sponge effectiveness

In order to identify potential sponge mechanisms involving circRNA, different scores of reliability are calculated. We defined as the affinity score between a miRNA and its targets $S_{Affinity}^{miRNA-target}$ the context score calculated by TargetScan that we rescaled between 0 and 1: the closer to 1, the stronger the interaction affinity. For each miRNA-target interaction, we also considered the number of MRE sites that we named the score $S_{MRE}^{miRNA-target}$. If an interaction involves multiple MREs for the same miRNA and target of interest, the interaction with the maximum $S_{Affinity}^{miRNA-target}$ is retained. To reduce the potential bias in the number of predicted MREs due to the length of the circRNA target sequence, the number of MREs for miRNA-circRNA interactions was normalized by the length of the circRNA sequence as described in the Cerina tool [19] and referenced here as the score $S_{MRE/kb}^{miRNA-target}$. In order to identify circRNAs that are significantly enriched for MRE binding sites of specific miRNAs, we used a binomial model conditioned on the total number of MRE for the given miRNA on each circRNA and the number of all other miRNA-MRE sites on the given circRNA as background, as proposed in the recent scanMiR tool [31] and defined as $S_{Enrichment}^{miRNA-target}$. A circRNA-miRNA interaction is kept if the Binomial enrichment test adjusted p-value (Benjamini Hochberg (BH)) is lower than 0.05.

In order to take into account the expression level information, different scores are calculated: the target expression fold change between conditions 1 and 2 ($S_{log(FC)}^{target}$), the median expression of each miRNA under all conditions ($S_{Expression}^{miRNA}$) and the highest mean expression of each target between the two conditions ($S_{Expression}^{target}$). Only networks with at least one circRNA and one mRNA with fold change values in the same direction are selected.

2.4.3. Calculation of a sponge score

For each miRNA-mRNA interaction, Cirscan calculates a global score defined as $SG_{Interaction}^{miRNA-mRNA}$ which integrates the different criteria described above ($S_{Affinity}^{miRNA-target}$, $S_{MRE}^{miRNA-target}$, $S_{log(FC)}^{target}$, $S_{Expression}^{miRNA}$ and $S_{Expression}^{target}$) using the TOPSIS method [24]. Briefly, the TOPSIS method is a multi-criteria decision analysis method which aims to determine the best alternative when different criteria need to be considered together. In our case, the best alternative is the miRNA-target interaction for which all criteria are maximized, e.g. highest affinity score and highly expressed miRNAs, circRNAs and mRNAs.

Then, a score for each miRNA-circRNA interaction is calculated and defined as a global sponge score, $SG_{Interaction}^{miRNA-circRNA}$. This score is also based on the TOPSIS method and uses the following criteria: $S_{Affinity}^{miRNA-target}$, $S_{MRE/kb}^{miRNA-target}$, $S_{Enrichment}^{miRNA-target}$, $S_{log(FC)}^{target}$, $S_{Expression}^{miRNA}$, $S_{Expression}^{target}$ and $S_{Enrichment}^{miRNA-miRNA}$, where $S_{Enrichment}^{miRNA-miRNA}$ corresponds to the enrichment score (NES) (fgsea R package [32]) calculated on the ranked global score $SG_{Interaction}^{miRNA-mRNA}$ of the mRNA targets defined above. In other words, for a given miRNA-circRNA interaction, if the mRNA targets of the miRNA have high global scores, this will contribute to increasing the sponge score. MiRNA-circRNA interactions are then ranked according to their sponge score, the top ranked interactions with the highest scores being the most relevant sponge circRNA candidates.

Finally, all this information is compiled into an “interaction score matrix” with 16 columns available and detailed in Cirscan (Fig. 1B), where $Rank_{miRNA-circRNA}$ is the rank of miRNA-circRNA interactions based on the global sponge score.

2.5. Visualization of the sponge mechanisms of interest

The identified circRNA-miRNA-mRNA networks involving potential sponge mechanisms can be visualized in the Network Visualization panel using the visNetwork package (v2.0.8, <https://github.com/datastorm-open/visNetwork>) (Fig. 1C). It is possible to visualize a network by

selecting an interaction from the “interaction score matrix” or by specifying the name of an RNA of interest: nodes correspond to the different types of RNAs (orange miRNAs, green mRNAs, and pink circRNAs) and edges to the miRNA-target interactions. The edges thickness is proportional to the global sponge score (the higher the value, the thicker the edge and the stronger the interaction affinity) and the RNA node of interest is larger than the others.

2.6. GO and KEGG enrichment analysis of mRNAs

In order to help the user in the biological interpretation of the visualized network, an enrichment of biological terms on the genes of the visualized network is performed using Enrichr tool [33] with KEGG (KEGG_2021_Human) and GO (GO_Biological_Process_2021) databases.

3. Application

We applied Cirscan on a public multi-level transcript expression dataset including mRNAs, miRNAs, and circRNAs of colorectal cancer (CRC) to infer sponge mechanisms involving circRNAs.

3.1. Pre-processing of input data

Microarray multi-level transcript expression data of 10 CRC samples and 10 normal adjacent samples were downloaded from the NCBI Gene Expression Omnibus database (accession number: GSE126095). Each dataset was imported into the RStudio (v1.4.1103) environment with R (v4.1.2) for pre-processing.

For transcripts belonging to the gene annotation, an expression average was applied. Quantile normalization and a log-transformation were applied to the mRNAs expression matrix. mRNAs microarray matrix was reduced to protein-coding genes, using the annotation file provided by the authors. Using the limma package [34] (v.3.50.1), 4,640 differentially expressed mRNAs with an adjusted p-value (BH) < 0.05 were selected. The circRNAs microarray matrix was also submitted to quantile normalization and a log-transformation. We selected 1,491 differentially expressed circRNAs with an adjusted p-value (BH) < 0.05 by using the limma package. Finally, the miRNAs microarray matrix was filtered to only include miRNA identifiers present in the annotation file, i.e. 2,055 miRNAs. Quantile normalization and log-transformation were also applied to the miRNAs expression matrix. These different pre-processed expression matrices were given as input to Cirscan.

In the sample annotation file, we considered the names of the 10 normal adjacent samples as “condition_1” and the 10 colorectal cancer samples as “condition_2”.

3.2. Results

Using the Cirscan tool on the multi-level transcript expression data from colorectal cancer described above, we identified 12,850 potential circRNA-miRNA sponge mechanisms involving 1,413 unique circRNAs. Among them, we identified 3 sponge mechanisms already described in the literature [22] (one sponge mechanism active in the normal condition and two in the tumor condition) significantly enriched in the top ranked sponge mechanisms (GSEA enrichment from fgsea R package [32], p-value = 0.00012). A similar result is observed using the TCGA colorectal cancer miRNA signature available internally in Cirscan (GSEA enrichment, p-value = 0.00015). Among the two subnetworks already described in the literature and specifically active in the tumor condition, we identified the hsa-circ-0001955:hsa-miR-145-5p sub-network, ranked 117 (in the top 1%) out of all identified mechanisms (Fig. 2A) and described as well by Ding *et al.* (2020) [22] in colorectal cancer. The biological term enrichment analysis of the mRNAs of this subnetwork revealed biological pathways associated with oncogenesis, such as Proteoglycans in cancer or MAPK signalling pathway (Fig. 2B). This result is consistent with the involvement of this sponge mechanism in the oncogenesis of colorectal cancer.

We next focused on the best ce-circRNA candidates having a sponge role in the tumor condition. Interestingly, within the top 10 ce-circRNA candidates, the first ce-circRNA has been already shown to have a sponge role in gastric or colorectal cancer in several studies (hsa_circ_0001658 [35–37]). Cirscan highlighted potential novel sponge mechanisms for this circRNA, involving for example the miRNA hsa-miR-665 (Fig. 2C) targeting genes associated to cell division and angiogenesis as

illustrated in Figure 2D (e.g. MAPK and VEGF signaling pathways). Four ce-circRNAs within the top 10 ce-circRNA candidates have been identified in other cancers as hepatocellular carcinoma and lung cancers (hsa_circ_0034326 [38], hsa_circ_0072088 [39–41], hsa_circ_0000517 [42,43], hsa_circ_0000326 [44]). The other best ce-circRNA candidates (hsa_circ_0087961, hsa_circ_0012283, hsa_circ_0007582, hsa_circ_0000254 and hsa_circ_0008720) have not yet been described in the literature as associated with cancer, making them interesting subjects for further experimental validations.

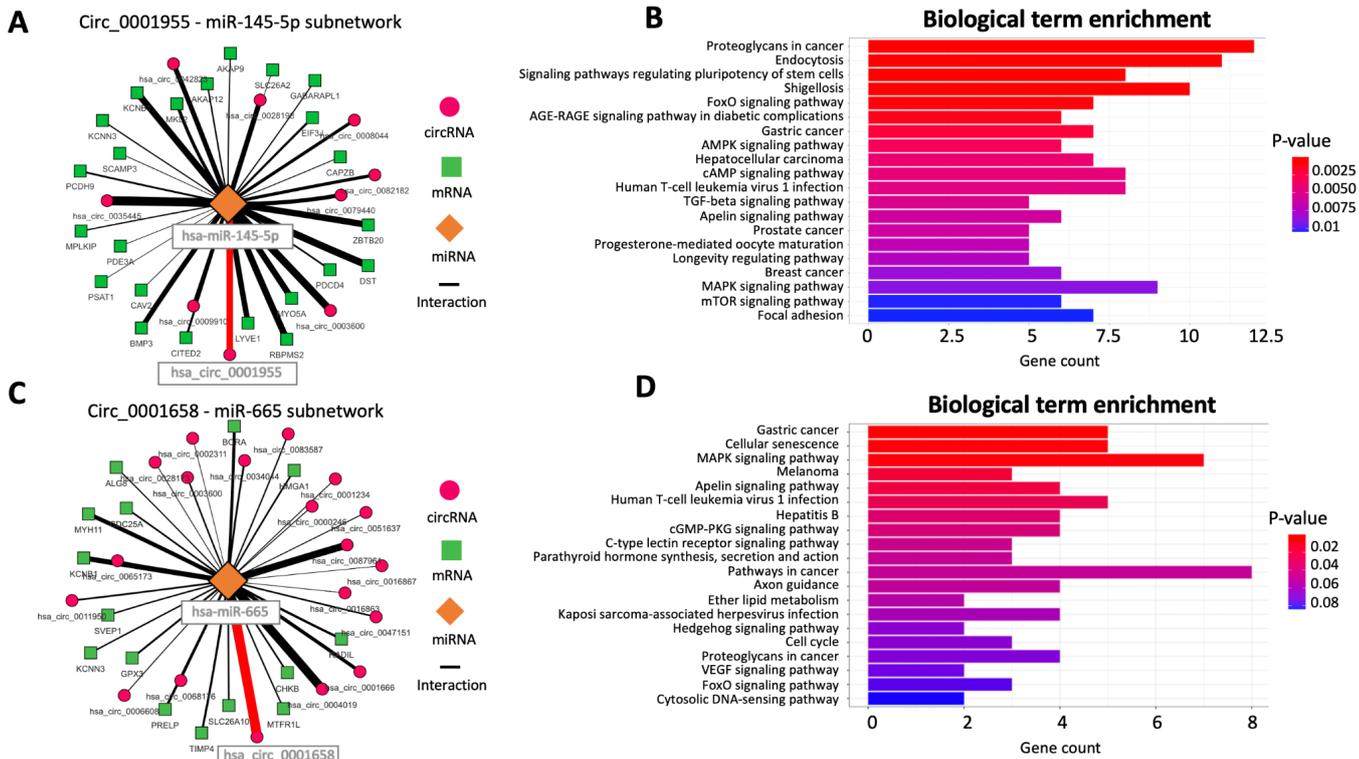


Fig. 2 Subnetworks identified by Cirscan using colorectal cancer data. (A) Circ_0001955-miR-145-5p subnetwork. The nodes represent the different RNA types (orange miRNAs, green mRNAs and pink circRNAs), and the edges represent miRNA-target interactions (targeting mRNAs or circRNAs). The red edges represent the interactions found in the literature and the thickness of the edges is proportional to the interaction score values. For a better visibility, only 10% of the targets are represented. (B) Top 20 mRNA-enriched pathways in the Circ_0001955-miR-145-5p subnetwork. (C) Circ_0001658-miR-665 subnetwork, with the same legend as panel A. (D) Top 20 mRNA-enriched pathways in the Circ_0001658-miR-665 subnetwork.

4. Conclusion and perspectives

Cirscan is a tool that takes as input any human multi-level transcript expression data (circRNAs, mRNAs, and optionally miRNAs) to identify and visualize condition-specific sponge mechanisms involving circRNAs. As shown using a public dataset from colorectal cancer tissues, Cirscan allows the identification of known and novel potential sponge mechanisms that may be further investigated and validated experimentally. This tool can be considered as a companion tool for biologists, facilitating their ability to prioritize sponge mechanisms for experimental validations. The mechanisms revealed by Cirscan could open new avenues for the development of novel RNA-targeted therapies. In particular, it would be possible to use antisense oligonucleotides, which can bind by complementarity to circRNA sequences of interest, to inhibit the sponge mechanisms active in a specific condition [23]. Finally, the framework established in this study could be extended to other species.

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