Analysis of common targets for circular RNAs
Ya-Chi Lin,‡† Yueh-Chun Lee,‡† Kai-Li Chang and Kuei-Yang Hsiao

Abstract
Background: The studies of functions of circular RNAs (circRNAs) are heavily focused on the regulation of gene expression through interactions with multiple miRNAs. However, the number of predicted target genes is typically overwhelming due to the synergistic complexity caused by two factors — the binding of multiple miRNAs to a circRNA and the existence of multiple targets for each miRNA. Analysis of common targets (ACT) was designed to facilitate the identification of potential circRNA targets.

Results: We demonstrated the feasibility of the proposed feature/measurement to assess which genes are more likely to be regulated by circRNAs with given sequences by calculating the level of co-regulation by multiple miRNAs. The web service is made freely available at http://lab-x-omics.nchu.edu.tw/ACT_Server.

Conclusions: ACT allows users to identify potential circRNA-regulated genes and their associated pathways for further investigation.

Keywords: Circular RNA, microRNA, Common targets, miRNA sponge

Background
Circular RNA (circRNA) is a newly recognized class of single stranded regulatory RNA molecules with ends covalently closed through a backsplice between a downstream splice donor and an upstream splice acceptor. Recent discoveries through sequencing technology and computational analyses have revealed the widespread existence of circRNAs in animal cells and many other organisms [1–3].

CircRNAs contribute to transcriptional activation, post-transcriptional modulation, translation, and protein interactions [4–8]. Among these, the most popularly studied function of circRNA is that of a miRNA sponge that regulates the gene expression network [9–11]. Pioneering studies have made great contributions dissecting and archiving these relationships among miRNAs, circRNAs, and associated pathological phenotypes [12–15]. However, studies investigating the biological functions of circRNAs are largely limited to the scope of a single miRNA linked to a single gene [16–18]. Thus, how to identify a manageable gene list length and to consider its role as a whole for further functional characterization has become a critical task.

In this study, we developed and tested an intuitive concept that genes targeted by more circRNA-associated miRNAs are more likely to be modulated by a given circRNA. We established and provided a web service for the analysis of common targets (ACT) for circRNAs to facilitate the molecular characterization of the biological functions of various circRNAs.

Implementation
The circRNA-associated miRNA-gene network typically involves many genes targeted only once by a miRNA (Additional file 1: Figure S1, gray nodes), and thus these genes may be less efficiently regulated by a given circRNA. The central idea of ACT is to identify target genes with high binding numbers for circRNA-associated miRNAs (Additional file 1: Figure S1, blue nodes). To implement this analysis, miRNA-binding sites in circRNAs were first extracted (Fig. 1a - Step
1), followed by the identification of target genes for each circRNA-associated miRNA (Fig. 1 - Step 2). Finally, the targeting number for each gene was calculated (Fig. 1 - Step 3). The flowchart for the sequent processes is summarized in Fig. 1b. The ACT server takes circular RNA sequences in FASTA format. The default miRNA sequences are downloaded from miRBase (Release 22) [19, 20]. First, the user-inputted sequence is extracted from the 5′ and 3′ ends (30 nt) and reverse-joined to produce a backsplice junction. Then the list of miRNAs that potentially bind to the given circRNA is generated by miRanda software (version 3.3a, Aug 2010) [21] using mature miRNA sequences from miRBase. In order to reduce the number of predicted miRNA binding sites, the parameter ‘-strict’ is applied when using miRanda. The position of miRNAs spanning the backsplice junction is calibrated to the beginning of the original inputted sequence, and the number of miRNA binding sites ($nbs$) is recorded for further calculations. A list of the target genes for each miRNA with binding site(s) on the given circRNA is generated using miRTarBase (Release 7.0) [22]. The gene list is then collapsed for unique entries, and for each gene (g), the number of targets for circRNA-associated miRNAs (from the last step) is calculated as the sum of $nbs$ (see Fig. 1a – Step 3 for example). The genes in the list are ranked by the common targeting time (CT).

**Results**

ACT-selected genes are enriched in specific biological pathways

ACT performs a distinct assessment compared to other metrics that measure the binding energy or pairing score between miRNA and circRNA. Compared to the density of miRNA binding sites, the binding energy and pairing score given by miRanda for these predicted miRNAs on circRNAs, CT for genes provides a more dynamic range to distinguish circRNAs with/without identified miRNA sponge activity and a background dataset (Additional file 1: Figure S2A-D and Table S1). The web interface for ACT is neat and the output files are annotated with detailed information (Fig. 2a and b). CircHIPK3, a previously identified circRNA that targets multiple miRNAs [10], was used as an example (provided for users in the web interface, Fig. 2a). Raw analysis using only the miRNA-target relationship revealed 7350 genes, and approximately half of these genes were targeted by one or two miRNAs (Fig. 3a and b; 3842 out of 7350, 52.27%). ACT is aimed at identifying common targets that are targeted by several circRNA-associated miRNAs. The top 100 genes were exported and are listed in Fig. 3a. A few
known circRNAs with and without known sponge activity to multiple miRNAs were used for comparison. Cytoplasmic circRNAs including circHIPK3, circCCDC66, circPVT1 and circIRAK3 [11, 23, 24], previously reported to function as molecular sponges for multiple miRNAs, and nuclear circRNAs from FLI1 and UBR5 genes with distinct molecular functions other than as miRNA sponges in the nuclei [25, 26] were applied to the ACT pipeline. To characterize whether the ACT-predicted circRNA-regulated genes play biological roles, we adapted the concept of co-regulation or the convergence of regulation. We assumed that the circRNA-targeted genes are more likely to be conserved and involved in the same pathways during evolution. The ACT-selected genes were subjected to pathway enrichment analysis. The results of pathway enrichment analysis of the genes selected by ACT from these cytoplasmic circRNAs with miRNA sponge activity demonstrated that these genes tended to be enriched or clustered in the same pathways (Fig. 3c). In sharp contrast, the ACT-selected genes from two nuclear circRNAs (either top- or bottom-ranked ones) showed no pathway enrichment (gene lists provided in Additional file 1: Table S2). The lack of convergence in the regulation of the pathways implied that the molecular functions of these nuclear circRNAs were less likely to be as regulators than as miRNA sponges.

ACT enables the distinction of circRNAs with or without potential miRNA sponge activity

To further elucidate the performance and potential application of ACT, we evaluated the convergence of pathway regulation among different metric-derived gene lists. The target genes of the top 10% of miRNAs according to the
pairing score or binding energy in the given circRNA sequence were subjected to pathway enrichment analysis. While enrichment analyses from the gene lists derived from the ranked energy or scores failed to distinguish circRNAs with/without sponge activity from multiple miRNAs (Fig. 4, left and central panels), pathway analyses with ACT-selected gene lists showed significantly more convergent pathways (Fig. 4, right panel). This implied that the genes targeted multiple times by circRNA-associated miRNAs tend to be more biologically significant. ACT is a novel tool to dissect the molecular and cellular functions of circRNAs. Compared to other pioneer databases for annotating circRNA/miRNA interactions (Additional file 1: Table S3) [13, 14, 27], ACT not only provides a list of interacting miRNAs, but also a ranked gene list in a manageable length ready for further functional and/or experimental characterization.

**Fig. 3** ACT-selected genes identified important biological pathways. **a** The exported ACT results for circHIPK3. The top 100 genes ranked by their common targeting times (parenthesized) are shown at the bottom. **b** The distribution of the common targeting times of circHIPK3-regulated candidate genes is shown as a pie chart. **c** The ACT-prioritized genes (top 100) and low ranked genes (bottom) from circHIPK3, circCCDC66, circPVT1 and circIRAK3 were subjected to pathway enrichment analysis.
Conclusion
Taken together, analysis using ACT-selected genes provided a novel and intuitive method to differentiate the molecular and biological functions of circRNAs. Incorporating the concept of co-regulation by multiple circRNA-associated miRNAs provides a straightforward method for assessing the potential targets of circRNAs and will help prioritize the candidates as well as identify major pathways for the functional study of circRNAs.

Availability and requirements
Project name: ACT
Project home page: http://lab-x-omics.nchu.edu.tw/ACT_Server/
Operating system(s): Platform independent (Web-based service)
Programming language: Perl 5 and R 3.4.3
Other requirements: N/A
License: GNU GPL; non-academic user: license needed

Additional file
Additional file 1: Figure S1. A schematic illustration of miRNA–gene interaction. Figure S2. Metrics comparison for circRNA-associated miRNAs. Table S1. The miRNA-related metrics for circRNA. Table S2. Gene lists from ACT for pathway analysis. Table S3. The comparison of platforms/tools for circRNA–miRNA–gene network. (PDF 270 kb)

Abbreviations
ACT: Analysis of common targets; circRNA: Circular RNA; CT: Common targeting time; miRNA: MicroRNA

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Authors’ contributions
YCLi, YCLe and KLC developed the concept and algorithm. YCLi and KYH integrated the databases, implemented the codes and established the web service. YCLI, YCLe and KLC analyzed and evaluated the results. YCLi and YCLe drafted the manuscript. KYH revised the manuscript and supervised the project. All authors have read and approved the final version of the manuscript for publication.

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Availability of data and materials
Web service is made freely available at http://lab-x-omics.nchu.edu.tw/ACT_Server.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
