2022 IEEE 4th International Conference on BioInspired Processing (BIP) | 978-1-6654-7048-3/22/$31.00 ©2022 IEEE | DOI: 10.1109/BIP56202.2022.10032479

miR-let-7a-2, miR103a-2 and CREB1-TF as therapeutic targets to regulate the transcription of DISC1 and PDE4D in the transcriptional regulation pathway by DISC1/ATF4 complex

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***Abstract*—Disrupted In Schizophrenia 1 (DISC1) is considered a multifunctional protein implicated in various signaling pathways with neurological relevant outcomes, with the Disrupted In Schizophrenia 1/Activating Transcription Factor 4 (DISC1/ATF4) transcriptional regulation pathway being a pathway of interest since it regulates Phosphodiesterase 4D (*PDE4D)* transcription. Alterations in the transcription levels of *DISC1* and *PDE4D* have been implicated in neurodegenerative processes and the identification of therapeutic targets that regulate these processes can be of great help to promote neuroprotective processes. By using a System Biology approach, we created a mathematical model of the transcriptional regulation of the DISC1/ATF4 pathway and using this model we conducted *in-silico* experiments to identify *potential* therapeutic targets with robust control on the network. As a result, miR-let-7a-2 is reported as a transcriptional regulator of *DISC1* as well as miR103-a2 and CREB1-TF as transcriptional regulators of the *PDE4D*. These three regulators are identified as *in-silico* therapeutic targets to promote neuroprotective processes against neurodegenerative mental disorders.**

Keywords— DISC1, PDE4D, System biology, microRNA, miR-let-7a-2, miR103a-2, CREB1

I Introduction

Schizophrenia is considered a multifactorial disorder due to the contribution of multiple susceptibility genes, which can interact with epigenetic processes and environmental factors [1]. For instance, when performing studies with cytogenetic markers, a translocation was reported in a proband with adolescent conduct disorder and in members of four generations of his extended family [2]. A follow-up of this family for 20 years showed an increased incidence of major psychiatric disorders among family members harboring that translocation, including diagnoses of schizophrenia and recurrent major depression. Furthermore, they pointed out that in affected subjects, the balanced chromosomal translocation t(1;11) (q42.1;q14.3) and, therefore, the dysregulation of these segments, could be associated with schizophrenia [3].

At that translocation level, an open reading frame for a coding gene was found with a predicted coding sequence consisting of 13 exons spanning 2565 nucleotides until the termination signal. This gene has been named Disrupted in Schizophrenia 1 (DISC1) [4]. DISC1 has been reported to play an important role in early development [5] and has been implicated in various signaling pathways with neurologically relevant outcomes, such as processes of neuronal maturation, neuronal memory, plasticity, neuronal migration, and proliferation of progenitor neurons [6].

DISC1 is often referred to as a scaffold protein due to its ability to bind to many proteins, especially proteins involved in important processes for proper neuronal development [7], which is why it is considered a multifunctional protein that transports and facilitates the interaction between other proteins [8]. Indeed, the main regulatory activity of DISC1 seems to be to inhibit enzymes by direct contact [7] or by the recruitment of repressive cofactors [9].

Nevertheless, the DISC1 interactome proposed by Camargo et al. [10], lists more than 200 possible interactions involving DISC1. The network of interactions has shown potential therapeutic targets by showing the affectation of DISC1 transcription levels in mouse brains using atypical therapeutic antipsychotic drugs [11]. Similarly, mouse models with mutations in DISC1 present phenotypes related to schizophrenic characteristics, which are partially reversed by the administration of antidepressants and antipsychotics [12].

On the other hand, one *in-silico* approach has been reported to discovery microRNA-Transcriptional Factors (miRNA-TF) regulations of DISC1 interactome [13], however, this approach focuses on interacting proteins involved on neuronal migration regulation and does not included the Disrupted In Schizophrenia 1/Activating Transcription Factor 4 (DISC1/ATF4) transcriptional regulation pathway. The ATF4 transcriptional activity is repressive while Activating Transcription Factor 4 (ATF4) is bound to DNA target sites by recruiting the transcriptional repression factor Nuclear Receptor Co-Receptor (N-CoR) [9]. Furthermore, this process is regulated by Protein Kinase A (PKA) signaling which reduces nuclear localization of DISC1 [14]. In addition, the DISC1/ATF4 complex directly affects PED4 transcription which is also phosphorylated and regulated by PKA and interacts with specific protein complexes to influence local cyclic Adenosine Monophosphate (cAMP levels) [14].

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Despite these observations about the transcriptional activities of DISC1, there are no reports of miRNA-mediated interactions potentially controlling *DISC1* expression levels or their closely related interaction partners for the DISC1/ATF4 transcriptional regulation pathway.

Given the extent of the DISC1 interactome, the identification of therapeutic targets is complex for traditional molecular biology research methods [6], so it is necessary to adopt a computational strategy that allows the study of biological processes. The development of mathematical models and simulations facilitate the possibility of predicting the properties, behaviors and responses to alterations of the systems, which may not be evident with direct observation, improving the understanding of how biological properties emerge from the interactions between the components of biological systems, facilitating the identification of new therapeutic targets, drug repositioning as well as the implementation of drug combinations [15].

In this paper, we present a transcriptional based model focusing on the interactions of DISC1/ATF4 transcriptional regulation pathway of Phosphodiesterase 4D (PDE4D), a key component in the Protein Kinase A/Phosphodiesterase 4D (PKA/PDE4) signaling pathway which regulates the cyclic Adenosine Monophosphate (cAMP) degradation. This model enables the *in-silico* identification of the network nodes with the highest potential control to regulate the inhibition of *PDE4D* transcription, through the transcriptional regulation pathway of DISC1/ATF4, thereby downregulating the degradation of cAMP by PKA/PDE4 signaling.

Our results present the construction of a mathematical model for the transcriptional regulation pathway by DISC1/ATF4 and the identification of miR-let-7a-2, miR103-a2 and CREB1-TF as *in-silico* therapeutic targets to regulate the transcription of *DISC1* and *PDE4D*. This search enables the identification of possible new therapeutic targets to promote protective processes against neurodegenerative mental disorders.

II Materials and Methods

A. Constructions and Visualization of the Network Topology

The construction of the network topology was done with the use of the BioNetUCR v1.3.0 program [16]. A list of genes of interest was selected including the main genes involved in the transcriptional regulation pathway of the DISC1/ATF4 complex and the PKA/PDE4 signaling pathway. This list consists of the DISC1, ATF4, PDE4D, Phosphodiesterase 4B (PDE4B), Nuclear Receptor Corepressor 1 (N-CoR1), Nuclear Receptor Corepressor 2 (N-CoR2), and NudE Neurodevelopment Protein 1 (NDE1) [7].

Once the topology was created, the model was fed with experimental expression and copy number data of the cell lines included in the NCI60 panel [17]. The network was visualized using the Cytoscape v3.9.1 program [18]. In addition, the range of differential expression of DISC1 for the NCI60 panel was confirmed by the network visualization of the expression levels for a cell line with the highest expression level of DISC1 and a cell line with its lowest expression level.

B. Parameter estimation and network simplification

Next, the network was automatically converted into an ODE mathematical model using BioNetUCR and its parameters were fitted using the COPASI v.4.35.258 program [19] with the Hooke & Jeeves method. We adjusted the limits for the values of each parameter and simplified the network using sensitivities to identify the nodes without control over our interest genes, these nodes were removed and then, a new fitted process was started. This was done iteratively until a simplified model was reached and a target value close to one, was done. Fitting results were evaluated using t-student's for paired samples tests (α=0,05) and by visualization of differences between experimental expression data and *in-silico* results.

C. Identification of therapeutic targets by in-silico experiments

Finally, *in-silico* experiments were carried on using COPASI tools, "Parameter Scan" and "Time Course" to identify the best control points representing potential therapeutic targets and evaluate the effect of their perturbation on a time lapse.

For this, optimal alteration values were defined for each candidate target using the parameter scan and then perturbations were applied to the model by modifying the global quantities of each candidate target and visualizing its effects on the gene of interest using the same parameter scan tool. In this way, *DISC1*, experiments were performed perturbing miR-let-7a-2, miR93a, TRIM28-TF, as well as combinations of miR-let-7a-2+miR93a, miR-let-7a-2+TRIM28-TF, miR93a+TRIM28-TF and miR-let-7a-2+miR93a+TRIM28-TF. Similarly, PDE*4D* experiments were performed perturbing miR26-a2, miR181-b2, miR122, miR103-a2, miR18-b, miR16-2, CREB1-TF and ESR1-TF, as well as combinations of miR103-a2+miR18, miR103-a2+miR16-2, miR18-b+miR16-2, miR103-a2+miR18-b+miR16-2, CREB1-TF+ESR1-TF and miR103-a2+CREB1-TF.

Next, to rule out candidate targets that generate adverse effects on the model, we assessed the stability of the model using a Time Course.

Finally, to determine the absence of off-target effects between *DISC1* and PDE4D regulation, *in-silico* experiments were performed combining the selected species.

All the *in-silico* results were plotted in a heatmap for analysis and comparative purposes.

III Results

A. Characterization for the new DISC1/ATF4 transcriptional regulation pathway network topology

In order to identify the best control points for the DISC1/ATF4 pathway network, first, we need to create a network topology for the DISC1/ATF4 transcriptional regulation pathway. To achieve that, we introduced our seven genes of interest into the BioNetUCR v1.3.0 program [16] using the "Add Forward <node>" and "Add Backward <node>" commands to include the interactions, in both ways, for each of the selected genes, with microRNA (miRNA), transcriptional factors (TF) and other interacting genes. In addition we used the "Delete autoregulated edges" and the

"Delete source nodes" commands at the end of the process to create our initial topology. Finally, we visualize the initial topology using Cytoscape v3.9.1 program [18]. As result, we constructed an initial topology containing 11 genes, 21 TFs, 56 miRNAs, 88 nodes and 347 edges (Figure 1a).

We next aimed to simplify the network deleting nodes without evidence of control over any of the genes of interest. To achieve this, first, we fed the model with experimental expression and copy number data of the cell lines included in the NCI60 panel [17]. Next, the network was converted into an ODE mathematically model with BioNetUCR and exported to COPASI where iterative fitting is performed using the Hooke & Jeeves method and sensitivities tests as previously described, until a simplified model was reached. At the end of this process, a final simplified topology was achieved, as Figure 1b shows, the final network has 4 genes, 7 TF, 9 miRNA, 20 nodes and 31 edges.

Moreover, to show the feasibility and ability of the model to explain experimental data and perform *in-silico* experiments, kinetic stability, t-test and differential expression analysis was made. First, the kinetic stability analysis shows the model as asymptotically stable, without evidence of oscillation, bistability or bifurcations. Next, t-student's for paired samples tests (α=0,05) shows a good fitting for almost all the species with HIF1A-TF and PDE4B being the exception (Figure 1c). Therefore, the data corresponding to these species cannot be explained by the model and were therefore excluded from the model. Also, *PDE4D* did not reached the threshold but we considered that the model is capable of explain most of its behavior as only several experimental cell lines were outliers in our analysis. In addition, graphical comparison of model and experimental data can be seen in Figure 1d, showing indeed a mathematical model able to describe the experimental data of our target genes, with PDE4B, as mentioned before, been the exception. Finally, Figure 1e confirms the existence of a range differential expression of *DISC1* for the NCI60 panel were a high level *DISC1* expression line (LE-SR) shows high expression of multiple nodes compared to a low level *DISC1* expression line (CNS-SF 539).

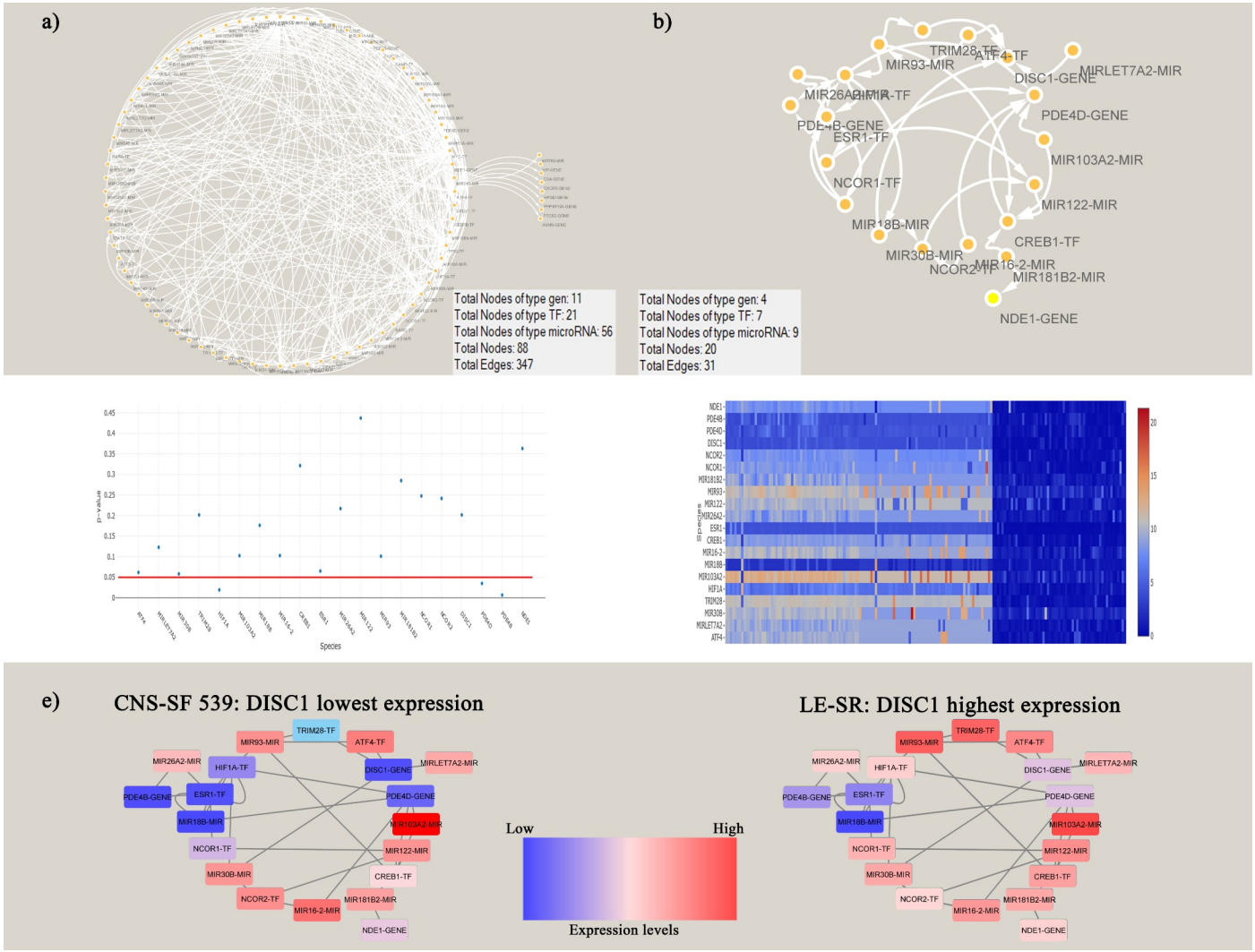

Fig. 1. Characterization of a new DISC1/ATF4 transcriptional regulation pathway network topology.
a) Initial topology without fitting nor simplification.
b) Final topology after several processes of fitting and sensitivities simplifications.
c) T-Test for all the species in the final topology.
d) Graphical comparison of modeled and experimental data.
e) Graphical visualization of differential expression of *DISC1* with a low expression *DISC1* vs high expression *DISC1* in the final topology using two different expression lines from the NCI60 panel. (CNS-SF 539 line has no expression data for TRIM28-TF)

B. *Identification of initial candidate targets as possible control points for the DISC1/ATF4 transcriptional regulation pathway by sensitivity analysis*

To determine potential candidate targets as control points on the DISC1/ATF4 transcriptional regulation pathway model we ran sensitivity analysis using COPASI tasks [19]. Figure 2 shows which target genes are affected by which parameters. In total the model identifies nine miRNA and four TFs, however, miR30b and HIF1A have low impact on the target genes, thus these two were excluded from further analysis. These results suggest that miR-let-7a-2, miR93a and TRIM28-TF may be control points for *DISC1* transcription. Furthermore, miR103-a2, miR18b, miR16-2, miR26-a2, miR122, miR181-b2 as well as CREB1-TF and ESR1-TF can control the *PDE4D* expression.

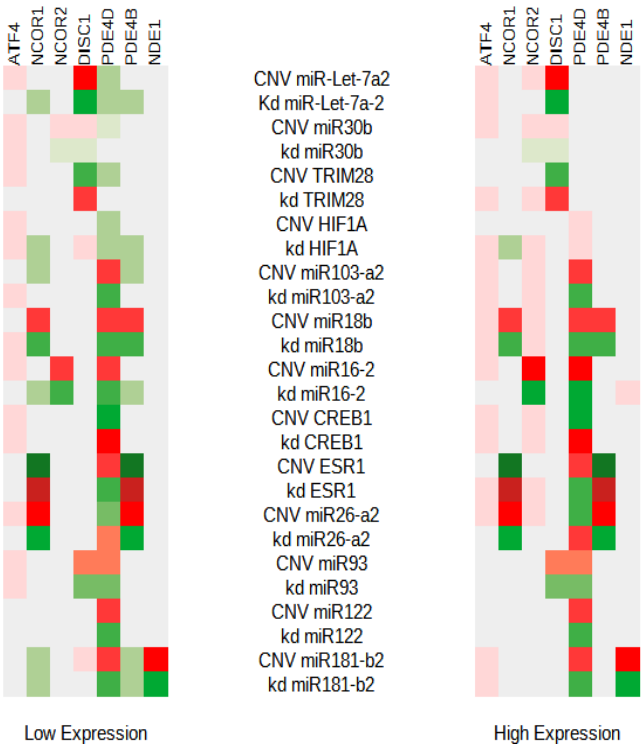

Fig. 2. Identification of candidate targets as possible control points for the DISC1/ATF4 transcriptional regulation pathway by sensitivity analysis. (Columns: Interest target genes, Rows: Candidate target parameters)

C. *Identification of non-candidate targets by detecting unharmonized regulatory effects with time course plot after model perturbation*

Despite previous results, miR18b, miR16-2, miR26-a2, miR122, miR181-b2 and ESR1-TF have effects over other target genes such as *NCOR1*, *NCOR2*, *PDE4B* and *NDE1*. Although this is not necessarily negative, effects in opposite directions to those desired for a harmonized regulation of the pathway were verified by perturbing the model with changes in these species and visualizing their effects in a time course plot. Figure 3 depicts the influence of a decreased expression in *PDE4D* with on the remaining six species, a perturbation that is also generated by decreasing the expression of *NCOR2*. However, NCOR proteins are necessary for the assembly of the ATF4 inhibition complex by DISC1 [4], therefore, these types of effects should be absent in suitable regulatory targets.

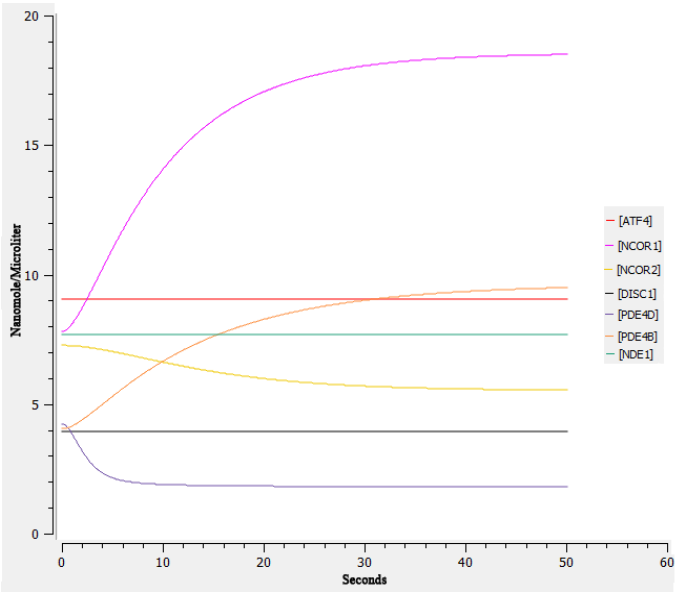

Fig. 3. Identification of non-candidate targets by detecting unharmonized regulations effects with time course plot after model perturbation.

D. *In-silico experiments with the mathematical model led to the identification of miR-let-7a-2, miR103a-2 and CRB1-TF as therapeutic control points for the DISC1/ATF4 transcriptional regulation pathway*

To identify control points that allow either the regulation of *DISC1* expression or *PDE4D* expression, a series of *in-silico* experiments were performed using COPASI's parameter-scan tool, generating perturbations in the copy number variant (CNV) or degradation constant (kd) of each species identified as a candidate in the sensitivity analysis. Each experiment was performed using data from both cell lines, LE-SR for the highest level of *DISC1* expression and CNS-SF 539 data from the cell line with the lowest level of expression for *DISC1*. Thus, assuming a downward dysregulation of *DISC1* in the line with CNS-SF 539, we sought to increase the expression of *DISC1* and decrease the expression of *PDE4D*, while, on the other hand, we sought to generate the opposite effect in the cell line LE-SR, assuming an upward dysregulation, we aim to detect a control point capable of decreasing *DISC1* expression and increase *PDE4D*.

To achieve our aim, in the case of increasing the expression of *DISC1*, the kd levels for miR-let-7a-2 and miR93 were increased, as well as the CNV levels for TRIM28-TF. In contrast, to decrease the expression of *DISC1*, the CNV levels of miR-let-7a-2 and miR93 as well as the kd of TRIM28-TF were increased. In addition, experiments were performed making combinations of the candidate targets.

Figure 4a has evidence that both, TRIM28-TF and miR93, do not influence the expression of *DISC1* in both cell lines, however, miR-let-7a-2 does generate a strong impact on the expression of *DISC1*. Moreover, the combined experiments confirmed these effects only when miR-let-7a-2 is included, while in its absence, the expression levels of *DISC1* remain stable. Together, these results demonstrate that miR-let-7a-2 is a control point that regulates *DISC1* transcription *in-silico*.

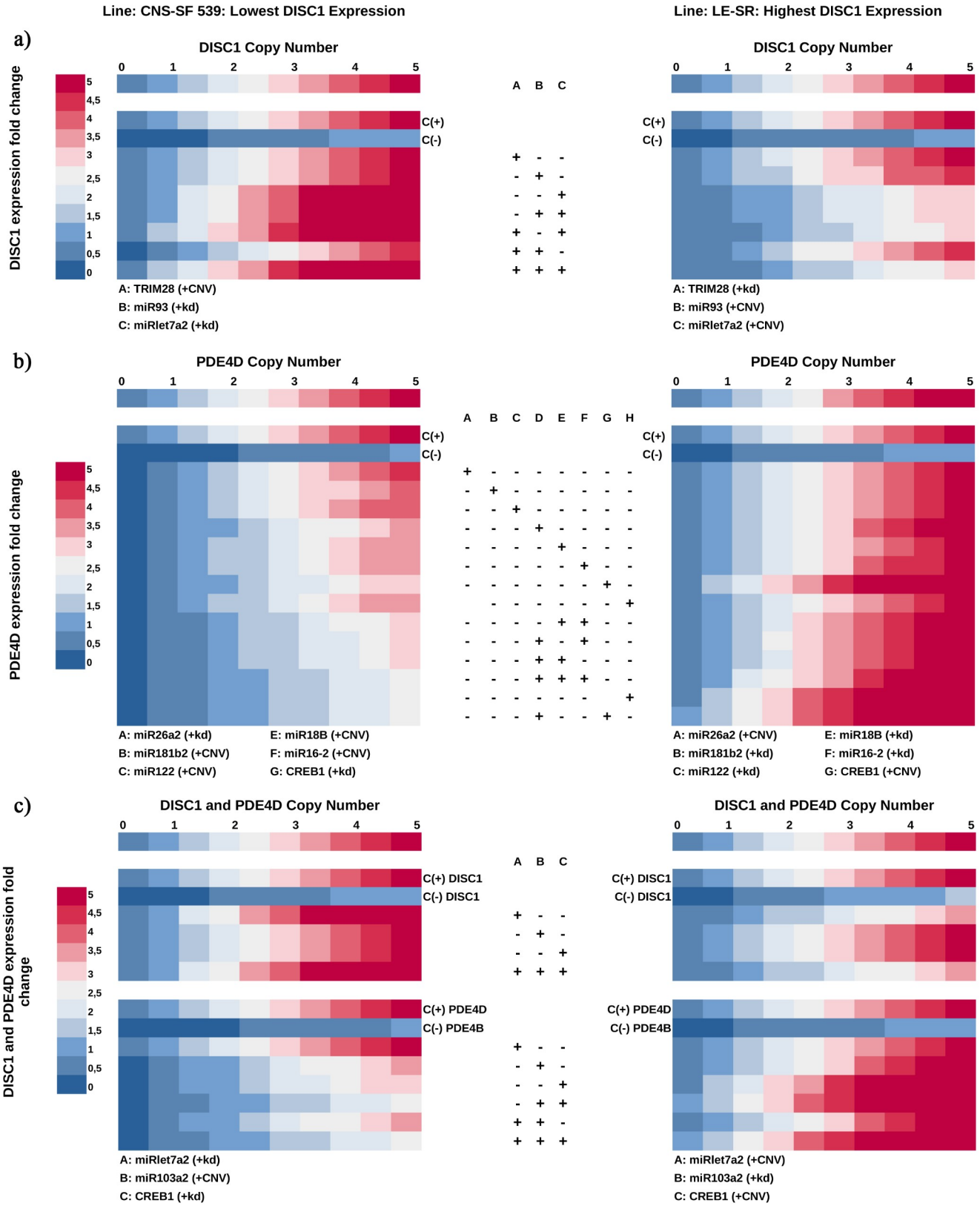

Fig. 4. *In-silico* experiments with the mathematical model led to the identification of miR-let-7a-2, miR103-a2 and CRB1-TF as therapeutic control points for the DISC1/ATF4 transcriptional regulation pathway. a) DISC1 experiments, b) PDE4D experiments, c) DISC1 and PDE4D cross-effect experiments.

Next, to decrease the expression of *PDE4D*, the CNVs of miR181b2, miR122, miR103a2, miR18B, miR16-2 and ESR1-TF were increased, as well as the kd of CREB1 and miR26a2. Meanwhile, to increase the expression of *PDE4D*, the levels of kd for miR181b2, miR122, miR103a2, miR18B, miR16-2 and ESR1-TF were increased, as well as the CREB1 and miR26a2 CNVs. In addition, experiments were also performed making combinations of the candidate targets. Figure 4b shows that miR26-a2, miR181-b2 and miR122 have no effect on *PDE4D* expression in both cell lines, while, on the other hand, miR103-a2, miR18-b, miR16-2, CREB1- TF and ESR1-TF have impacts on *PDE4D* expression. Furthermore, the combined experiments of the three microRNAs that show effects allowed us to identify a greater effect on expression with the combination of those two microRNA perturbations, regardless of which combination is involved, and the effect is further increased when perturbing the three microRNAs, which indicates an additive effect of microRNAs in the regulation of *PDE4D* expression. Moreover, the perturbation of the transcription factors also show effects on the expression of both cell lines, which increases when both transcription factors are perturbed in the same experiment. Despite this, the occurrence of unwanted effects, as mentioned above, on other model species rules out miR18-b, miR16-2, and ESR1-TF as control points. However, CREB1-TF and miR103-a2 are free from these effects and, furthermore, the combination of both generates a regulatory effect as strong as the other combinations tested for *PDE4D*. All these results demonstrate that miR103a-2 and CREB1-TF are checkpoints that regulate *PDE4D* transcription in the DISC1/ATF4 transcriptional regulation pathway.

Finally, to rule out effects of miR-let-7a-2 on *PDE4D* expression, and effects of miR103a-2 as well as CREB1-TF on *DISC1* expression, experiments were performed combining them. Figure 4c results confirm that there are no cross-effects of the control of one gene over the other.

IV DISCUSSION

We present a model for the DISC1/ATF4 transcriptional signaling pathway, constructed with a system biology approach, capable of identifying therapeutic targets *in-silico* for the regulation of *DISC1* and *PDE4D* transcription.

Since its discovery, *DISC1* have been associated with different neuropsychiatric phenotypes as a risk factor [20] and has been implicated in various neurologically relevant signaling pathways [6]. Furthermore, DISC1 downregulation in mice generates anxiety-like behavior, sociability impairment and damage at both synaptic plasticity and recognition memory in mice. Moreover, mice transcriptome analyses showed that there were evident transcriptional changes [21]. These results support the importance of transcription regulation of *DISC1* and our model selection of miR-let-7a-2 as a target for *DISC1* regulation.

Despite the important role of microRNAs to repress gene expression, few attempts have been made to report microRNA regulation of *DISC1*. Polymorphisms in the *DISC1* 3'UTR predict an allele specific regulation of *DISC1* levels by miR135b-5p [22]. Nevertheless, the most important attempt used an *in-silico* approach to identify miRNA-TF regulation of DISC1 interactome and predicts three miRNA-TF feedback loops, miR233-TAL1, miR320a-TCF3 and miR155-STAT3, with the capacity of regulating DISC1 interactome [13]. However, the study was made considering the neuronal migration roles of the DISC1 interactome and does not include the DISC1/ATF4 transcriptional regulation pathway, therefore, to our knowledge, there are no previous reports on miR-let-7a-2 regulation of *DISC1*.

In general, let-7 miRNA family is one of the first discovered miRNAs and has a high level of evolutionary conservation. Furthermore, let-7 family members promote differentiation on development stages and tumor suppression in various cancers [23]. Despite been more studied in lung Cancer [24] and as a biomarker of efficacy prognosis of radiotherapy [25], a role of let-7 activating Toll-like receptor 7 is associated with neurodegeneration, inducing a dose time dependent neuronal loss [26]. These reports show their key roles of on neurological pathways. These reports also agree with our identification of miR-let-7a-2 as a target to promote neuroprotective processes.

On the other hand, PDE4D is known for playing an important role mediating memory via the control of intracellular cAMP signaling [27]. Mice test associates deficient PDE4D levels with enhanced memory, hippocampus neurogenesis and increase of phosphorylated CREB, suggesting that the search for a selective inhibitor is promising to treat neurodegenerative disorders such as Alzheimer´s [27]. Furthermore, using Bioinformatics approaches, eight miRNA were predicted to target *PDE4D* in mouse (miR139-5p, miR335-5p, miR340-5p, miR7-5p, miR103a-3p, miR19b-3p, miR18a-5p, and miR16-5p) with miR139-5p with the most significant difference. Interestingly, miR103a-3p also indicates significance differences (p=0,01). However, further analysis with this miRNA was not carried on [28]. Moreover, an *in-vitro* study suggests neuroprotective effects when a decrease in *PDE4D* is achieved by the upregulation of miR219a-5p [29]. Finally, miR103 has been found to be at low levels in patients with Alzheimer's and Parkinson's and has been recommended as a biomarker of disease risk and progression in Alzheimer's [30]. These findings evidence the role of *PDE4D* expression levels in neurodegeneration and how the use of miRNAs can help to promote neuroprotective effects. Moreover, these results are in agreement with our finding of miR103-a2 as a control point and therapeutic target for neurodegenerative disease since our model demonstrated the capacity of miR103-2a to regulate *PDE4D* in both directions, decreasing or increasing *PDE4D* expression.

Furthermore, CREB1 polymorphisms have been associated with increased risk of mayor psychiatric disorders [31] [32] and brain data expression analysis observed significant downregulation of CREB1 in psychiatric patients [32] suggesting an important role of CREB1 conferring risk or generating protection against psychiatric disorders. These results support our CREB1-TF

identification as a control point target to promote neuroprotective processes by enhancing its expression and improving its haplosufficiency.

In conclusion, using a systems biology approach, we present the construction of a mathematical model for the transcriptional regulation pathway of DISC1/ATF4. Furthermore, using this mathematical model, we identified targets that allow inhibiting the synthesis of *DISC1* as well as the transcription of *PDE4D*, emerging miR-let-7a-2, to regulate *DISC1* synthesis, and miR103-a2 and CREB1-TF, to alter *PDE4D* transcript levels, as the best therapeutic targets *in-silico* to promote protective processes against mental disorders. Finally, the use of transcriptional data from patients with major mental disorders and healthy controls could be used to feed the model and obtain a more accurate fitting to determine the effects over cAMP and PKA, which are important new directions for future *in-vivo* studies to validate the model.

A. Limitations of the study

The candidate's identifications are limited to the information contained in the NCI60 data set.

The regulatory networks constructed depend on the experimentally reported interactions available. Due to this, future inclusions of interactions could reveal new nodes that expand the network and allow considering novel candidate targets.

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