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ULSAN NATIONAL INSTITUTE OF SCIENCE AND TECHNOLOGY

Master's Thesis

# A study on the regulatory mechanism of DSCR1 expression

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Department of Biological Sciences

Ulsan National Institute of Science and Technology

2021

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# A study on the regulatory mechanism of DSCR1 expression

A thesis/dissertation submitted to
Ulsan National Institute of Science and Technology
in partial fulfillment of the
requirements for the degree of
Master of Science

Jiwon Park

12 / 02 / 2020 of submission

Approved by

Advisor
Jae-Ick Kim

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# A study on the regulatory mechanism of DSCR1 expression

Jiwon Park

This certifies that the thesis/dissertation of Jiwon Park is approved.

12 / 02 / 2020 of submission

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## Abstract

Common features or prominent complications of Down Syndrome (DS) patients who have part of chromosome 21 as trisome made researchers interested in studying the DS related proteins. DSCR1 (Down Syndrome Critical Region 1) was found as the one of the critical proteins which markedly affects DS patient's abnormality. A number of studies have been carried out to reveal the cellular functions of DSCR1, focusing on its duality of functions (beneficial or detrimental) in diverse conditions when this protein is either up-regulated or down-regulated. Although there have been some efforts in the past to interrogate the regulatory mechanisms of DSCR1 expression, our overall understanding of this mechanism remains fragmentary. In this study, we aimed to understand the regulatory mechanisms of DSCR1 expression, which might be utilized to develop therapeutic strategies for various disease conditions including leukemia, heart defects, and Alzheimer's diseases. Here, we found that RALY isoform 2 protein may play a potential role in the regulation of DSCR1 expression as a suppressor in the endothelial cell. This study definitely requires further research for better understanding of detailed regulatory mechanisms and molecular players implicated in this process. However, our data still provides insight into potential strategy of DSCR1 regulation for gene therapy for DS.

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# Abbreviations

| | |
|---|---|
| 5xFAD | 5 familial Alzheimer's disease mouse model |
| AAV | Adeno-associated virus |
| ABC | Ammonium bicarbonate |
| ACN | Acetonitrile |
| AD | Alzheimer's disease |
| ANOVA | Analysis of variance |
| AP-MS | Affinity purification coupled with mass spectrometry |
| ATF4 | Activating transcription factor 4 |
| ATP | Adenosine triphosphate |
| BPs | Biological processes |
| BRE | TFIIB recognition element |
| Chr 21 | Chromosome 21 |
| CID | Collision-induced dissociation |
| CN | Calcineurin |
| CNS | Central Nervous System |
| DAPI | 4', 6-diamidino-2-2phenylindole |
| DNA | Deoxyribonucleic acid |
| DS | Down Syndrome |
| DSCR1 | Down Syndrome Critical Region 1 |
| DSCR1 KO | DSCR1 knock-out mouse |
| DSCR1 TG | DSCR1 transgenic mouse |
| DTT | Dithiothreitol |
| eIF2 | Eukaryotic initiation factor 2 |
| FA | Formic acid |
| FBS | Fetal bovine serum |
| FC | Fold change |
| FDR | False discovery rate |
| Fluc | Firefly luciferase |
| gDNA | Genomic DNA |
| GO | Gene ontology |
| GTF | General transcription factor |
| IAM | Iodoacetamide |

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| | |
|---|---|
| IHC | Immunohistochemistry |
| Inr | Initiator |
| ISR | Integrated stress response |
| LC-MS/MS | Liquid chromatography with tandem mass spectrometry |
| MOAB-2 | Monoclonal amyloid beta peptide |
| mRNA | Messenger RNA |
| NRC | Nucleosome-remodeling complexes |
| PFA | Paraformaldehyde |
| PCR | Polymerase chain reaction |
| Pi | 150 mm x 25 mm culture dish |
| PIC | Pre-initiation complex |
| Poly(A) | Poly-adenine |
| qRT-PCR | Quantitative reverse transcription PCR |
| Rluc | Rnilla luciferase |
| RNA | Ribonucleic acid |
| RNA Pol II | RNA polymerase II |
| RRM | RNA recognition motif |
| RT | Room temperature |
| SDS-PAGE | Sodium dodecyl sulfate poly acrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SVEC4-10 | Lymphatic endothelial-like cell |
| TSS | Transcription start site |

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# I. Introduction

**Down syndrome and DSCR1 (Down Syndrome Critical Region 1).**

The condition of Down syndrome (DS) was first described by John Langdon Down in 1866 [1]. About one century later, Lejeune found DS is caused by the triplication of human chromosome 21 (chr21) [2]. DS patients generally have different length of chr21-genome triplication and these differences result in a varying degree of symptoms from mild to severe among DS individuals [3]. However, there are common distinctive features with DS patients, which are intellectual disabilities, learning and memory defect, characteristic facial features, and low muscle tone (hypotonia) in infancy. In addition, DS has accompanying complications, including heart defects [4], leukemia [5], obesity [6], and early-onset Alzheimer's diseases [7]. To search for therapeutic strategies for the common features and prominent complications of DS, lots of research have been actively conducted to understand the functions and related molecular pathways of the genes on chr21.

Among many genes on chr21, Down Syndrome Critical Region 1 (DSCR1) was identified in human chr21 from region 21q22.1-21q22.2 [8] and mouse chromosome 16 [9] by Fuentes. DSCR1 gene is composed of 7 exons and there are two identified isoforms of DSCR1 protein translated from two different mRNA transcripts, DSCR1.1 (exon 1, 5, 6, and 7) and DSCR1.4 (exon 4, 5, 6 and 7) (Figure 1A, B). Both isoforms have the same C-terminal 196 amino acids including calcineurin (CN) inhibitor domain, RNA recognition motif (RRM), phosphorylation of a serine/proline-rich motif (SP), and proline-rich SH3 binding domain (Figure 1C) [10-15]. Owing to these structural characteristics of DSCR1, it contributes to diverse transcriptional regulation and signal transduction [16-19]. The functional domain of the DSCR1 N-terminal region, however, remains not well understood, partly due to its random structural features.

**The diverse functions of DSCR1 in central nervous system.**

DSCR1 was not included in the DS critical region at first. However, it is now considered as a part of the DS critical region because its diverse functions are associated with many phenotypes of DS [20]. DSCR1 is highly expressed in brain and heart of both human and mouse [21]. One of distinctive features in DS is the defect in learning and memory and it was demonstrated that the expression level of DSCR1 is related the intellectual disability [22]. Kyung-Tai Min previously found that Nebula, the Drosophila homolog of DSCR1, plays a key role for mitochondrial function [23] and synaptic development [24]. It has been also reported that DSCR1 functions to regulate spine morphogenesis and local protein synthesis [25], axon growth and steering [26], and hippocampal neurogenesis [13] in the mouse central nervous system (CNS).

DSCR1 has been studied to be implicated not only in the physiological regulation of CNS, but also

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in the pathological regulation of CNS, especially in Alzheimer's diseases (AD) [27-29]. The major hallmarks of AD is the accumulation of amyloid plaques and neurofibrillary tangles in the brain [30]. Interestingly, DS patients have an increased risk of early-onset AD [7]. While the evidence is indirect, it appears that DSCR1 is involved in the modulation of AD pathology [27-29, 31]. In the preliminary data, we found that the manipulation of DSCR1 expression significantly reduced the amyloid plaques formation in the 5xFAD (Familial Alzheimer's Disease) mouse model of Alzheimer's disease (Figure 2). The precise molecular mechanism of how DSCR1 is related to AD pathology is still unclear. However, it seems very likely that the modulation of DSCR1 expression level is able to attenuate certain aspects of AD pathology.

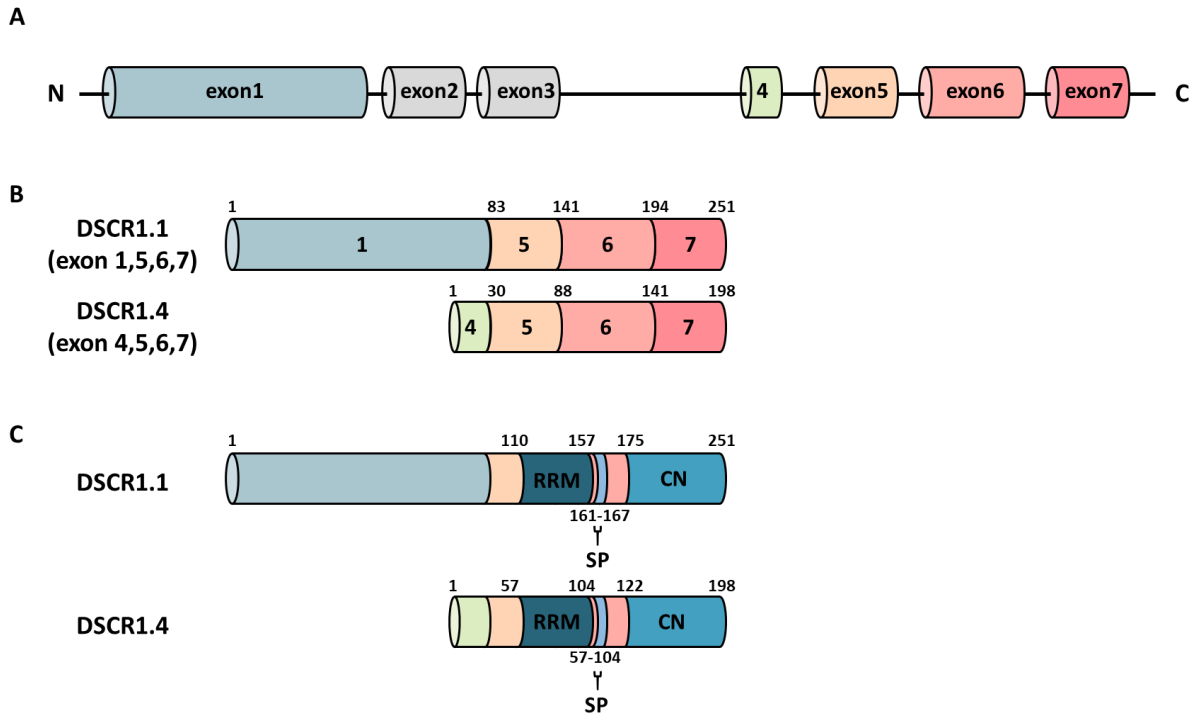


**Figure 1. Schematic diagram of DSCR1.** (A) Schematic diagram of DSCR1 components. DSCR1 consists of 7 exons. DSCR1 has an intergenic promoter before exon4 region. (B) Schematic diagram of DSCR1 isoforms, DSCR1.1 and DSCR1.4. (C) Schematic diagram of functional domains of DSCR1. Both DSCR1 isoforms have RNA Recognition Motif (RRM), phosphorylation of a serine/proline-rich motif (SP), and C-terminal calcineurin inhibitory domain (CN).

**The regulatory mechanism of DSCR1 gene expression**

Many molecular and cellular events are required for the proper regulation of gene expression. For example, separation of two strands of DNA in the target gene is necessary for the access of

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transcriptional machinery. Unwound DNA sequences, which have a recognition site for transcriptional activator, recruit the transcriptional activator and other transcriptional machinery. Essential transcription factors and associated cofactors bind to each other through protein-protein interactions and form the pre-initiation complex (PIC). Initially formed PIC is remodeled by DNA helicase, and transcription start site (TSS) is revealed. Next, bound transcriptional machinery leaves the promoter region and early elongation step of transcription is initiated, resulting in the synthesis of mRNA. Once mRNA is produced, translational process is initiated for the protein expression of the target gene (mRNA) (Figure 3) [32, 33].

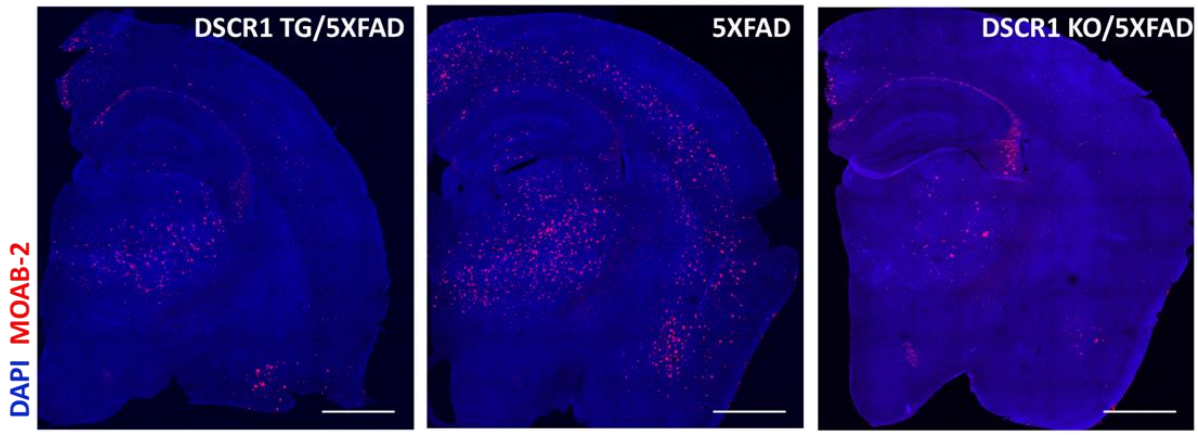


**Figure 2. The potential role of DSCR1 in the regulation of AD pathology.** Immunohistochemistry images from 5-6 months old 5xFAD mice. Left: DSCR1 TG/5xFAD(transgenic mice), Center: 5xFAD (B6SJL background 5xFAD, crossed with C57BL/6J for background match with DSCR1 mutant/5xFAD mice), Right DSCR1 KO/5xFAD (knockout mice). Red signal is MOAB-2 (anti-amyloid beta peptide), and blue signal is DAPI. Scale bars: 1000 μm. Each brain section was 40 μm in thickness. N = 3 per each group.

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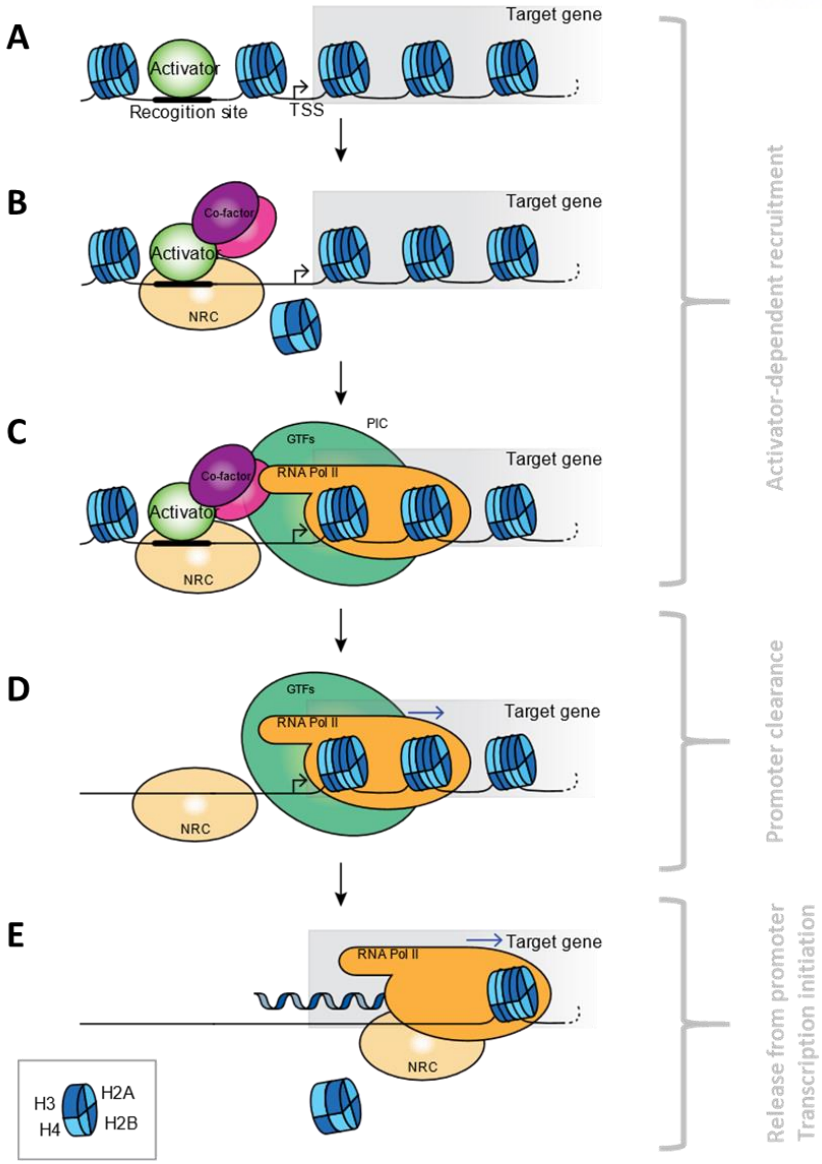


**Figure 3. Schematic diagram of transcription process.** (A) Transcriptional activator binds to the specific DNA sequences (recognition sites) close to the target gene. (B) Bound transcriptional activator recruits large multi-subunit protein co-activator complexes through protein-protein interaction. ATP-dependent nucleosome-remodeling complex such as NRC, is also recruited to remove the histones at the promoter. (C) Displacement of histones facilitate the recruitment of other associated cofactors and general transcription factors; GTFs, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, and RNA polymerase II (RNA Pol II). The association of transcriptional machinery through protein-protein interaction forms the pre-initiation complex (PIC) at the core promoter. Processes from (A) to (C) are activator-dependent recruitment. (D) Formed PIC is modified by transcription factors and unwound transcription start site (TSS). PIC also interacts with RNA Pol II. (E) RNA Pol II dissociates from some GTFs and leave the promoter region. Early transcriptional elongation is initiated, and mRNA is produced.

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The regulation of the specific gene expression has been emerged as a potential strategy for gene therapy and there are currently some developed methods that can be used for the regulation of gene expression, by using pharmacology or/and adeno-associated virus (AAV) [34, 35]. To successfully regulate the expression of target gene, fundamental and detailed knowledge about the target gene is required, including promoter sequence, transcription factors, and associated endogenous signal pathways. Considering the previous researches of the cellular functions of DSCR1, DSCR1 can be a potential target for developing therapeutic application for diverse human diseases. It has been shown that the alteration of DSCR1 expression, by either up-regulation or down-regulation, rescues the defects in learning and memory [13], and suppressed the tumor growth in the Ts65Dn mouse model of Down syndrome [36]. Despite these findings, however, more in-depth knowledge for the practical utilization of DSCR1 is still limited and we do not clearly understand at present how the expression of DSCR1 is fundamentally regulated.

In this study, we tried to decode the essential principle of how the expression of DSCR1 is regulated. Furthermore, we aimed to identify the signaling pathways via which DSCR1 is tightly controlled. Here, we demonstrate the core promoter region of each DSCR1 isoforms, DSCR1.1 and DSCR1.4. Using affinity purification coupled with mass spectrometry (AP-MS), we listed the candidate proteins that can bind potentially and specifically to core promoter region of DSCR1. Among the selected candidate proteins, we observed that RALY regulates the expression of DSCR1. Importantly, we confirmed that RALY protein interacts with core promoter region of DSCR1, while the regulation of DSCR1 via RALY occurs at the translational level. Although much needs to be further investigated, this study establishes fundamental insights into the regulatory mechanism of DSCR1 expression.

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# II. Results

**Identification of the DSCR1 core promoter region.**

Principal components of core promoters, which are TATA box, initiator (Inr), and TFIIB recognition elements (BRE), were considered when designating the candidate promoter sequences of DSCR1 [37-39]. Candidate promoter sequences (target sequences) firstly selected were 1-kb each from TSS, 92,466,061~92,467,198 and 92,467,199~92,468,227 for DSCR1.1 and 92,399,970~92,401,107 and 92,401,108~92,402,136 for DSCR1.4 on mouse chromosome 16. To obtain the selected target sequences, genomic DNA from C57BL/6J mouse was used as a template. To identify the core promoter for transcriptional activation among the target sequences, we generated bicistronic reporters and inserted the target sequence to the system. In bicistronic reporter system, the transcription and translation of first cistron, *Renilla* luciferase (Rluc), are directed by T7 promoter and isolated from the second cistron, *Firefly* luciferase (Fluc) by a random sequence and a poly(A) sequence. Transcription and translation of Fluc depend on the transcription ability of inserted target sequence (Figure 4). Reporter assay was performed in SVEC4-10 cells, which are derived from mouse endothelial cells of axillary lymph node vessels. The ultimate goal of this study is to establish the foundational knowledge for gene therapy targeting DSCR1. Thus, we performed our experiments in this endothelial system, aiming to regulate the gene expression in endothelial cells [40, 41]. SVEC4-10 cell lysates transfected with reporter vector were used to measure Fluc/Rluc ratio and this ratio was used as the indicator of transcription activity of target sequence. To confirm the core promoter region among the target sequences, we narrowed down the sequences up to 100-bp [42]. The target sequences that showed the highest transcriptional activity in DSCR1.1 and DSCR1.4 included TSS and their sizes were about 450-bp and 400-bp, respectively (Figure 5A, B). Transcriptional activity of DSCR1.1 target sequence showed the highest signal at 434-bp upstream (-) to 17-bp downstream (+) from TSS. Among the DSCR1.4 target sequences, -379-bp to +12-bp was the highest. These data suggest that both DSCR1 isoforms contain the core promoter region for transcriptional activation, which are about 420-bp upstream from the TSS.

**Preparation for the interacting proteins with DSCR1 promoter region.**

Transcription initiation process requires many protein factors and co-factors. We hypothesized that among the core promoter interacting proteins, there would be a key protein which can critically regulate the expression of DSCR1. To test this hypothesis, we first constructed the scrambled sequence of DSCR1.1 (-434/+17) and DSCR1.4 (-379/+12) into the bicistronic reporter system. The reporter vector inserted with scrambled sequence was transfected to SVEC4-10 cells and transcriptional activity was examined after 24 hours incubation. We confirmed that scrambled

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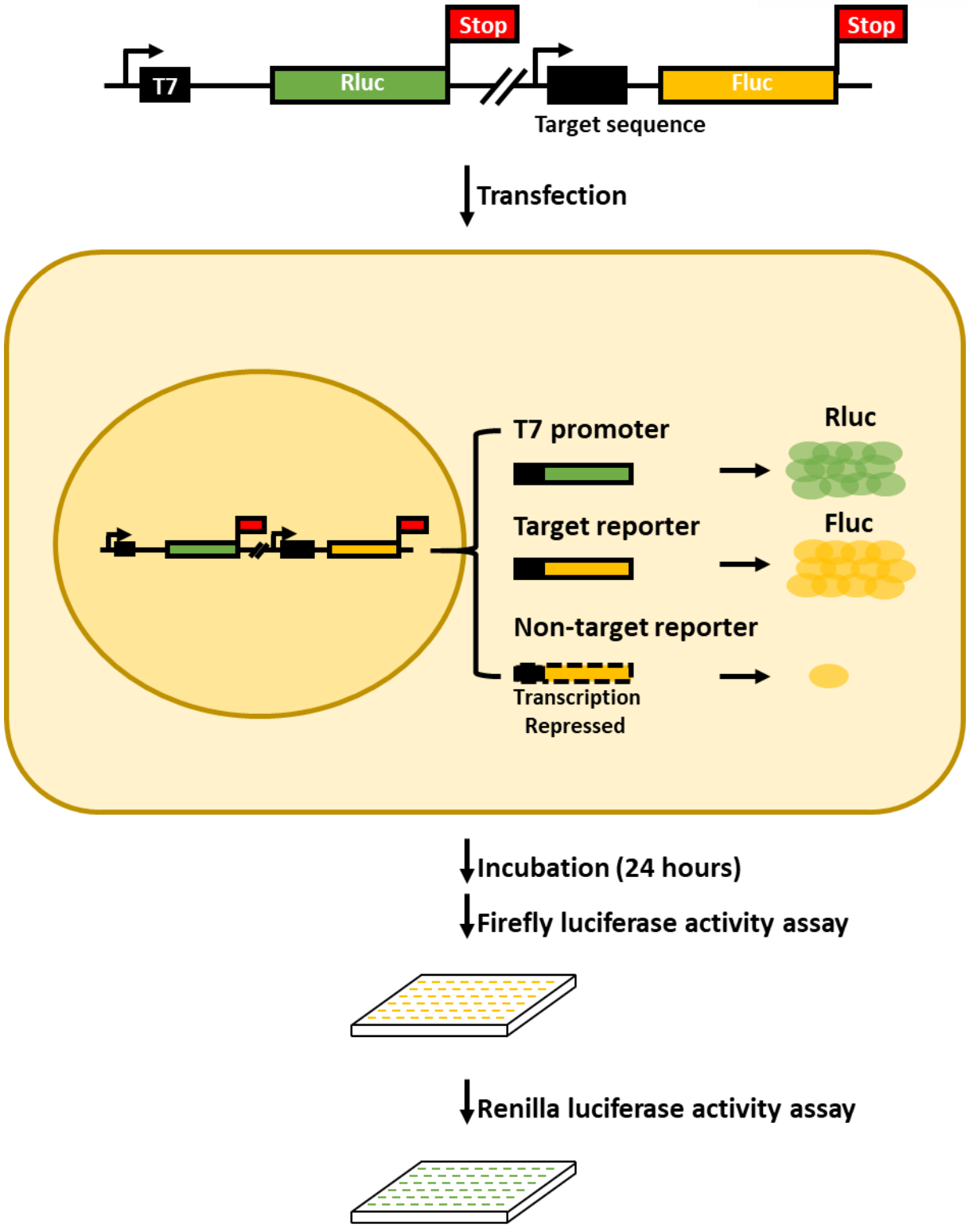


**Figure 4. Schematic diagram of bicistronic reporter system.** Schematic representation of bicistronic reporter system. In the scheme, // represents random sequence and poly(A) sequence for the transcription termination. Second cistron, Fluc, is transcribed only if the inserted target sequence has transcriptional activity.

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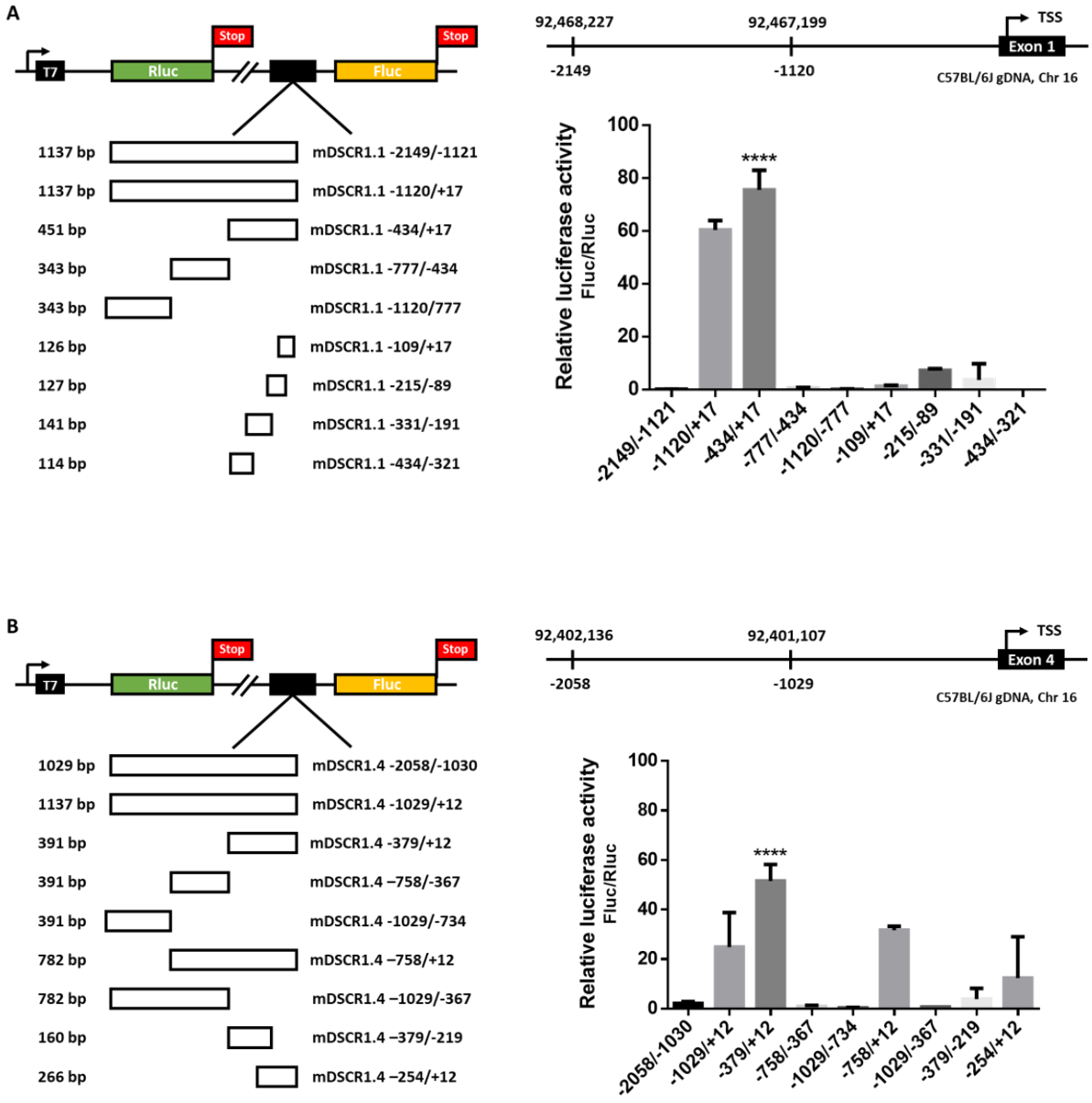


**Figure 5. Identification of DSCR1 core promoter region by bicistronic reporter system.**

(A, B) Transcriptional activity of target sequences is measured by using bicistronic reporter system. Single vector is transfected to SVEC4-10 cells and incubated for 24 hours. Fluc and Rluc luciferase activities is measured by using dual-luciferase assay (Promega). The Fluc signal is normalized by the Rluc signal. Values are mean ± SEM and statistical significance is tested one-way ANOVA with Bonferroni *post hoc* test; N = 3. ****P < 0.0001.

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sequence did not have any transcriptional activity and was used as a negative control for further experiments (Figure 7A, C). Identification of promoter interacting proteins was carried out by AP-MS (Affinity Purification Mass Spectrometry) with biotin-streptavidin pull-down (Figure 6A, B). We biotinylated the target DNA sequence and its scrambled sequence via PCR using 5' biotinylated primers [43]. We incubated the biotinylated DNA with streptavidin beads (Dynabeads M-280) and then added SVEC4-10 cell lysate to DNA-biotin-streptavidin complex for interaction. Target sequence specifically interacting with proteins would only remain after washing this complex. We eluted the complex from beads and sampled the purified proteins for SDS-PAGE gel separation (Figure 6A). After SDS-PAGE gel separation, there are two ways to check interacting proteins, which are silver staining and mass analysis. Silver staining shows the overall proteins from the loading sample. Owing to its chemical properties, however, it is hard to conduct silver staining in parallel with mass analysis. For the identification of interacting proteins, proteins in SDS-PAGE gel were digested with trypsin and in-gel digestion and shotgun LC-MS/MS analysis were performed. Proteins by non-specific, charge-dependent binding could be excluded by comparison with a negative control (Figure 6B). When comparing between the DSCR1.1 promoter sequence and its negative control, the overall proteins detected were similar in the silver staining. However, there were some bands that did not show up in the control sample (about 13, 18, and 33 kDa size) (Figure 7B). The slightly upper band at 35 kDa was identified when comparing the DSCR1.4 promoter sequence with its control sequence. (Figure 7D).

**Identification of the interacting proteins via LC-MS/MS.**

DNA-biotin-streptavidin complex was incubated with 150 pi SVEC4-10 cell lysate per sample. Eluted proteins from complex were separated by SDS-PAGE gel. Then SDS-PAGE gel was cut into 10 pieces by its size and the in-gel digestion was performed for LC-MS/MS sample preparation (Figure 6A, B). The processes of in-gel digestion are de-staining, reduction, alkylation of cysteine residues, and digestion via trypsin. We could identify the proteins that potentially interact with DSCR1.1 promoter region, through shotgun LC-MS/MS analysis. Identified proteins were listed by *Scaffold 4.10.0* and preliminarily classified by species. All identified *mus musculus* species proteins are analyzed via *CRAPome* for scoring the true interaction of our samples [44]. Fold change (FC), t-test (p-value), and SAINT probability (FDR; false discovery rate [45, 46]) were run in parallel by *CRAPome*. The identified proteins were sorted by FC (Figure 8A) and in our CRAPome analysis, FDR less than 0.08% equals to the SAINT probability higher than 0.66. The graph shows the top 10 biological processes (BPs) identified from the satisfied proteins of *CRAPome* estimations (Figure 8B). As the results were produced from the promoter DNA interacting proteins, RNA processing-related BPs generally were positioned at high ranking. 40 proteins out of 584 identified proteins satisfied all

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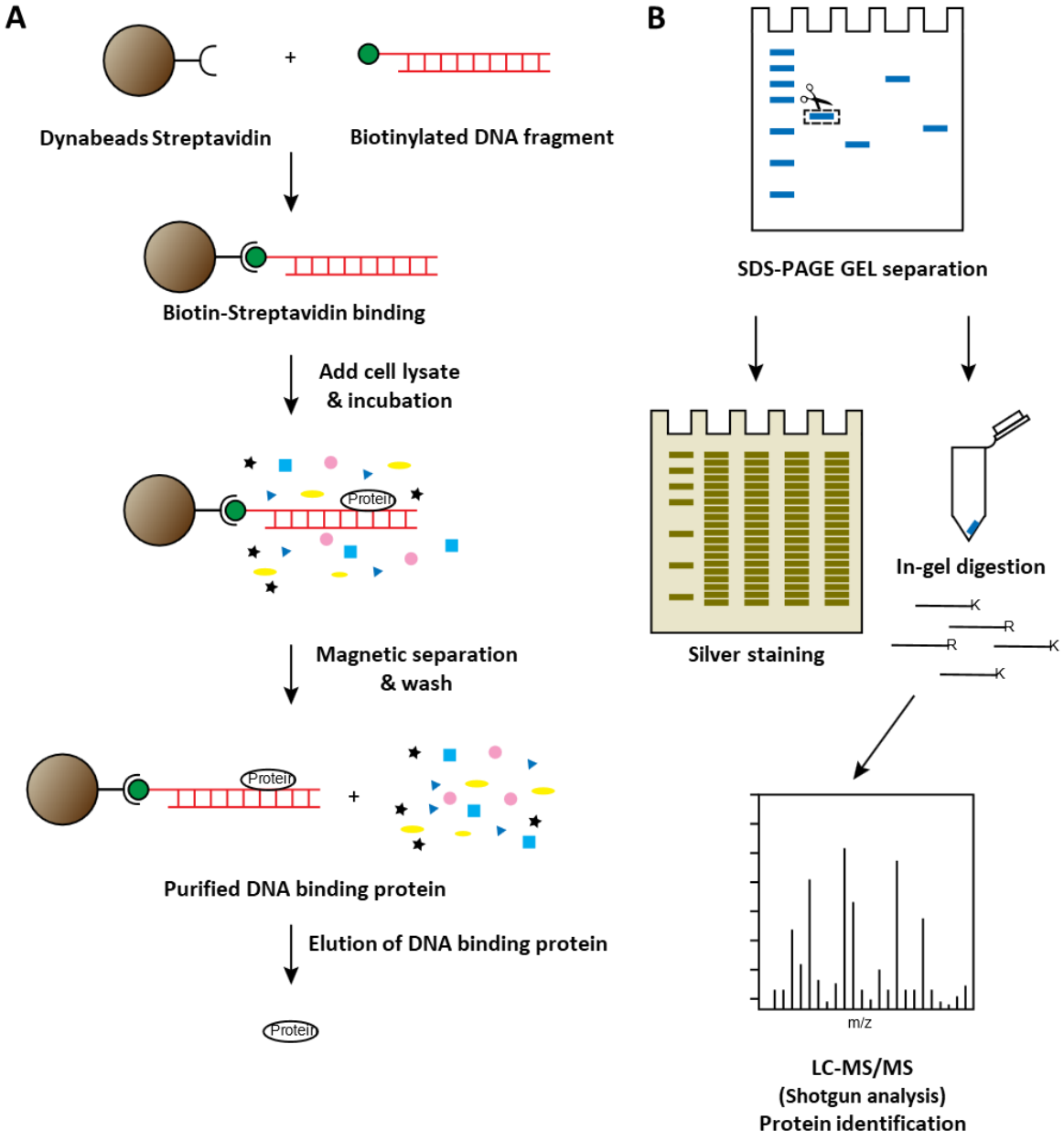


**Figure 6. Schematic diagram of Biotin-Streptavidin pull down and LC-MS/MS procedure.** (A) Target sequence is biotinylated by PCR using 5' biotin labeled primers. Biotinylated target sequence interacts with magnetic streptavidin beads. Incubation and washing step of the biotin-streptavidin complex with cell lysate will only leave streptavidin-biotinylated DNA-specific interacting protein complex. Purified proteins can be eluted by adjusting the conditions (high salt, heating, or low pH). (B) Eluted proteins are prepared for SDS-PAGE gel and separated. We can directly check SDS-PAGE gel separated proteins by silver staining or digest the proteins in the gel into peptides for the identification by LC-MS/MS analysis.

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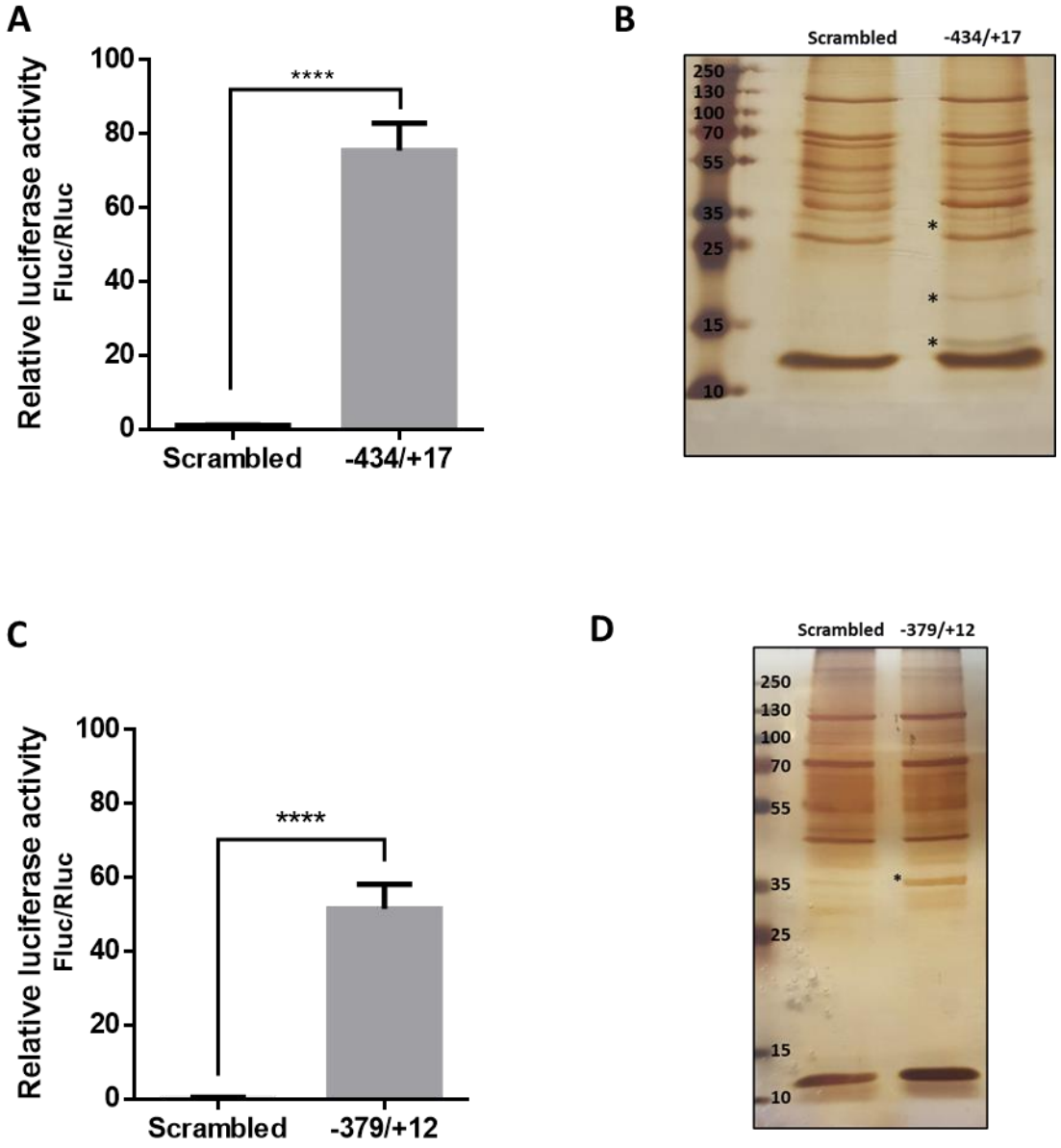


**Figure 7. Comparison of scrambled control sequence and promoter sequence.**

(A, C) Transcriptional activity of DSCR1 promoter sequence and its scrambled sequence are measured by bicistronic reporter system. Single vector is transfected to SVEC4-10 cells and incubated for 24 hours. Firefly (Fluc) and Renilla (Rluc) luciferase activities are measured by dual-luciferase assay (Promega). Fluc signal is normalized by Rluc signal. Values are shown as mean ± SEM and statistical significance is evaluated by unpaired t test. N = 3. ****P < 0.0001. (B, D) Silver staining of separated SDS-PAGE gel. Streptavidin-biotin-DNA complex interacting proteins are eluted through pull down and used as an SDS-PAGE gel loading samples.

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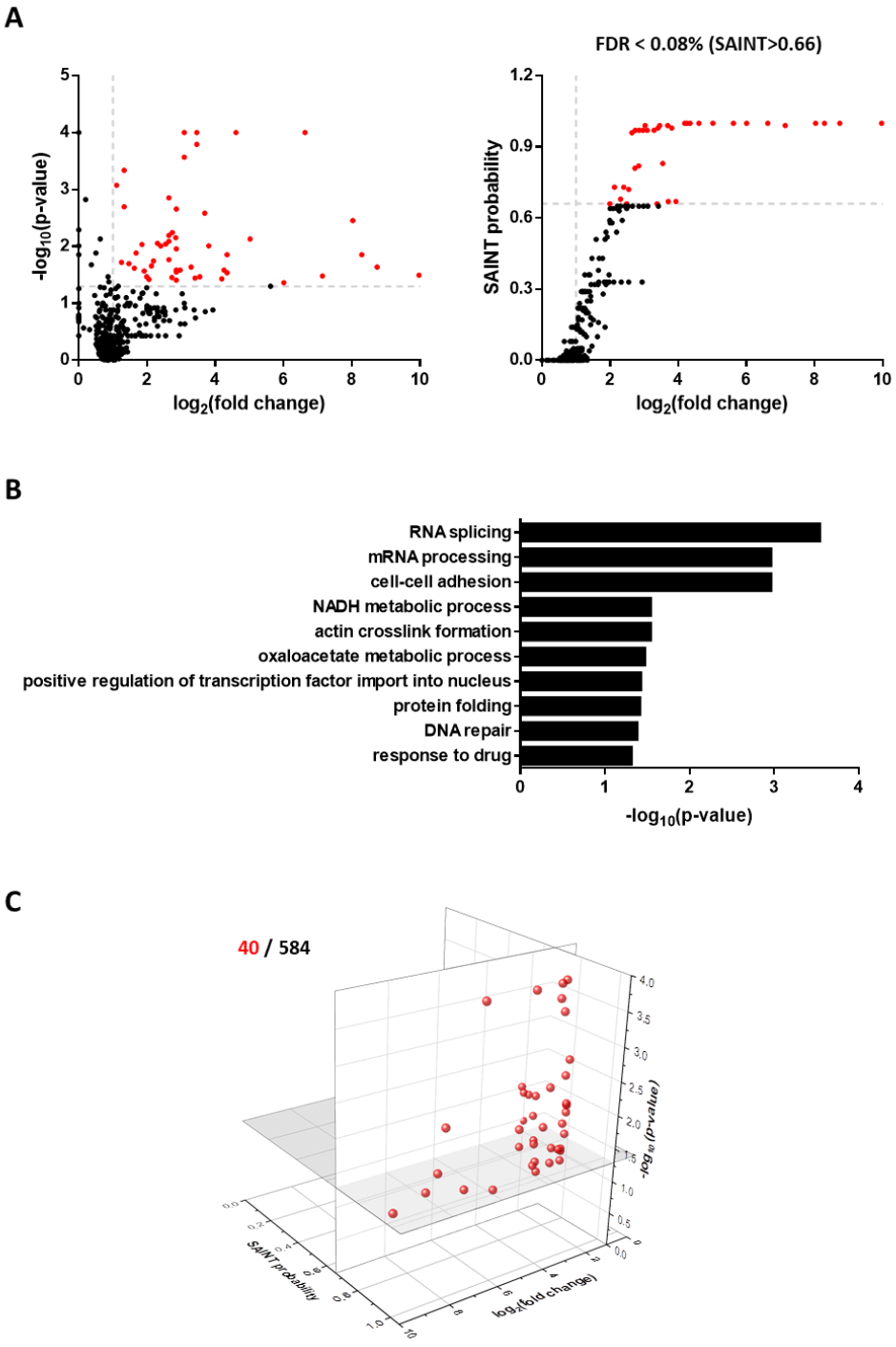


**Figure 8. Identification of the interacting proteins by LC-MS/MS.** (A) Scatter plots of DSCR1.1 promoter sequence binding proteins. Multiple unpaired *t*-test (P-value) followed by FDR (SAINT probability) analysis. (B) Co-annotations of interacted proteins to common Gene Ontology (GO) terms in biological processes (BPs). *DAVID: Functional Annotation Tool 6.8 ver*. (C) A 3D scatter plot visualizing the proteins satisfied with all three different conditions: P-value < 0.05, FDR < 0.08%, and Fold change > 2. (A-C) N = 3. The *CRAPome* database is used for analysis.

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| BAIT | PREY | GENE | MW [kDa] | FC_A | SAINT | T-test |
|---|---|---|---|---|---|---|
| 1_1 | P08249 | MDH2 | 36 | 6.63 | 1 | 0.0001 |
| 1_1 | Q7TPR4 | ACTN1 | 103 | 4.61 | 1 | 0.0001 |
| 1_1 | P26040 | EZR | 69 | 8.03 | 1 | 0.0035 |
| 1_1 | P09405 | NCL | 77 | 5.02 | 1 | 0.0074 |
| 1_1 | Q01853 | VCP | 89 | 8.29 | 1 | 0.014 |
| 1_1 | P37889 | FBLN2 | 132 | 4.35 | 1 | 0.014 |
| 1_1 | P11087 | COL1A1 | 138 | 8.74 | 1 | 0.023 |
| 1_1 | P11103 | Parp1 | 113 | 4.26 | 1 | 0.026 |
| 1_1 | Q9QXS1 | PLEC | 534 | 4.35 | 1 | 0.029 |
| 1_1 | P27773 | PDIA3 | 57 | 9.97 | 1 | 0.032 |
| 1_1 | Q80X90 | FLNB | 278 | 4.19 | 1 | 0.037 |
| 1_1 | Q64727 | VC+C2:C41L | 117 | 6.01 | 1 | 0.043 |
| 1_1 | P09103 | P4HB | 57 | 5.62 | 1 | 0.05 |
| 1_1 | Q3V3R1 | MTHFD1L | 106 | 3.46 | 0.99 | 0.0001 |
| 1_1 | E9Q555 | RNF213 | 585 | 3.46 | 0.99 | 0.00016 |
| 1_1 | P05202 | GOT2 | 47 | 3.69 | 0.99 | 0.0026 |
| 1_1 | P26039 | TLN1 | 270 | 7.14 | 0.99 | 0.033 |
| 1_1 | P10107 | ANXA1 | 39 | 3.03 | 0.99 | 0.068 |
| 1_1 | P63101 | YWHAZ | 28 | 3.81 | 0.98 | 0.0097 |
| 1_1 | P17751 | TPI1 | 32 | 3.41 | 0.98 | 0.036 |
| 1_1 | P62259 | YWHAE | 29 | 3.09 | 0.97 | 0.0001 |
| 1_1 | Q8QZT1 | ACAT1 | 45 | 3.09 | 0.97 | 0.00027 |
| 1_1 | P46061 | RANGAP1 | 64 | 2.86 | 0.97 | 0.0022 |
| 1_1 | P61979 | HNRNPK | 51 | 2.74 | 0.97 | 0.0057 |
| 1_1 | P49312 | HNRNPA1 | 34 | 2.86 | 0.97 | 0.011 |
| 1_1 | Q9DBG3 | AP2B1 | 105 | 3.29 | 0.97 | 0.023 |
| 1_1 | O70157 | TOP3A | 112 | 2.97 | 0.97 | 0.026 |
| 1_1 | Q62465 | VAT1 | 43 | 2.86 | 0.97 | 0.026 |
| 1_1 | P13020 | GSN | 86 | 2.86 | 0.97 | 0.028 |
| 1_1 | Q62318 | TRIM28 | 89 | 2.86 | 0.97 | 0.039 |
| 1_1 | P97855 | G3BP1 | 52 | 3.09 | 0.97 | 0.1 |
| 1_1 | Q64012 | RALY | 33 | 2.64 | 0.96 | 0.0014 |
| 1_1 | P26231 | CTNNA1 | 100 | 2.64 | 0.96 | 0.0064 |
| 1_1 | P43274 | HIST1H1E | 22 | 2.64 | 0.96 | 0.0081 |
| 1_1 | P25206 | MCM3 | 92 | 2.64 | 0.96 | 0.017 |
| 1_1 | Q8BMK4 | CKAP4 | 64 | 3.55 | 0.83 | 0.034 |
| 1_1 | Q8VDN2 | ATP1A1 | 113 | 2.84 | 0.82 | 0.007 |
| 1_1 | Q8VHX6 | FLNC | 291 | 2.73 | 0.81 | 0.035 |
| 1_1 | Q68FD5 | CLTC | 192 | 2.4 | 0.73 | 0.0098 |
| 1_1 | Q61029 | TMPO | 50 | 2.13 | 0.73 | 0.022 |

**Table 1.** The list of proteins satisfying all three conditions from *CRAPome* results.

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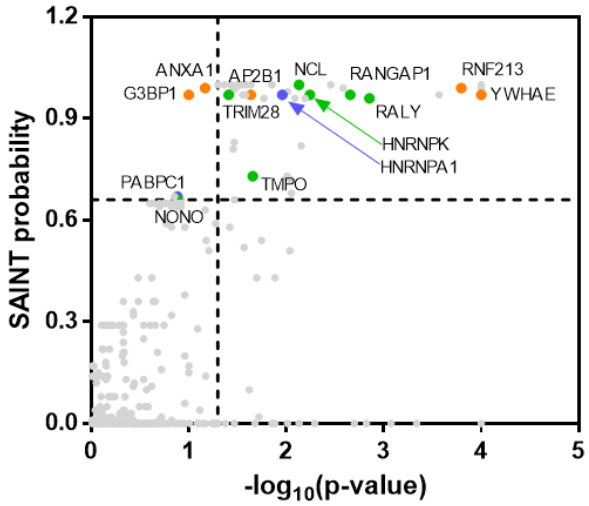
A




B

| | |
|---|---|
| Transcription regulation | NCL<br>RALY<br>HNRNPK<br>TRIM28<br>TMPO |
| | (p-value > 0.05)<br>NONO |
| Nuclear factor | HNRNPA1 |
| | (p-value > 0.05)<br>PABPC1 |
| Signal transduction | YWHAE<br>RNF213<br>RANGAP1<br>AP2B1 |
| | (p-value > 0.05)<br>ANXA1<br>G3BP1 |

**Figure 9. The list of proteins categorized by their functions from DSCR1.1 sample.** (A) A scatter plot of DSCR1.1 promoter sequence binding proteins. Multiple unpaired t-test (P-value) versus FDR (SAINT probability) analysis. (B) Candidate proteins list categorized by their functions.

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three conditions (p-value, FDR, and FC) (Figure 8C, Table 1) and we categorized these proteins according to their functions (Figure 9A, B). Among them, we selected the candidate proteins in terms of 3 categories that are related to transcriptional process.

**Regulation of DSCR1 expression via RALY isoform 2 protein in endothelial cells**

To confirm our proteomics data (Figure 8, 9), we first generated the Flag-tagged candidate protein constructs. The size of Flag-tagged RALY isoform 1, isoform 2, and YWHAE proteins were about 37 kDa, 39 kDa, and 35 kDa, respectively. Next, we transfected the constructed candidate proteins into SVEC4-10 cells and conducted biotin-streptavidin pull-down assay with the transfected cell lysate. We confirmed that RALY isoform 1 and RALY isoform 2 proteins actually interact with DSCR1 promoter region (Figure 10A). However, YWHAE protein did not interact with DSCR1 promoter region, so we used YWHAE protein as a negative control for western blot and qRT-PCR. Among the candidate proteins, we observed that RALY isoform 2 (RALY iso2) significantly down-regulates the expression level of DSCR1 (Figure 10B). We hypothesized that the regulation of DSCR1 expression may occur at the transcriptional level because the candidate proteins were potentially able to interact with the core promoter region of DSCR1. To demonstrate this possibility, we performed qRT-PCR experiment and designed qPCR primers targeting the region between exon6 to exon 7 in DSCR1 gene to check both DSCR1.1 and DSCR1.4 mRNA levels. Surprisingly, our qRT-PCR analysis revealed that DSCR1 mRNA levels did not change (Figure 10C) and any differences in DSCR1 mRNA levels were not detected. Both DSCR1.1 and DSCR1.4 mRNA levels remained unchanged even when potentially interacting proteins were overexpressed. In conclusion, it is likely that RALY iso2 binds to DSCR1 promoter region, but also down-regulates the DSCR1 protein synthesis through unknown mechanism that should be further investigated.

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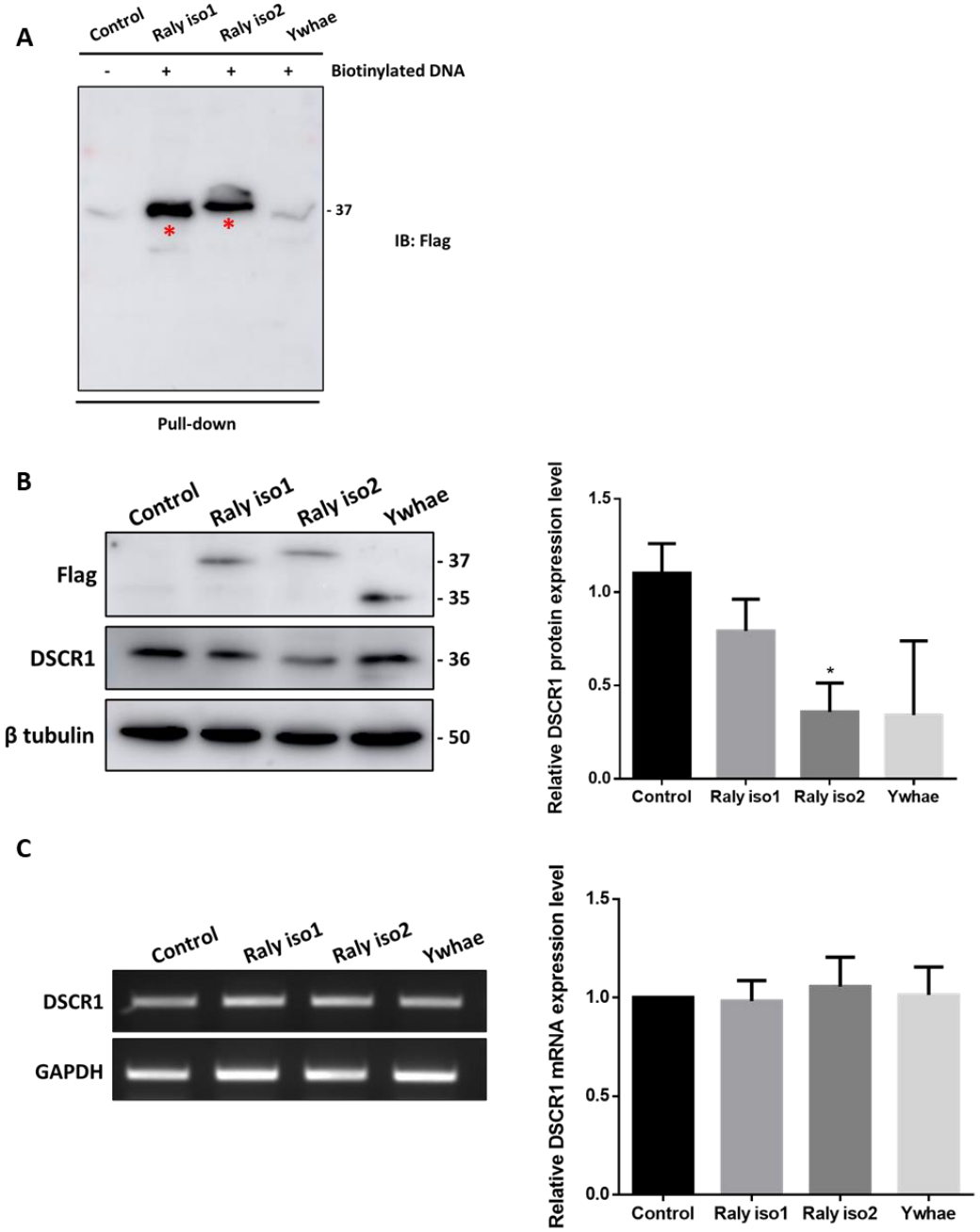


**Figure 10. Raly and Ywhae protein down-regulates DSCR1 expression at the translational level.** (A) Biotinylated DNA-streptavidin beads immunoprecipitation with Flag-tagged candidate proteins were performed using Magnetic Dynabeads. Candidate proteins interaction with DSCR1 promoter region (red asterisk) by immunoprecipitation. (B) Raly isoform 2 significantly decreases the expression level of DSCR1. Beta-tubulin is used as a loading control. (C) DSCR1 mRNA level is not altered even when potentially interacting proteins are overexpressed. The expression of each mRNA is normalized by GAPHD. Values are shown as mean ± SEM and statistical significance is evaluated by unpaired t test. N = 3. *P < 0.05

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## III. Discussion

DSCR1 is an interesting protein because the dysregulation of its expression causes two opposing effects, either defective or beneficial depending on specific conditions. DSCR1 overexpression ameliorates Alzheimer's disease phenotypes (Figure 2) [27, 36, 47]. On the other hand, it functions as a risk factor in AD pathology [5, 29]. Similarly, DSCR1 knock-out conditions also caused either detrimental or beneficial phenotype [13, 48]. Considering these opposite findings about DSCR1 functions, it is likely that the expression level of DSCR1 and/or the regulation of DSCR1 expression may be crucial for producing diverse cellular effects. Together, the fundamental knowledge about how DSCR1 expression is regulated remains poorly understood.

In this study, we identified the core promoter region of each DSCR1 isoform and candidate proteins which can interact with DSCR1 core promoter region. Through LC-MS/MS analysis, we identified DSCR1 promoter interacting proteins and listed the candidate proteins by their functions. Within our candidate proteins list, RALY iso2 protein binds to DSCR1 core promoter region and significantly down-regulates the expression of DSCR1 protein. Thus, we hypothesized that the regulatory mechanism of DSCR1 via RLAY iso2 protein, which interacts with DSCR1 core promoter, would occur at the transcriptional level. However, unexpectedly, DSCR1 mRNA level was not affected by those candidate proteins. Considering this result, it seems that the regulation of DSCR1 protein expression via RALY iso2 does not occur at the transcriptional level. It is hard to interpret our data because RALY iso2 binds to the DSCR1 promoter region. One hypothesis we can propose is that RALY iso2 protein might be one of the transcriptional co-factors. Thus, RALY iso2 might be by itself not crucial for transcriptional initiation, but it can play a modulating role in the key factors affecting translation. Additionally, YWHAE, used as a negative control, also showed its potential for regulating the DSCR1 protein expression. YWHAE protein is well known for its potential roles in the development of hippocampus and cortex [13, 22, 24-26] and has a similarity with DSCR1 signal transduction mechanism that mediates the signal transduction by protein phosphorylation [10, 49]. Although YWHAE does not directly interact with DSCR1 promoter region, the expression of DSCR1 can be also regulated by YWAHE through unknown, indirect mechanism that should be further investigated.

Taken together, we aimed to dissect out the regulatory mechanism of DSCR1 expression and related signaling pathways through this study. In this study, we provided a preliminary finding that DSCR1 expression can be regulated by the interaction with DSCR1 promoter-binding proteins. However, we have to further demonstrate the precise regulatory mechanism of DSCR1 expression through RALY iso2 and YWHAE. Despite this limitation of our study, as mentioned above, the

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regulation of DSCR1 expression could be an important target for developing therapeutic application for diverse disease conditions.

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# IV. Materials and Method

**Animals**

Animals were used in accordance with protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the Ulsan National Institute of Science and Technology. DSCR1 knock-out and transgenic mice were obtained from K. Baek at Sungkyunkwan University. DSCR1 mutant mice have C57BL/6J background. 5xFAD mice, which have SJL:C57BL/6J background were obtained from Inhee Mook-Jung at Seoul National University. For the hybrid mice of 5xFAD and DSCR1 mutant mice genetic background match, 5xFAD mice were crossed with C57BL/6J.

**Cell culture.**

SVEC4-10 from ATCC was cultured in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 4.5 g/L glucose and 0.11 g/L sodium pyruvate (Welgene; LM 001-05) with 10% fetal bovine serum (FBS) (Gibco; 16000044) and 1% penicillin-streptomycin (Gibco). Lipofectamine 2000 (Invitrogen) was used for transfection.

**Plasmid Construction.**

The mouse DSCR1.1 and DSCR1.4 promoter target sequences were amplified from C57BL/6J gDNA using Phusion High Fidelity polymerase (NEB; B0519S) and were confirmed by sequencing (Solgent, Bionics). All PCR products from DSCR1.1 were digested with XmaI/HindIII restriction enzymes (NEB) and DSCR1.4 products were digested with XmaI/NcoI restriction enzymes (NEB). PCR products were inserted into bicistronic reporter vector that contains both renilla luciferase (Rluc) and firefly luciferase (Fluc). The bicistronic reporter vector was constructed by combined the pRL-TK (Rluc reporter vector) and pGL3 (Fluc reporter vector) bought from Promega. Scrambled sequence of each DSCR1 isoform's promoter sequence was synthesized (Bionics) and amplified and inserted to bicistronic reporter vector for further experiments. PCR used primers are listed in Table 2.

| Oligo name | Oligo sequence (5' → 3') |
|---|---|
| F:_T7-Rluc_KpnI | GGGGTACCTAATACGACTCACTATAGGCTAG |
| R:_T7-Rluc-poly(A)-random_XmaI | TCCCCCCGGGCACATTTCCC |
| F:_1.1_-1120_p.c.1_XmaI | tcccCCCGGGGAGCCACCGGGGGCAG |
| R:_1.1_+17_p.c.1_HindIII | cccAAGCTTGCCACGCCGTCCTCCATGC |
| F:_1.1_-2149_p.c.2_XmaI | ccCCCGGGATCACGGGGTTGGCAAC |
| R_1.1_-1121_p.c.2_HindIII | cccAAGCTTACTTCCTCCAGGAAAG |
| F:_1.1_-434_XmaI | tcccCCCGGGGCCTCCGGGTTACCG |
| F:_1.1_-777_XmaI | tcccCCCGGGCGAGGGAGGGCGATG |
| F:_1.1_-109_XmaI | tcccCCCGGGCGGGCACTGGAAGGC |

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| | |
|---|---|
| F:_1.1_-215_XmaI | tcccCCCGGGTGGGCCGACCTTAAAG |
| R:_1.1_-89_HindIII | cccAAGCTTGACGCCGCCTTCCAG |
| F:_1.1_-331_XmaI | tcccCCCGGGCAAATATGGCCGCCC |
| R:_1.1_-191_HindIII | tcccAAGCTTGAACGTTCCCTTTAAGGTCGGC |
| F:1.1_scrambled_-434_XmaI | tcccCCCGGGTCGGCCCTCTTGG |
| R:1.1_scrambled_+17_HindIII | cccAAGCTTGCCATTGGACGCCGC |
| F:_1.4_-1029_p.c.1_XmaI | tcccCCCGGGCAGACTGGTAGTTACAAGTAGG |
| R:_1.4_+12_p.c.1_NcoI | catgCCATGGCCTAAAATGCATTCTGCTTTT |
| F:_1.4_-2058_p.c.2_XmaI | tcccCCCGGGCAAGTACCGCTCCTGC |
| R:_1.4_-1030_p.c.2_NcoI | catgCCATGGAATCAGTCAGTCAGCTCCC |
| F:_1.4_-379_XmaI | tcccCCCGGGGAACTATGCCGCAAG |
| F:_1.4_-758_XmaI | tcccCCCGGGTGGGTGCTCTTCCATCA |
| R:_1.4_-367_NcoI | catgCCATGGGCGGCATAGTTCCCACTGG |
| R:_1.4_-734_NcoI | catgCCATGGCAGCGGATGATGGAAGAGC |
| R:_1.4_-254_NcoI | catgCCATGGGGAAAGAGGTGACGTCAAC |
| R:_1.4_-219_NcoI | catgCCATGGCTCGGGCAGACGGGG |
| F:_1.4_-254_XmaI | ccCCCGGGAGTAGAAATTTACACTC |
| F:_1.4_scrambled_-379_XmaI | TCCCCCCGGGTACGAAATATGCTTCATC |
| R:_1.4_scrambled_+12_NcoI | CATGCCATGGTCGCCCCACTTCAG |

**Table 2.** Forward and reverse primers used in PCR.

**Immunohistochemistry**

Collected mouse brain tissue was fixed with 4% paraformaldehyde (PFA, Thermo; AC416785000) for overnight at 4 °C and for the preservation of tissue morphology, soak the brain tissue in 30% sucrose until it sank to the bottom. Brain tissue was frozen with cryo-embedding media (Leica; 14020108926) and section the frozen tissue block into a desired thickness (typically 40-45 μm) through cryostat (Leica; CM1950). Wash the brain sections with PBS three times for 10 min each and break the protein cross-link and unmask the antigens and epitopes through 10 mM citric acid (pH 6.0) incubation, 30 min at 80 °C. Cool the brain sections in the ice and wash the sections with PBS three times. Block the tissue with blocking buffer (0.3% triton X-100, 5% normal horse serum with PBS solvent) for 1 hour at room temperature (RT). Primary antibody, MOAB-2 (NOVUS; NBP2-13075), was incubated for overnight at 4 °C. PBS wash for three times, incubate the secondary antibody, Alexa Fluor 568 (Thermo; A-11031), for 2 hours at RT. DAPI (Thermo; 62248) was used for nuclear counterstaining and stained tissues were mounted with Prolong Gold Antifade Mountant (Thermo; P36930). Images were acquired via Zeiss LSM 780 confocal microscope.

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**Luciferase assay**

Bicistronic reporter vectors were transfected to SVEC4-10 cells and harvested in Passive lysis buffer (Promega) after 24 hours. Firefly luciferase and renilla luciferase activities were measured by Dual-luciferase assay system (Promega), using GloMax system of luminometer. Each firefly luciferase activities were normalized each renilla luciferase activity. Normalized firefly activity was defined as transcriptional activity of inserted DNA sequence.

**Western Blot**

SDS-PAGE GEL consists of 4% stacking gel and 10% running gel. 60 Watt (W) for 35min, 100 W for 90min running the gel and transferred to a PVDF membrane (Millipore; #IPVH00010). Antibodies against OctA-Probe (Santa Cruz; sc-166384), DSCR1 (Santa Cruz; sc-377507), and beta-tubulin (abcam; ab6046) were used. Amersham Imager 680 was used for the membrane development.

**Biotinylation and biotin-streptavidin pull down**

5' biotin primer was used to biotinylate the amplicon of interest via PCR. Components are 5x Phusion HF buffer, 5% DMSO, 100 μM dNTP, 0.5 μM forward and reverse primers, and 2 units of Phusion polymerase (NEB) with following conditions: initial denaturation at 98°C for 30sec, followed by 35 cycles of 98°C for 10sec, primers annealing temperature for 30sec, and 72°C for 45sec, final extension at 72°C for 10min. Biotinylated target sequences were incubated with pre-washed Dynabeads M-280 streptavidin (Invitrogen; 11205D) with gentle agitation. For 15min incubation at RT, SVEC4-10 lysate was added and incubated at 4°C overnight on a rotary shaker. Washed the beads complex with B&W buffer (0.5 M Tris-HCl, 250 mM EDTA, and 2.5 M NaCl) three times, target sequence interacting proteins were eluted and analyzed for silver staining and LC-MS/MS. Used primers for biotinylation are listed in Table 3.

| Oligo name | Oligo sequence (5' → 3') |
|---|---|
| F:_1.1_-434_p.c.1 | GCCTCCGGGTTACCGTTCC |
| R:_1.1_+17_p.c.1_5'Biotin | GCCACGCCGTCCTCCATGC |
| F:_1.1_scrambled_-434 | TCGGCCCTCTTGGACCCGG |
| R:_1.1_scrambled_+17_5'Biotin | GCCATTGGACGCCGCGTG |
| F:_1.4_-379_p.c.1 | GAACTATGCCGCAAGAG |
| R:_1.4_+12_p.c.1_5'Biotin | CCTAAAATGCATTCTGCTTTT |
| F:_1.4_scrambled_-379 | TACGAAATATGCTTCATCGTCTG |
| R:_1.4_scrambled_+12_5'Biotin | CGCCTGAAGTGGGGCG |

**Table 3.** Forward and reverse primers used for biotinylation via PCR.

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**Silver staining**

Separated SDS-PAGE GEL was directly used for the silver staining. First, Fix the gel with fixing buffer (50% methanol, Millipore-Merk; 106007, and 50% acetic acid, Merck; 100063) for 20min and wash with 50% methanol for 10min and water for 10min at RT. Sensitizing the fixed gel with 0.02% sodium thiosulfate (Sigma; 217263) for 1min and wash with water for 1min. Repeat the sensitizing step for two times. Incubate the gel with 0.1% silver nitrate (Sigma; 209139) with 0.08% formalin (Sigma; F8775) for 20min at RT. Rinse the gel with water for 1min. Repeat the silver reaction step for two times. Develop the reacted gel with 2% sodium carbonate (Merck; 106392) with 0.04% formalin until desired intensity of staining occurs. Replace with fresh developer solution when developer solution turns yellow. Stop the gel development with 5% acetic acid for 10min incubation. Rinse the gel with water for 5min, and permanent storage is possible in 8.8% glycerol (AMRESCO; 0854) solution.

**In-gel digestion**

Cut the SDS-PAGE GEL to 1 mm x 1 mm size and destained the gel with 0.1 M Ammonium bicarbonate (ABC, Sigam; A6141), 0.1 M ABC : acetonitrile (ACN, Millipore; 100030) 1:1, and ACN, successively. Reduce the disulfide bonds to the thiol by incubate 1hr at 56°C with 10 mM dithiothreitol (DTT, Sigma; 43817) in 0.1 M ABC on ThermoMixer, 800rpm (Eppendorf). Irreversibly alkylate the thiol group with 55 mM iodoacetamide (IAM, Sigma; I1149) in 0.1 M ABC through vortexing for 30min at 25°C. Wash the alkylated protein contained gel via same process of destaining. Remove every solvent via speed vacuum. Dissolve 20 ug lyophilized trypsin (Pierce; 90057) to 160 μl of 50 mM acetic acid. Dilute the trypsin stock with 50 mM ABC to 1/10. Add diluted trypsin solution and incubate for 45min in ice. Add 50 mM ABC and overnight incubation at 37°C with vortexing. Add ACN : 5% formic acid (FA, Pierce; 28905) and incubate for 15min with vortexing. Collect the solution and get dried peptides by speed vacuum.

**Liquid Chromatography with tandem mass spectrometry (LC-MS/MS)**

Mass analysis was performed using LTQ-Orbitrap mass spectrometer (Thermo, Bremen, Germany) equipped with a nano-electrospray ion source. A C18 reverse-phase HPLC column (500mm x 75 μm ID) was used to separate the peptide mixture with 2.4% ACN in 0.1% FA to 24% ACN in 0.1% FA gradient for 60min at 300 nL/min flow rate. For tandem mass analysis, precursor ion scan MS spectra (400 ~ 2000 m/z) were acquired using the 60,000 resolution Orbitrap spectrometer at 400 m/z with an internal lock mass. The most intensive 20 ions were isolated and fragmented in the linear ion trap by collision-induced dissociation (CID).

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**Protein Construction**

Selected candidate proteins of LC-MS/MS results were constructed as tagged by Flag. All constructed proteins were checked via western blot using Flag-tag antibody (Santa Cruz; sc-166384).

| Oligo name | Oligo sequence (5' → 3') |
|---|---|
| F:_Raly_tv1-3_NheI | CTAGCTAGCATGTCCTTGAAGATTCAGAC |
| R:_Raly_tv1-4_EcoRI | CCGGAATTCCTGCAAGGCTCCATCTTC |
| F:_Raly_tv4_NheI | ctaGCTAGCAtgtccttgaagattcagacc |
| F:_Ywhae_NheI | CTAGCTAGCATGGATGATCGGGAG |
| R:_Ywhae_EcoRI | CCGGAATTCCTGATTCTCATCTTC |

**Table 4.** Forward and reverse primers used for candidate proteins construction.

**Quantitative reverse transcription PCR (qRT-PCR)**

Isolate the total RNAs from cultured cells with TRIzol reagent (Invitrogen; 10296028), 2 μg of total RNA was used for cDNA synthesis with High-Capacity RNAto-cDNA kit (Thermo Fisher; 4388950). The mRNA transcripts expression levels were analyzed by RT-qPCR with PowerUp SYBR Green Master Mix (Thermo Fisher; A25741). qPCR condition was followed the standard cycling mode from SYBR Green guidelines. Used primers for RT-qPCR are listed in Table 4.

| Oligo name | Oligo sequence (5' → 3') |
|---|---|
| F:_DSCR1_exon6_qPCR | GATGCCACCCCCGTCATAAA |
| R:_DSCR1_exon7_qPCR | CACTGGGAGTGGTGTCTGTC |
| F:_mGAPDH_CDS_qPCR | GCCATCAACGACCCCTTCATT |
| R:_mGAPDH_CDS_qPCR | GCTCCTGGAAGATGGTGATGG |

**Table 5.** Forward and reverse primers used for RT-qPCR.

**Statistical analysis**

Statistical significance was measured by unpaired Student's t-test, one-way ANOVA followed by Bonferroni *post hoc* test using *GraphPad Prism 6.01* software. Data from LC-MS/MS was analyzed its statistical significance through p-value, FDR, and FC using *CRAPome 1.1* (accessible by https://www.crapome.org/) at and *Scaffold 4.10.0* software. GO terms in BPs were measured by *DAVID 6.8* (accessible by https://david.ncifcrf.gov/) Functional Annotation program.

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