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Identification of protein-interaction partners of ZBED6

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Abstract

ZBED6 was recently discovered as a novel transcriptional repressor of *IGF2* because a mutation disrupting one of its binding sites in porcine *IGF2* intron 3 leads to greater postnatal *IGF2* expression in skeletal muscle and makes pig grow more muscle. ZBED6 is unique to placental mammals and derived from domesticated DNA transposon. However, the mechanism of ZBED6-mediated transcriptional regulation is still unknown. The study of the protein interacting partners of ZBED6 is a straightforward way to help us answer the question. By using the cells with stable overexpression of ZBED6, we perform one-step pull down assay and tandem affinity purification. A protein complex, SFPQ-Nono, is identified as the protein-interaction partners of ZBED6 by using mass spectrometry.

Contents

Introduction

Zbed6

The *Zbed6* gene encodes a novel nuclear transcription factor, which is evolutionarily conserved in placental mammals[1-3]. *Zbed6* is an intronless gene located in the first intron of *Zc3h11a*, which is a member of a large zinc-finger protein family. It consists of an ORF (open reading frame) of more than 900 codons showing no sequence similarity with *Zc3h11a*[1]. Bioinformatics analysis of amino acid sequence of ZBED6 reveals that it belongs to hAT transposase superfamily[6] that is named after hobo from Drosophila melanogaster, Activatior from zea maize and Tam3 from Antirrhinum majus and includes many DNA transposons in different species. The ZBED6 protein contains one hAT dimerization domain at the carboxy-terminal and two BED (chromatin-boundary-element-binding) domains responsible for DNA binding at the amino-terminal[4]. As its name indicates, it is the sixth identified mammalian protein containing BED domains. The two zinc-finger BED sequences in ZBED6 show more sequence similarity to one another than to any other BED domains in mammalian genomes. This implies a duplication event occured after *Zbed6* integration to the genome[1,2]. The *Zbed6* gene is co-transcribed with *Zc3h11a* by using a common promoter. When the first intron containing *Zbed6* is present in the transcript, only ZBED6 protein is translated, because it contains the first start codon of the transcript and a termination codon. The *Zc3h11a* gene will be expressed into proteins only when the first intron is spliced out[1,2].

The *Zbed6* mRNA is expressed in a broad range of tissues including brain, stomach, intestine, liver, lung, kidney, ovary, skeletal muscle, thymus and placenta[1]. In addition, ZBED6 is highly enriched in the nucleolus, detected by using anti-nucleophosmin antibody as a nucleolar marker. This indicates that ZBED6 is associated with transcriptional regulation of ribosomal RNA gene, since the nucleolus is a place for its transcription[5]. Furthermore, ZBED6 proteins, particularly the BED domains, are highly conserved among all the available genome sequences

of placental mammals and it is determined to be located at the same site in all these genomes[1,3]. The *Zbed6* pseudogene is found in the genomes of monotremes, but not in those of reptiles and birds, so it can be inferred that the *Zbed6* ancestor was integrated into a primitive mammalian genome before the divergence of monotremes with other mammals.

Approximately 2500 putative downstream targets of ZBED6 were identified in mouse genome by ChIP-seq[1] (Chromatin immuno-precipitation followed by next generation sequencing). The consensus binding motif of ZBED6 is 5'-GCTCGC-3' based on the bioinformatics analysis of all the putative target regions. About 1200 genes including *Igf2* contain at least one ZBED6 binding site within 5kb of the transcription start site[**1**]. A few studies have showed that ZBED6 acts as a repressor for the *IGF2* P3 promoter in pig[7-10]. The ontology analysis of the 1200 putative target genes shows that they are of great importance in muscle development, cell-cell signaling, neurogenesis, morphogenesis, cell differentiation, transcriptional regulation, and regulation of biological processes. Silencing of ZBed6 in mouse C2C12 cells leads to faster myotube formation, promoted cell proliferation and increased transcription of IGF2[**1**]. Mild ZBED6 overexpression on the other hand, leads to growth retardation in C2C12 cells[3]. The underlying mechanism for ZBED6-mediated transcriptional repression is an important question that remains to be explored.

BAC TransgeneOmics

BAC TransgeneOmics is a high-throughput technology to study protein function in mammalian tissue culture cells based on tagging genes of interest by recombineering in BAC (Bacterial Artificial Chromosome) constructs[**12**]. Due to the large capacity of BAC, coding region as well as all the endogenous regulatory elements of a transgene can be contained in the construct, so the genomic environment of BAC transgenes closely resembles that of endogenous genes. BAC transgenes can thereby be expressed in physiological pattern and level. In addition, BAC libraries for most model organisms has been established and mapped in genome sequencing projects, so they can be directly used to tag genes of interest in BAC TransgeneOmics[**13, 14**]. The development of recombineering technology ensures accurate and efficient modification of BAC constructs in bacteria. In order to place the protein of interest in an appropriate position of cellular biochemical pathways, tandem affinity purification and protein localization can be performed after stable transfection of BAC transgenes into mammalian tissue culture cells.

The general procedure of BAC TransgeneOmics is described as follows (**Fig. 1**). An appropriate BAC clone containing the gene of interest is selected from BAC libraries. Subsequently, the selected BAC clone is tagged in *Escherichia coli* by recombineering[15-18]. The tagged BAC transgene purified from BAC clone culture is transfected stably into mammalian tissue culture cells. After that, protein-protein interaction and protein localization studies can be conducted using BAC transgenic cell pool or clonal cell line grown from a single cell.

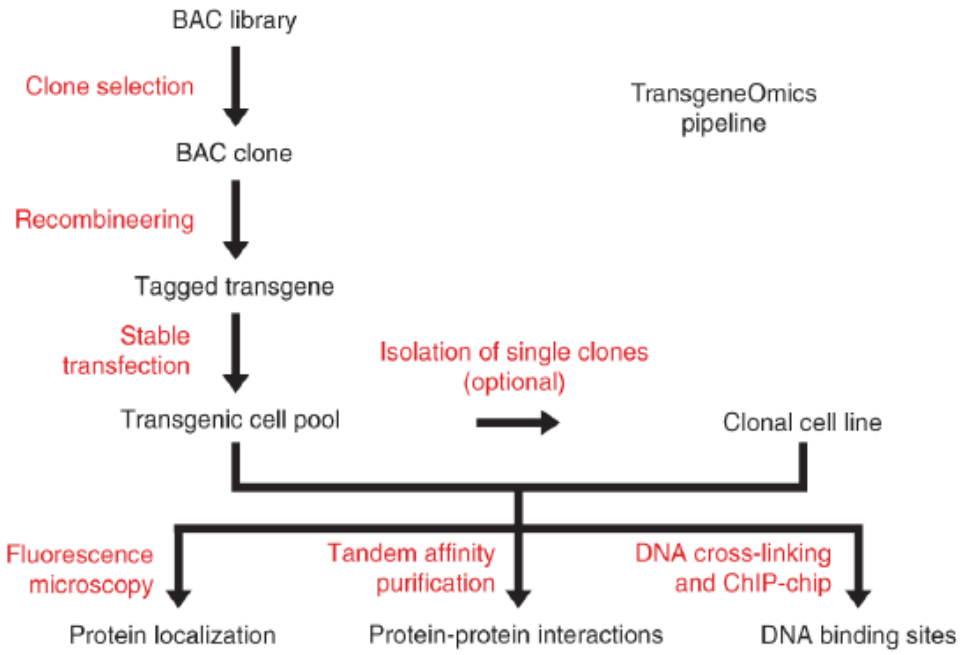

Figure1. The pipeline of BAC TransgeneOmics

The LAP (Localization and Affinity Purification) tag used in BAC TransgeneOmics contains EGFP (extended green florescent protein) for protein localization and the first step of tandem affinity purification, a PreScission cleavage site for the first-step elution, S-protein for the second step of tandem affinity purification and a TEV (Tobacco etch virus) protease cleavage site for the second-step elution (**Fig. 2**). The LAP can be added to either the N terminus or C terminus of the target protein by recombineering. The N-terminal tag contains a neomycin-kanamycin resistance gene located in an artificial intron of EGFP and regulated by a gb2 (a bacterial promoter)-PGK (phosphoglycerate kinase) promoter (**Fig. 2a**). This selection cassette can be eliminated by Cre recombinase from LoxP sites. The antibiotic resistance gene of C-terminal tags is located downstream of the tag-encoding sequence and is regulated by gb3 (a bacterial promoter) and translated from an IRES[19-21] (internal ribosomal entry site) (**Fig. 2b**).

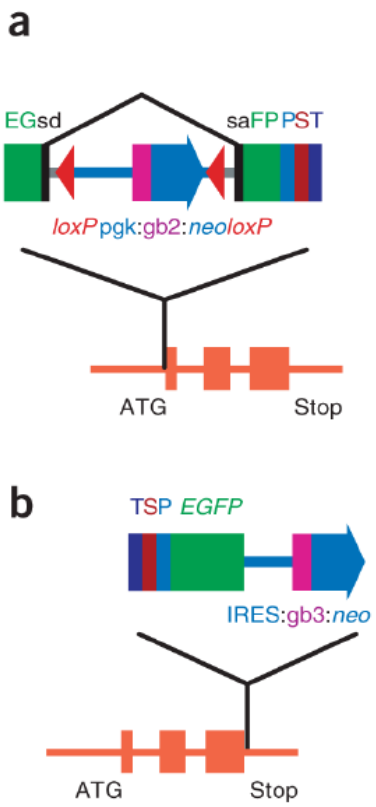

Figure2. LAP tags used in BAC transgeneOmics.
(a) The N-terminal tag.
(b) The C-terminal tag

Tandem affinity purification of TAP- tagged proteins with BAC Transgene Omics

In most cases, cellular biochemical processes are conducted by protein complexes. The identification of protein-interacting partners is indispensable to discern their function and place them in an appropriate position in cellular biochemical pathways. The recent development of BAC TransgeneOmics, which is a fast and reliable pipeline to study protein function in mammalian cells based on protein tagging in bacterial artificial chromosomes (BAC). It contains most of the native regulatory elements and ensures endogenous expression of the transgene. Therefore, it can avoid many artificial protein-protein interactions resulted from a strong over-expression driven by a typical plasmid-based method. Here we used a previously generated BAC transgenic C2C12 myoblast cell line with stable overexpression of tagged ZBED6 protein at the endogenous level. The tagged ZBED6 protein complex was purified from cell extracts by two-step affinity purification and further analyzed by SDS-PAGE and silver staining to assess their purity and yield. The detected bands were cut and submitted for mass spectrometry (MS) analysis. Sequences attained from MS are searched against protein database to identify specific protein-interaction partners[22-24].

Results

Validation of BAC transgenic overexpression cell lines

The expression level of ZBED6 in BAC transgenic overexpression cell lines was measured by reverse transcription real-time PCR. It showed that *Zbed6* mRNA is significantly overexpressed in ZBED6^{+} and ZC3H11A^{+} transgenic cell lines, while the transcription level of *Igf2* for these two cell lines is, as expected, much lower than that for the control one (GFP^{+} line)(**Fig. 3**). Our result is consistent with the fact that ZBED6 acts as a repressor for the *Igf2* transcription. As stated above, *Zbed6* is located in the first intron of *Zc3h11a* and uses its promoter for transcription, which could be the reason why ZBED6 is co-overexpressed in the ZC3H11A^{+} transgenic cell line.

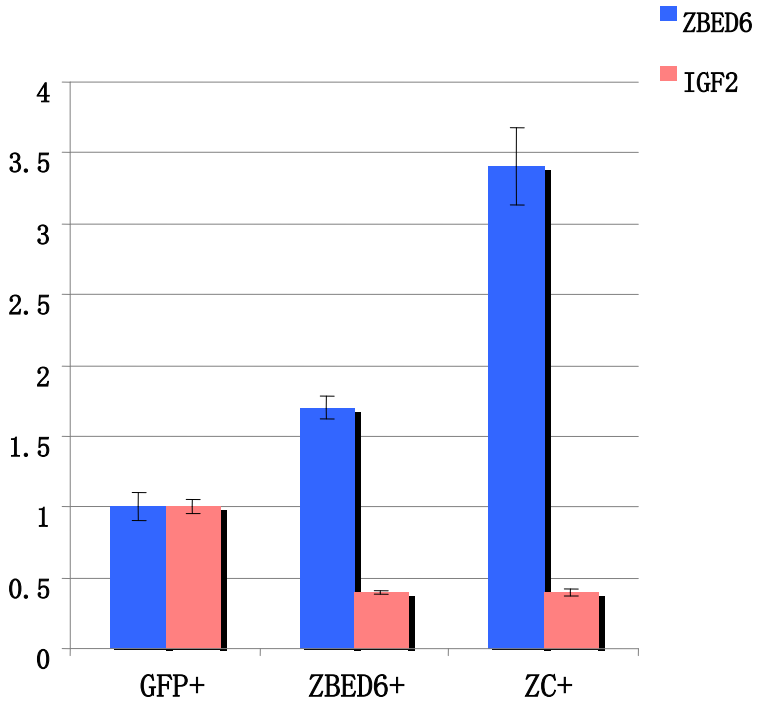

Figure3. Reverse transcription real-time PCR showing the transcription level of *Zbed6* and *Igf2* in BAC transgenic cell lines. ZBed6^{+} and Zc^{+} represent the ZBED6^{+} transgenic overexpression cell line and the ZC3H11A^{+} transgenic overexpression cell line, respectively. GFP^{+} is used as a negative control BAC transgenic cell line.

One-step pull down assay of ZBED6 interaction complex

To identify protein-interaction partners of ZBED6, we first carried out one-step pull down assay, in which only the EGFP tag in the ZBED6^{+} transgenic protein was used to purify proteins. Several protein bands on the silver staining gel were found to be specific for the ZBED6^{+} transgenic overexpression cell line, but not present in the control cell line[25] (**Fig. 4**). A protein complex, SFPQ-Nono, co-purifying with ZBED6 proteins was identified by mass spectrometry (**Table 1**). SFPQ-Nono heteromers are involved in several important nuclear biochemical processes including pre-mRNA splicing, DNA repair and transcriptional regulation, which could help us to understand the mechanism by which ZBED6 acts as a nuclear transcription factor.

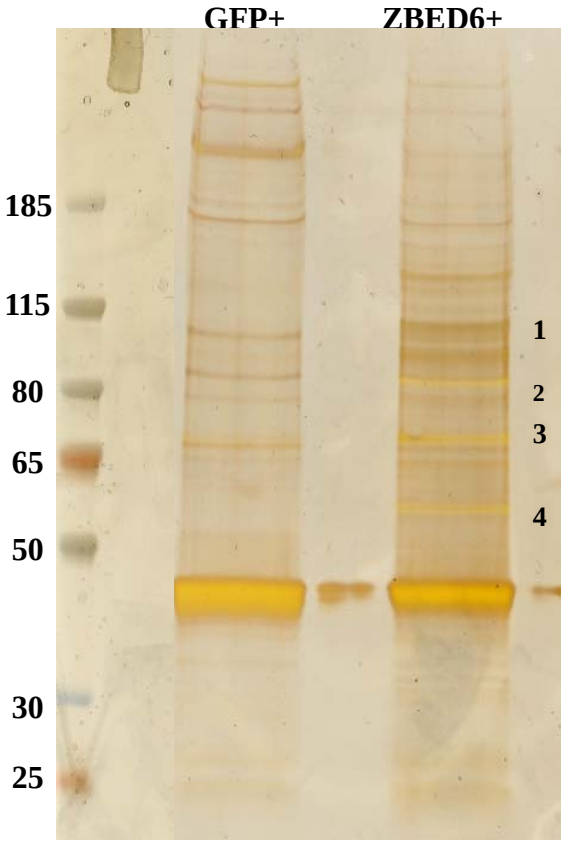

Figure4. Silver staining of the purified proteins. Protein ladder is shown on the left. Numbers in the figure refer to identified proteins in Table 1.

Table1. List of identified proteins interacting with ZBed6

	Accession number	Protein name and description	Score/Exp
1	CAT02779	Zinc finger BED domain-containing protein 6 (Muscle growth regulator) (MGR)	1.3×10^{-4}
2	NP076092	Splicing factor, proline-and glutamine-rich (DNA-binding p52/p100 complex, 100 kDa subunit) (Polypyrimidine tract-binding protein-associated-splicing factor) (PSF) (PTB-associated-splicing factor)	6.7×10^{-8}
3	AAH04534	SFPQ protein	2.8×10^{-3}
4	NP075633	Non-POU domain-containing octamer-binding protein (NoNo protein)	1.8×10^{-5}

Tandem affinity purification of ZBED6 interaction complex

For higher specificity of protein purification we subsequently performed tandem affinity purification, in which both the EGFP tag and the S-protein tag are employed to pull down specific protein-interaction partners. The western blot analysis using antibodies against ZBED6 detected a distinct LAP tagged ZBED6 protein band of expected size in the lane for two-step immuno-precipitation, showing that ZBED6 was successfully pulled down by this method (**Fig. 5b**). After the first step purification with the EGFP tag, a protein band shift of ZBED6 was observed on the western blot, since the EGFP tag was cut off at the PreScission protease cleavage site. Purified proteins were separated by SDS-PAGE and visualized by silver staining. On the silver staining gel, no protein bands are found on the lane for the negative control cell line (GFP^+), which means that almost all the proteins pulled down through tandem affinity purification specifically interact with LAP-tagged proteins (**Fig. 5a**). In order to obtain the threshold yield for mass spectrometry, we repeated the experiment seven times and attained the same protein pattern on silver staining gels. The last time we used culture cells grown in eighteen 25-cm tissue culture plates with 80% confluence to perform tandem affinity purification, but the protein yield is still not sufficient for mass spectrometry[28-29].

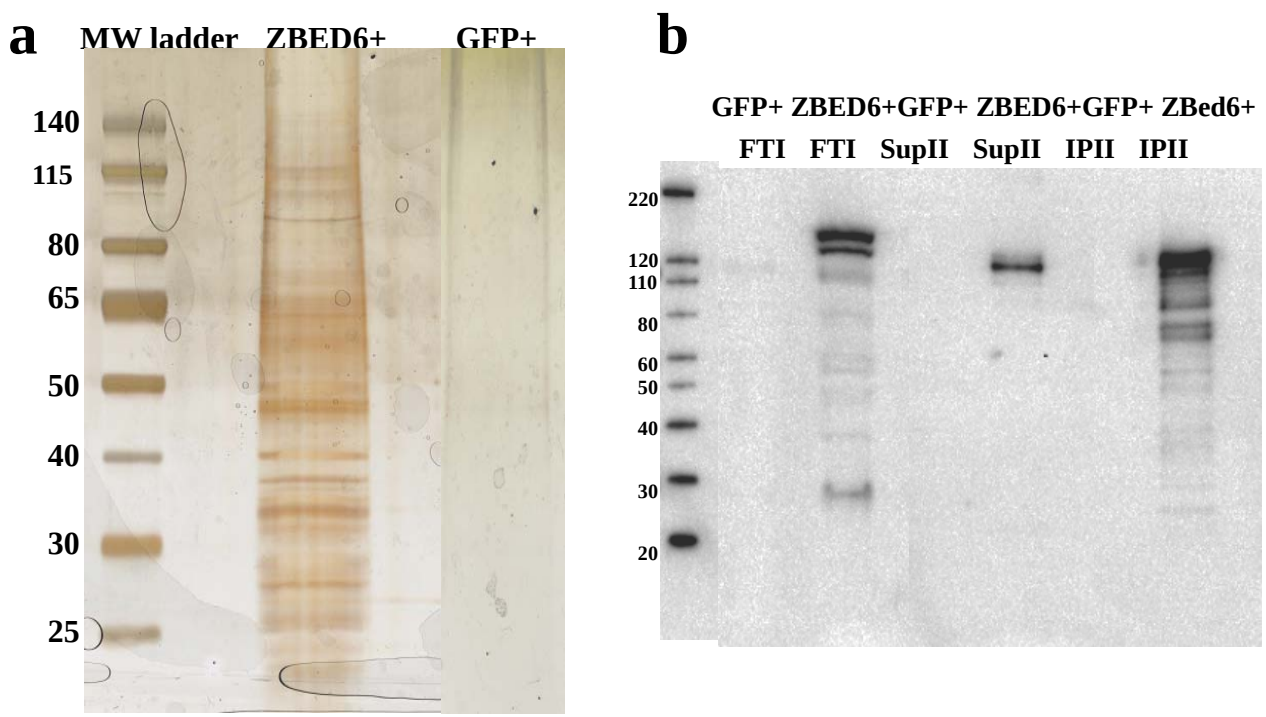

Figure5. Tandem affinity purification (a). Silver staining of the purified proteins. (b). Western blot assay using antibodies against ZBED6. FTI represents the flow-through in the first step purification; SupII refers to the supernatant in the second step purification; IPII is the final immunoprecipitates. Molecular weight ladder is shown on the left of the figures.

Discussion

In most cases, proteins with certain purity and quantity are required for studying protein structure and investigating their mechanism of action. As a result, protein purification is often essential before studying proteins in detail. In this study, both one-step pull down assay and tandem affinity purification are employed to co-purify the ZBED6 protein with its interaction partners from approximately 10,000 different proteins in mouse C2C12 cells. Purified proteins were separated by SDS-PAGE, visualized by silver staining, and identified by mass spectrometry. Comparing the results of the two protein purification approaches, we could conclude that tandem affinity purification has higher specificity but lower purification efficiency and less protein yield than one-step pull down assay[30-32]. For one-step pull down assay, we used culture cells grown in two 25cm tissue culture plates with 80% confluence, while for tandem affinity purification, cells of eighteen plates are used, but the protein yield is still not sufficient for mass spectrometry. Subsequent purification with more cells is needed.

The study of protein-protein interaction and protein localization provide important knowledge about the functional role of a target unknown protein. For the ZBED6 protein used as target in this study, we already knew that it is a transcriptional repressor expressed throughout the nucleus and that it is highly enriched in the nucleolus[1]. In the analysis of protein-protein interaction, three proteins were identified to be the interaction partners of ZBED6, which can form a protein complex, SFPQ-Nono which plays important roles in pre-mRNA splicing, DNA repair and transcriptional regulation. The complex can bind to the promoter of CYP17 and control the basal and cAMP-dependent transcription. The SFPQ protein also attaches to SIN3A and subsequently recruits histone deacetylases (HDACs), which modify chromatin state and negatively regulate gene transcription[33-35]. There is a probability that ZBED6 represses transcription through the interaction with SFPQ and further recruitment of HDACs. The interaction between ZBED6 and SFPQ complex needs to be confirmed by other methods including co-immunoprecipitation.

Materials and Methods

Materials

Harvest C2C12 cells

- C2GM (C2C12 Growth Medium): DMEM (Dulbecco's Modified Eagle Medium) complemented by 10% FBS (fetal bovine serum), 100ug/ml penicillin, 100ug/ml streptomycin and 0.2 mM L-glutamine. Add 500ug/ml G-418 (Geniticin) for the selection of BAC transgenic overexpression cell lines.
- 0.25% Trypsin-EDTA
- Wash buffer: 1×PBS

Cell lysis and pull down assays

- LAP buffer: 150 mM KCl, 5mM EDTA, 50 mM HEPES-KOH, 10% (v/v) glycerol, pH 7.5
- 10×PIM (Protease Inhibitor Mix): one tablet of complete mini protease inhibitors (Roche) per 10mL LAP buffer
- Lysis buffer: LAP buffer added by 1mM DTT, 10mM NaF, 1×PIM, 1% (v/v) TritonX-100
- CLB (Cell lysis buffer): 0.01M Tris-HCl pH 7.5, 0.01M KCl, 0.2% (v/v) NP-40
- Wash buffer: LAP buffer added by 1mM DTT, 10mM NaF, 1×PIM, 0.5% (v/v) TritonX-100
- Cleavage buffer: LAP buffer added by 1mM DTT, 10mM NaF, 0.5% (v/v) TritonX-100
- PreScission protease (GE healthcare)
- S-protein agarose beads (Novagen)
- Elution buffer: 4×Sample buffer

SDS-PAGE and Western blot

- Precast tris-glycine SDS-PAGE gel (BioRad)
- 20×MOPS SDS-PAGE running buffer (BioRad)
- PageRuler™ Plus Prestained Protein Ladder, PageRuler™ Plus Unstained Protein Ladder, MagicMark™ XP Western Protein Ladder
- Wash buffer: 1×TBST
- Stripping buffer: 4% (v/v) NaOH
- 1:3000 anti-GFP mouse antibodies, 1:500 anti-ZBed6 rabbit antibodies

Silver Staining

- Gel fixing solution I: 50% Methanol, 5% glacial acetic acid
- Gel fixing solution II: 50% Methanol
- Sensitizing solution: 0.02% Sodiumthiosulfate
- Staining solution: 0.1% Silvernitrate
- Developing solution: 2% Sodiumcarbonate
- Stop solution: 5% glacial acetic acid

Methods

C2C12 cell culture

C2C12-BAC cells were cultured on monolayer in 10% CO_2 at 37℃. C2C12 cells grown in C2GM were harvested until 80% cell confluence was attained. Sufficient CLB was used to lyse the cell membrane. Cell suspension was centrifuged at 2500rpm for 5min. We collected the supernatant as the cytoplasm of culture cells. Pellets were further lysed with enough Lysis buffer, passed through 20G needle twenty times, and centrifuged at maximum speed for 30min. The supernatant was collected as the nuclear lysate of culture cells and stored at -20℃.

Tandem affinity purification

The first step of tandem affinity purification was carried out with μMACS™ Epitope Tag Protein Isolation Kit. The nuclear lysate was thawed on ice and added with 50μL anti-GFP microbeads to pull down the

LAP-tagged proteins. After 30-minute incubation, we applied the cell lysate to μ Column in a strong magnetic field. Unspecific proteins were washed away with sufficient Wash buffer. Subsequently, the μ Column was removed from the magnetic field. We added 1:50 PreScission proteases (2U/μl, GE Healthcare) and incubate the solution at 4℃ for 1hour.

The μ Column was placed back to the magnetic field. We applied the cleavage solution to the μ Column and collected the flow-through to start with the second step of tandem affinity purification. 50uL S-protein agarose beads were added to the flow-through, rotated end over end for 1hour, and washed with Wash buffer for 6 times. We incubated S-protein agarose beads with 4×Sample buffer at 95℃ for 5min to directly elute proteins.

Mild silver staining

SDS-PAGE was fixed in Gel fixing solution I for 20min, in Gel fixing solution II for 10min, and washed in water for 10min. Subsequently, we sensitized the gel in Sensitizing solution for 1min, washed it twice with pure water. Then the gel was stained with Staining solution at 4℃ for 20min. After washing it twice, we developed the gel with Developing solution for 5-10min and terminated the reaction in Stopping solution for 5min.

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