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Purification of an Intact Human Protein Overexpressed from Its Endogenous Locus *via* Direct Genome Engineering

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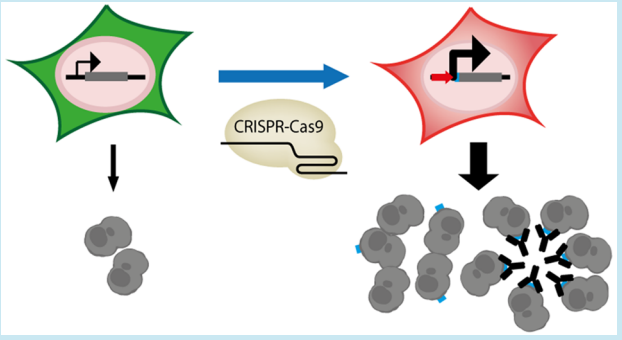
ABSTRACT: The overproduction and purification of human proteins is a requisite of both basic and medical research. Although many recombinant human proteins have been purified, current protein production methods have several limitations; recombinant proteins are frequently truncated, fail to fold properly, and/or lack appropriate post-translational modifications. In addition, such methods require subcloning of the target gene into relevant plasmids, which can be difficult for long proteins with repeated domains. Here we devised a novel method for target protein production by introduction of a strong promoter for overexpression and an epitope tag for purification in front of the endogenous human gene, in a sense performing molecular cloning directly in the human genome, which does not require cloning of the target gene. As a proof of concept, we successfully purified intact human Reelin protein, which is lengthy (3460 amino acids) and contains repeating domains, and confirmed that it was biologically functional.



KEYWORDS: *protein purification, CRISPR-Cas system, genome engineering, Reelin, molecular cloning*

The human genome includes about 20 000 protein-coding genes, from which more than one million proteins are expressed. The overproduction and purification of various human proteins is crucial not only in basic research for *in vitro* functional studies, protein structure analysis, and protein engineering, but also in medical applications such as those involving therapeutic proteins. Since the first establishment of molecular cloning techniques in the 1970s,[1,2] many recombinant human proteins have been purified from various heterologous expression systems including *E. coli*, yeast, insect cells, and mammalian cells. However, production of the majority of human proteins in intact form by established methods is still difficult for mostly unknown reasons.

An expression system using human embryonic kidney (HEK) 293 cells has been widely used to overproduce intact human proteins of interest, because human cells are expected to have the proper cellular factors required for protein expression, folding, modification, and secretion. Although this system has several disadvantages, including a relatively high cost and low expression level compared with other systems, it is often the only available method for the overproduction and purification of proteins that require human-specific lipid environments and/or post-translational modifications for functional expression. However, this method, like all other conventional expression systems, also requires subcloning of the target gene into a specialized plasmid vector that enables high protein expression, high plasmid copy number, and/or virus generation. Although various cloning techniques have been developed to improve the DNA cloning processes, subcloning human genes in *E. coli* is sometimes difficult due to the instability of plasmids containing foreign DNA sequences, frequently limiting further progress. In particular, for long genes with multiple repetitive sequences, subcloning of the full-length gene and/or production of high quantities of the gene-containing plasmid can be extremely challenging. Among the human proteins registered in the Universal Protein Resource (UniProt) database, a total of 1235, including medically and biologically important proteins such as Reelin, Ryanodyne receptors, ApoB-100, and Plectin, contain more than 3000 amino acids each, suggesting that studies on these proteins may have been hampered by difficulties in subcloning. Therefore, an alternative method that does not require any DNA subcloning would be a welcome advance for human protein purification.

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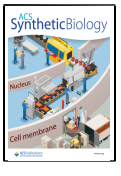

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Here we present a novel method that does not require cloning of a target gene coding sequence, but instead relies on direct genome engineering in human cells. To this end, we employed CRISPR-Cas9 endonucleases targeting an endogenous gene of interest, allowing us to insert a strong promoter along with a specific epitope tag upstream of the coding sequence, just like what occurs when a gene is subcloned into an expression plasmid in *E. coli*. This approach enables overexpression and direct purification of the target protein with both an intact structure and the appropriate post-translational modifications.

RESULTS AND DISCUSSION

To demonstrate our strategy of endogenous protein production (Figure 1A), we carefully chose human Reelin,

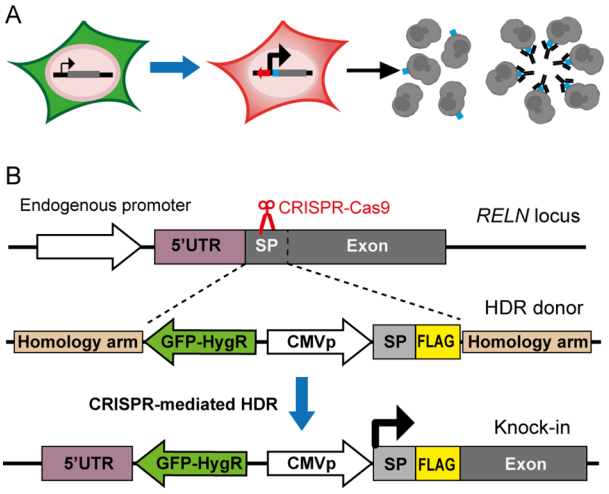

Figure 1. Genome engineering strategy for purification of intact human proteins. (A) To purify a target protein that is expressed at low levels endogenously, strong promoter and epitope tag sequences were introduced to the endogenous locus by CRISPR-mediated HDR repair. The resulting knock-in cells produced epitope-tagged proteins at milligram scale. Epitope-specific antibody resin was used to purify the intact endogenous protein from cells. (B) Precise donor integration by CRISPR-dependent HDR at the human *RELN* locus. Cas9 nuclease induced double strand breaks at the SP coding region in the *RELN* locus. The HDR donor contained a strong CMV promoter, the SP coding sequence with silence mutations in the sgRNA target site for prohibiting further cleavages after editing, a FLAG epitope tag, and two homology regions. HDR-mediated repair can precisely replace the original SP coding sequences with the epitope tag-fused protein expression cassette.

an extremely large extracellular signaling glycoprotein. This protein is encoded by the *RELN* gene, which is about 450 kb in length; the expressed mRNA is about 11.7 kb long.[3] The Reelin protein consists of 3460 amino acids and contains eight repeated epidermal growth factor (EGF) motifs.[4] Reelin has a role in neuronal migration and positioning during pre- and postnatal brain development and in synaptic plasticity in adult brains.[5,6] Reelin mutant mice, characterized by a "reeling" gait, exhibit disrupted cortical layer formation and abnormal morphology in structures, indicating a role for Reelin in brain development.[7−9] On the other hand, overexpression of Reelin in a mouse model of Alzheimer's disease was found to reverse cognitive impairment as well as ameliorate amyloid plaque formation in the cortex and hippocampus.[10] In addition, many studies have reported altered Reelin expression in neuropsychiatric illness and neurodegenerative disease, with abnormal levels found in the cortex, hippocampus, cerebellum, cerebrospinal fluid, and blood of patients.[8,11−14] Because Reelin may be implicated in the pathogenesis of a number of human brain diseases such as frontotemporal dementia, Alzheimer's disease, temporal lobe epilepsy, schizophrenia, bipolar disorder, and major depression, purification of intact Reelin protein is of biomedical interest. Although mouse Reelin cDNA was previously cloned and the full length of it has been purified in mammalian cells,[4,15] an intact version of the human Reelin protein has not yet been purified. To date, only a partial Reelin fragment has been purified from stably transfected Chinese hamster kidney (CHO) cells; it has been used for functional and structural studies of this protein.[16−19]

To overexpress and purify endogenous human Reelin proteins, we selected the HEK293E cell line as a protein production factory because of its relatively high transfecting and protein expression activities. We first designed six single guide RNAs (sgRNAs) that specifically target the endogenous signal peptide (SP) coding region of the *RELN* gene. After testing the gene editing efficacy of each sgRNA in HEK293E cells, we chose two sgRNAs (*i.e.*, sg4 and sg5) with higher activities (Supplementary Figure S1). We next established a CRISPR-mediated homology directed repair (HDR) method for precise DNA donor integration at the SP coding region of the *RELN* gene. The HDR donor plasmid was prepared to contain a strong promoter from cytomegalovirus (CMV) for overexpression, a FLAG epitope tag for purification, and the green fluorescent protein (*GFP*) gene linked to the hygromycin resistance (*hph*) gene for effective selection of knock-in cells (Figure 1B). The FLAG epitope tag, which was designed to link to the N terminus of the endogenous Reelin protein, will be exposed after post-translational processing of the SP. The HDR donor plasmid also contains two homology arm regions that are identical to 800-bp regions upstream and downstream of the CRISPR-Cas9 target site. Of note, we designed the main parts of the HDR donor construct so that they could be used in common for various secreted target genes, with the exception of the homology arm sequences, which vary according to the target gene.

We next simultaneously transfected three plasmids (Cas9 encoding, sgRNA4/5 encoding, and HDR donor) into HEK293E cells for gene engineering. Transfected cells were selected under hygromycin treatment and GFP-positive cells were further sorted by flow cytometry (Supplementary Figure S2). To confirm the expression of FLAG-tagged Reelin, we harvested the medium from each of the sorted bulk cell cultures, bulk-sg4 and bulk-sg5, and performed an immunoprecipitation assay using anti-FLAG M1 antibody resin followed by SDS-PAGE. In contrast to the harvested medium and the resin-unbound fraction, the immunoprecipitated fraction showed two clear bands over 250 kDa in size, which were both identified as human Reelin by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Supplementary Figure S3). Given this positive indication that intact Reelin proteins could indeed be purified from genetically engineered HEK293E cells, we further spread bulk populations of transfected cells and selected 21 single colonies. We confirmed that the 21 single cell-derived line contained a knock-in construct with a PCR assay involving a specific primer set that selectively amplifies DNAs between integrated HDR donor and flanking genomic sequences (Supplementary Figure S4). For each of the 21 clonal cell lines, we harvested

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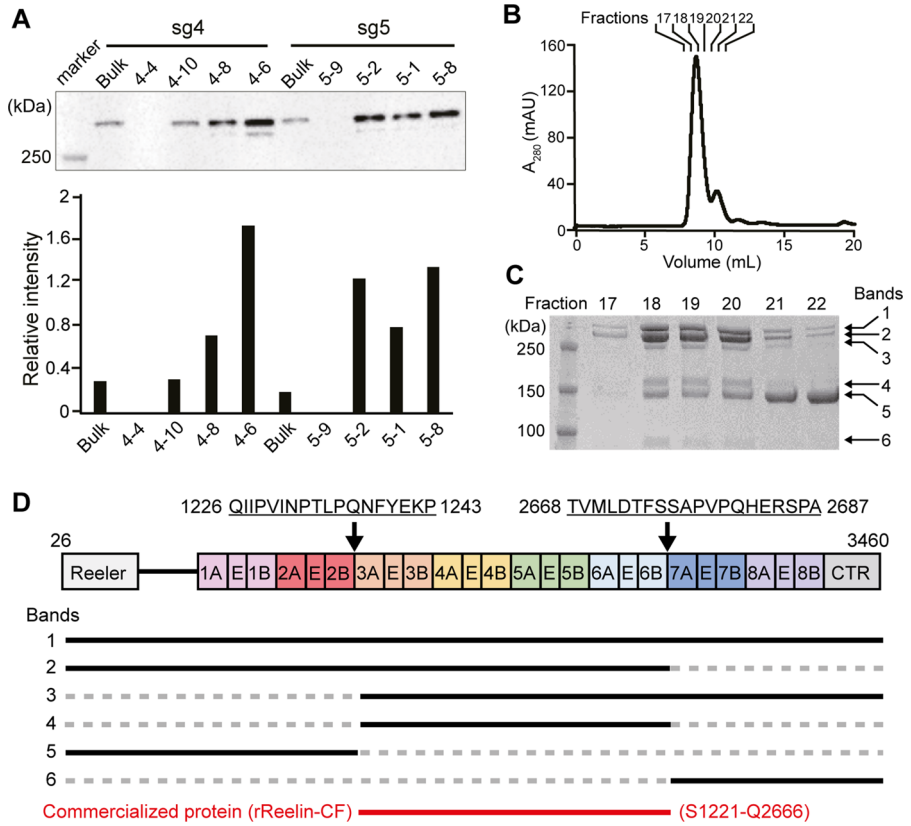

Figure 2. Identification and purification of full-length human Reelin and its derived fragments. (A) Overexpression of FLAG-tagged Reelin from its endogenous genomic locus. Two different sgRNAs (sg4 and sg5) were used to generate genome-edited, GFP-positive cells. Bulk cell populations were cultured and diluted to obtain single cell-derived colonies (Supplementary Figure S4). (Top) Samples of culture medium from the resulting clonal cell lines were subjected to Western blotting using the anti-FLAG M2 antibody to detect FLAG-tagged Reelin. (Bottom) The levels of Reelin produced by the different cell lines varied significantly. (B) Gel filtration chromatogram of purified Reelin. (C) Peak fractions from the gel filtration chromatography were analyzed by SDS-PAGE. The Image of complete SDS-PAGE gel is shown in Supplementary Figure S6. (D) (Top) Schematic of human Reelin domain architecture. Reelin has an N-terminal reeler domain and eight repeats of 300−350 amino acids. Each repeat contains two subrepeats, A (the BNR/Asp-box repeat) and B (the EGF-like domain), as well as an EGF motif between the subrepeats. The black arrows indicate the two major cleavage sites in Reelin, which were proved by the identification of the C-terminal peptides derived from the N-terminal and central fragments in the LC/MS-MS analysis (Supplementary Figure S7). (Bottom) The six solid black bars represent full-length Reelin and its derived fragments in the purified sample, which respectively correspond to the six bands in the SDS-PAGE gel in C. Bands 1 to 6 were directly identified by the LC-MS/MS analysis (Supplementary Figure S7). The red bar represents commercially available recombinant protein (rReelin-CF), which lacks both N- and C-terminal regions, used in this study.

the culture medium and performed Western blotting with the anti-FLAG M2 antibody. The blotting data showed that the expression level of FLAG-tagged Reelin varied substantially according to the cell line (Figure 2A and Supplementary Figure S5), possibly implying cell-to-cell variations in Reelin expression and/or secretion. Among bulk cells and clonal cells, we found that Clone #4−6 showed the highest Reelin protein expression level; thus, this clone was used for further experiments.

The Clone #4−6 line was cultured at large scale, after which the expressed Reelin proteins were purified from the harvested medium by anti-FLAG M1 antibody affinity chromatography and gel filtration chromatography. The resulting gel filtration chromatogram showed a major and a minor peak (Figure 2B), and the SDS-PAGE analysis of the peak fractions (fractions 17−22) identified six protein bands: three major (Band 1, 2, and 5 in Figure 2C and Supplementary Figure S6) and three minor protein bands (Bands 3, 4, and 6 in Figure 2C and Supplementary Figure S6). To determine the identities of the multiple bands, we performed LC-MS/MS analyses for Bands 1−6 (Supplementary Figure S7), and found that Band 1 was full-length Reelin and the other bands were truncated Reelin fragments. Previous studies have shown that *in vivo*, secreted Reelin undergoes proteolytic cleavage at two sites, located between the proline at position 1243 (Pro^{1243}) and the alanine at position 1244 (Ala^{1244}) and between the alanine at position 2687 (Ala^{2687}) and the asparagine at position 2688 (Asp^{2688}),[20,21] and that the resulting Reelin fragments have their own specific functions.[22,23] We also identified the precise Reelin cleavage sites in our purified protein sample based on the molecular weights of the C-terminal peptides derived from Bands 2 and 5 in the LC-MS/MS data (Figure 2D), confirming that the purified human Reelin in our study is processed at the same sites as seen in previous reports. Although Bands 3, 4, and 6 do not contain the N-terminal FLAG tag, they were copurified from gel filtration chromatography (Figure 2C,D). It seems that full-length Reelin (Band 1) and Reelin fragments with N-terminal FLAG tags (Bands 2 and 5) were initially bound to the FLAG M1 resin, but they underwent slow processing during purification to produce Band 3 from Band 1, Band 4 mainly from Band 2, and Band 6 mainly from Band 1.

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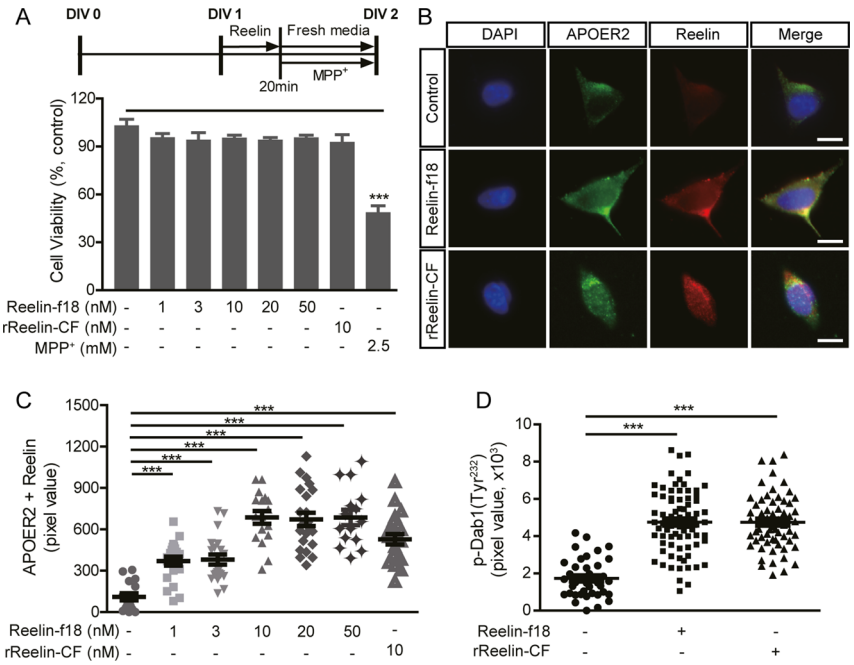

Figure 3. Assessment of the functionality of purified Reelin-f18. (A) (Top) Schematic of the experimental design. (Bottom) Cytotoxic effects of purified Reelin-f18 or rReelin-CF on SH-SY5Y cells, evaluated by measuring cell viability. Treatment with the neurotoxin MPP^+ (2.5 mM) was used as the positive control. Representative immunofluorescence images (B) and scatter graph (C) to assess Reelin-f18 (red, B, middle row) or commercial rReelin-CF (red, B, bottom row) colocalization with APOER2 (green) on the surface of SH-SY5Y cells. Scale bar: 10 μm. (D) Optical density measurements of phosphorylated Dab1(Tyr^{232})-positive cells following treatment with Reelin-f18 or rReelin-CF. (A) $n \geq 3$ for each group. (C) $n \geq 15$ for each group. (D) $n \geq 38$ for each group. ***$p < 0.001$. Statistical significance was analyzed by one-way ANOVA (Bonferroni *post hoc* test for multiple comparison).

After successful purification and identification of intact Reelin protein, we further investigated the functional activity and stability of purified Reelin in human neuroblastoma cells (SH-SY5Y). We purchased a central fragment of recombinant Reelin protein (rReelin-CF, hereafter) purified from CHO cells (RnD system, 8546-MR, S1221-Q2666) as a control and compared it with our purified Reelin protein, fraction #18 (Reelin-f18, hereafter), because Reelin-f18 contained the highest concentration of full-length Reelin. First, we measured cell viabilities after treating SH-SY5Y cells with either Reelin-f18 or rReelin-CF for 20 min.[10,14] We found that Reelin-f18 caused no significant cytotoxicity compared to rReelin-CF at the same concentration (Figure 3A). It has been reported that binding of Reelin to the apolipoprotein E receptor 2 (APOER2) and the very low density lipoprotein receptor in target cells can trigger a signaling cascade, resulting in phosphorylation of the Dab1 protein.[24] Hence, we next compared Reelin-f18 and rReelin colocalization with endogenous APOER2,[14,25] and their effects on the levels of phosphorylated Dab1 protein, by immunohistochemistry. Our results showed that Reelin-f18 clearly colocalized with APOER2 in SH-SY5Y cells (Figure 3B,C) and that exogenous treatment of the cells with the protein increased the level of phospho-Dab1 (Figure 3D and Supplementary Figure S8). These findings with Reelin-f18 are in line with previous results showing that rReelin-CF is biologically active.[17]

In this study, as a model of endogenous protein overexpression and purification from human cells, we successfully purified full-length Reelin, an extremely large, secreted protein containing repetitive modular structures. Naturally, human *RELN* mRNA has a very limited expression pattern; it is only expressed in certain types of neurons such as Cajal-Retzius cells and cerebellar granule cells in the pre- and postnatal brain and in GABAergic interneurons in the adult brain.[26−31] In addition, the promoter and transcription start site of the *RELN* gene contain abundant CpG dinucleotides,[32] implying that the spatiotemporal expression of *RELN* might be regulated by DNA methylation and that Reelin proteins would rarely be expressed in HEK293E cells.[32,33] Despite these constraints, however, endogenous human Reelin proteins were sufficiently expressed, *via* direct genetic engineering, for purification from HEK293E cells, indicating that our engineering system should be generally applicable to many human genes. The ability to directly purify intact proteins from human cells should aid broad research areas, including *in vitro* functional studies, protein structure analysis, protein engineering, and production of therapeutic proteins. Previously, to our knowledge, only one study has reported the purification of endogenous, tagged proteins from human cells,[34] but this approach is applicable only for proteins that are naturally highly expressed in cells. In summary, our method might be referred as a type of "direct molecular cloning in the human genome". However, our approach is not limited to human cells, but could be applied in other mammalian or plant cells, enabling direct purification of intact proteins from a wide variety of organisms.

METHODS

sgRNA Target Selection and Measurement of Editing Efficiency. Cas9 sgRNA targets within the SP coding sequence of the *RELN* gene were selected using Cas-OFFinder[35] (www.rgenome.net/cas-offinder/) and Cas-Designer[36] (www.rgenome.net/cas-designer/), web-based softwares. Each target sequence was cloned into an sgRNA expression plasmid, pRG2 (addgene 104174). The sgRNA

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expression plasmids were then transfected with a Cas9 expression plasmid, p3S-Cas9HN (addgene 104171), into HEK293E (HEK293 c18, ATCC-10852) cells. After 72 h of transfection, genomic DNA was extracted from cells using a Nucleospin tissue kit (Macherey-Nagel) according to the manufacturer's directions. DNA segments that encompassed the Cas9 target sites were amplified with Phusion polymerase (Thermo Scientific) using appropriate pairs of primers. PCR amplicons were subjected to paired-end read sequencing using Illumina MiniSeq The resulting next generation sequencing data were analyzed using Cas-Analyzer[37] (www.rgenome.net/cas-analyzer/).

HDR Donor Plasmid Construction. To construct the HDR donor plasmid, all fragments were amplified by PCR using Phusion polymerase (Thermo Scientific). Homology arm sequences were amplified from genomic DNA from HEK293E cells. The CMV promoter, hygromycin resistance gene, and GFP expression cassette were amplified from the pAAY-sfGFP plasmid. SP coding and FLAG tag sequences were obtained by PCR extension of annealed oligonucleotides (Macrogen). All fragments were assembled using isothermal assembly.

Generation of Knock-in Cell Lines. HEK293E (HEK293 c18, ATCC-10852) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humidified incubator at 37 °C with 5% CO_2. The Cas9 expression plasmid (750 ng), sgRNA expression plasmid (250 ng), and HDR donor plasmid (500 ng) were transfected into the cells using lipofectamine 2000 (Invitrogen). After culture for 3 days, transfected cells were treated with 50 μg/mL Hygromycin B (Invitrogen) for 2 weeks to select knock-in cells. Hygromycin resistant cells were then sorted by the presence of a GFP signal using a FACS Aria III.[33] GFP-positive, knock-in cells were spread in a 96-well plate for isolation of single cell-derived clones. The knock-in status of each clonal cell line was confirmed by knock-in specific PCR.

Protein Purification. The single cell-derived knock-in line that showed the highest Reelin expression level was cultured in DMEM with high glucose (Welgene, LM001−01), 10% fetal calf serum (FCS), and 4 mM L-glutamine. Confluent cells in 10 plates (100 mm in diameter, with 12 mL medium per plate) were subcultured into 40 plates (150 mm in diameter, with 30 mL medium per plate). After 120 h, the medium (∼1 L) was harvested and filtered through a nominal 0.22 μm pore-size membrane (Millipore, #SCGPU05RE). For the binding of N-terminal FLAG-tagged Reelin to anti-FLAG M1 resin (Sigma-Aldrich, #A4596), 5 mL of 1 M $CaCl_2$ solution was added to the filtered medium. Five mL of anti-FLAG M1 resin was packed into an open column (Bio-Rad, Econo-Column Chromatography Columns, 2.5 × 10 cm, #7372512) with a flow adaptor (Bio-Rad, #7380017). The medium was loaded into the column at a rate of 1 mL/min for ∼17 h. The resin was washed with a buffer (50 mL) containing 20 mM Tris pH 7.5, 250 mM NaCl, and 1 mM $CaCl_2$. For protein elution, 5 mL elution buffer containing 20 mM Tris pH 7.5, 250 mM NaCl, 1 mM $CaCl_2$, and 50 μg/mL FLAG peptides (Sigma-Aldrich, #F3290) was flowed into the column, and the resin was incubated with gentle mixing for 1 h. Fifteen mL elution buffer was additionally flowed into the column, and the eluted sample containing ∼2.1 mg protein was collected. The protein sample (15 mL) was concentrated by reducing its volume to <500 μL using a centrifugal concentrator (Millipore, 100 kD MWCO Amicon Ultra-15, UFC910024) and loaded into a gel filtration column (GE healthcare, Superdex200 increase 10/300). The peak fraction was cryo-cooled in liquid nitrogen and stored at −80 °C. The final yield was ∼0.77 mg per 1.2 L culture. Protein concentration was measured by the Bradford assay (Thermo Scientific, #23236).

Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS) Analysis. Excised gel bands were first destained and dehydrated in 50% ACN solution of 25 mM ammonium bicarbonate (ABC) buffer and then followed by in-gel reduction of disulfide bonds with 10 mM dithiothreitol in 25 mM ABC for 30 min at 56 °C. Subsequently the reduced cysteine residues were alkylated with 40 mM iodoacetamide in 25 mM ABC for 1 h at 37 °C in the dark. After three times washing out the excess reagents with 25 mM ABC, the resulting sample was digested by MS-grade trypsin (Thermo Scientific) at ratio of 1:50 (w/w) for overnight at 37 °C. The digested peptides were subjected to C18-SPE clean up using 10 μL of ZipTip (Millipore). The final peptides reconstituted with 25 mM ABC were analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) coupled with Dionex Ultimate 3000 RSLCnano system (Thermo Scientific), which was operated at a flow rate of 350 nL/min over 1 h with linear gradient ranging from 95% solvent A (H_2O with 0.1% formic acid) to 40% of solvent B (acetonitrile with 0.1% formic acid). Analytical capillary column (100 cm × 75 μm i.d.) and trap column (2 cm × 150 μm i.d.) were packed in-house with 3 μm Jupiter C18 particles (Phenomenex). The LC-MS/MS spectra were processed using MaxQuant program to obtain the intensity information for each peptide.[38]

SH-SY5Y Cell Culture. SH-SY5Y (human neuroblastoma) cells were maintained in DMEM/F12 medium supplemented with 10% FBS and 1% P/S (maintenance medium) in a humidified incubator at 37 °C with 5% CO_2. The culture medium was replaced every 2−3 days. When the cells reached 90% confluence, they were transferred to 48-well culture plates or to poly-D-lysine (0.1 mg/mL, Sigma) coated coverslip and incubated for an overnight. For experiments involving Reelin treatment, the culture medium was changed to high-glucose DMEM (with 10% FBS and 1% P/S) containing Reelin at different concentrations, after which the cultures were incubated for 20 min. For experiments involving exposure to the neurotoxin 1-Methyl-4-phenylpyridinium (MPP^+) iodide, cells were treated with 2.5 mM MPP^+, (Sigma) in high-glucose DMEM (with 10% FBS and 1% P/S) for 24 h.[39,40]

Reelin-f18 Cytotoxicity Assay. To assess whether the Reelin protein has a cytotoxic effect, SH-SY5Y cells were seeded in 48-well culture plates. Cell viability was evaluated with an ADAM-MC automated cell counter (Digital Bio Technology Co., Ltd., NanoEnTek Inc., Seoul, Korea) as described in the instruction manual. Briefly, after cells were exposed to Reelin or MPP^+, cells were washed with Dulbecco's phosphate-buffered saline, collected, centrifuged, and then resuspended in high-glucose DMEM (containing 10% FBS and 1% P/S). The cell suspension was then mixed with Accustain solution T or N (Digital Bio, Seoul, Korea) to measure the total or nonviable cells, respectively. The numbers of total or nonviable cells were automatically calculated by the ADAM-MC software.

Reelin-f18 Activity and Cell Surface Labeling Assays. To investigate whether purified Reelin-f18 is biologically active, we tested its effect on SH-SY5Y cells, which express APOER2. The cells were incubated with different concentrations (1−50 nM) of purified Reelin-f18 (FLAG tagged) or

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10 nM rReelin-CF (S1221-Q1226, 6xHis tagged, R&D Systems, 8546-MR) in maintenance medium for 20 min at 37 °C with 5% CO_2.[10,14] Cells were then washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at 4 °C, rinsed, permeabilized with 0.2% Triton X-100 in PBS containing 1% bovine serum albumin for 10 min at room temperature, and then incubated with antiphosphorylated Dab1 antibody (Sigma) for 1 h at room temperature. Cells were then washed and incubated in avidin−biotin complex solution (Vector Laboratories) for 1 h at room temperature. After washing in PBS, cells were incubated in 3−3′-diaminobenzidine (Sigma) in 0.1 M phosphate buffer that contained 0.003% H_2O_2. Cells were washed in PBS and then, viewed under a bright field microscope.

To detect Reelin-f18 or rReelin-CF on the cell surface, cells were respectively incubated with goat anti-FLAG M2 antibody (Abcam) or mouse anti-6xHis antibody (Abcam) in 0.5% bovine albumin contained blocking solution for 1 h at room temperature. For detection of the anti-FLAG and anti-6xHis antibodies, cells were then respectively incubated with antigoat or antimouse Alexa Flour 594 antibodies (Invitrogen) in blocking solution for 1 h at room temperature. To detect intracellular APOER2, cells were then washed, permeabilized, washed a second time, and incubated with rabbit anti-APOER2 C-terminal labeling antibody (Abcam) in blocking solution for 1 h at room temperature. To detect the anti-APOER2 antibody, cells were incubated with antirabbit Alexa Flour 488 antibody (Invitrogen) in blocking solution for 1 h at room temperature, viewed under Axio Imager M2 microscope. Image data were analyzed with Multi Gauge software (Fuji photo firm, version 3.0, Tokyo, Japan) for optical density quantification and with ImageJ (U.S. National Institutes of Health) with a colocalization plugin to quantify immunofluorescence and a color deconvolution plugin to quantify the chromogenic signal intensity in the image.

Statistics. All data are expressed as mean ± standard error of the mean (SEM). All experiments were repeated three times independently. Statistical significance ($P < 0.05$ for all analyses) was evaluated by one-way ANOVA followed by Bonferroni multiple comparison test.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00090.

Supplementary Figures: Design of sgRNAs and determination of gene editing efficiency; Knock-in cell sorting by flow cytometry; Identification of proteins immunoprecipitated with anti-FLAG antibodies from the medium harvested from bulk cell cultures; Confirmation of knock-in cell lines; Expression of FLAG-tagged Reelin protein in single cell-derived clonal cell lines; Image of complete SDS-PAGE gel that is shown in part in Figure 2C; LC-MS/MS analysis of purified Reelin proteins; Reelin-induced Dab1 phosphorylation (PDF)

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Author Contributions

¶J.Y., E.C., and Y.-G.C. contributed equally to this work. S.B. and J.-S.W. conceived this project; J.Y., E.C., Y.-G.C., and Y.K.J. performed the experiments; Y.N. and J.-S.K. performed LC-MS/MS analyses. J.Y., E.C., S.-R.C., J.-S.W., and S.B. wrote the manuscript with the approval of all other authors. S.-R.C., J.-S.W., and S.B. supervised the research.

Notes

The authors declare the following competing financial interest(s): S.B., J.-S.W., and J.Y. have filed a patent application based on this work.

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■ REFERENCES

(1) Cohen, S. N., Chang, A. C. Y., Boyer, H. W., and Helling, R. B. (1973) Construction of Biologically Functional Bacterial Plasmids *In Vitro*. *Proc. Natl. Acad. Sci. U. S. A.* 70 (11), 3240−3244.

(2) Jackson, D. A., Symons, R. H., and Berg, P. (1972) Biochemical Method for Inserting New Genetic Information into DNA of Simian Virus 40: Circular SV40 DNA Molecules Containing Lambda Phage

Genes and the Galactose Operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A. 69* (10), 2904−2909.
(3) Royaux, I., Lambert de Rouvroit, C., D'Arcangelo, G., Demirov, D., and Goffinet, A. M. (1997) Genomic organization of the mouse reelin gene. *Genomics 46* (2), 240−250.
(4) D'Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. (1997) Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J. Neurosci. 17* (1), 23−31.
(5) Segarra, M., Aburto, M. R., Cop, F., Llao-Cid, C., Hartl, R., Damm, M., Bethani, I., Parrilla, M., Husainie, D., Schanzer, A., Schlierbach, H., Acker, T., Mohr, L., Torres-Masjoan, L., Ritter, M., and Acker-Palmer, A. (2018) Endothelial Dab1 signaling orchestrates neuro-glia-vessel communication in the central nervous system. *Science 361* (6404), No. eaao2861.
(6) D'Arcangelo, G. G., Miao, G., Chen, S.-C., Scares, H. D., Morgan, J. I., and Curran, T. (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature 374* (6524), 719−723.
(7) Assadi, A. H., Zhang, G., Beffert, U., McNeil, R. S., Renfro, A. L., Niu, S., Quattrocchi, C. C., Antalffy, B. A., Sheldon, M., Armstrong, D. D., Wynshaw-Boris, A., Herz, J., D'Arcangelo, G., and Clark, G. D. (2003) Interaction of reelin signaling and Lis1 in brain development. *Nat. Genet. 35*, 270−276.
(8) Janusonis, S., Fau, G. V., and Rakic, P. (2004) Early serotonergic projections to Cajal-Retzius cells: relevance for cortical development. *J. Neurosci. 24* (7), 1652−1659.
(9) Smalheiser, N. R., Costa, E., Guidotti, A., Impagnatiello, F., Auta, J., Lacor, P., Kriho, V., and Pappas, G. D. (2000) Expression of reelin in adult mammalian blood, liver, pituitary pars intermedia, and adrenal chromaffin cells. *Proc. Natl. Acad. Sci. U. S. A. 97* (3), 1281−1286.
(10) Pujadas, L., Rossi, D., Andres, R., Teixeira, C. M., Serra-Vidal, B., Parcerisas, A., Maldonado, R., Giralt, E., Carulla, N., and Soriano, E. (2014) Reelin delays amyloid-beta fibril formation and rescues cognitive deficits in a model of Alzheimer's disease. *Nat. Commun. 5*, 3443.
(11) Impagnatiello, F., Guidotti, A. R., Pesold, C., Dwivedi, Y., Caruncho, H., Pisu, M. G., Uzunov, D. P., Smalheiser, N. R., Davis, J. M., Pandey, G. N., Pappas, G. D., Tueting, P., Sharma, R. P., and Costa, E. (1998) A decrease of reelin expression as a putative vulnerability factor in schizophrenia. *Proc. Natl. Acad. Sci. U. S. A. 95* (26), 15718−15723.
(12) Maloku, E., Covelo, I. R., Hanbauer, I., Guidotti, A., Kadriu, B., Hu, Q., Davis, J. M., and Costa, E. (2010) Lower number of cerebellar Purkinje neurons in psychosis is associated with reduced reelin expression. *Proc. Natl. Acad. Sci. U. S. A. 107* (9), 4407−4411.
(13) Saez-Valero, J., Costell, M., Sjogren, M., Andreasen, N., Blennow, K., and Luque, J. M. (2003) Altered levels of cerebrospinal fluid reelin in frontotemporal dementia and Alzheimer's disease. *J. Neurosci. Res. 72* (1), 132−136.
(14) Cuchillo-Ibanez, I., Mata-Balaguer, T., Balmaceda, V., Arranz, J. J., Nimpf, J., and Saez-Valero, J. (2016) The beta-amyloid peptide compromises Reelin signaling in Alzheimer's disease. *Sci. Rep. 6*, 31646.
(15) Yasui, N., Kitago, Y., Beppu, A., Kohno, T., Morishita, S., Gomi, H., Nagae, M., Hattori, M., and Takagi, J. (2011) Functional importance of covalent homodimer of reelin protein linked *via* its central region. *J. Biol. Chem. 286* (40), 35247−35256.
(16) Yasui, N., Nogi, T., and Takagi, J. (2010) Structural Basis for Specific Recognition of Reelin by Its Receptors. *Structure 18* (3), 320−331.
(17) Roberts, B. L., Bennett, B. J., Bennett, C. M., Carroll, J. M., Dalboge, L. S., Hall, C., Hassouneh, W., Heppner, K. M., Kirigiti, M. A., Lindsley, S. R., Tennant, K. G., True, C. A., Whittle, A., Wolf, A. C., Roberts, C. T., Jr., Tang-Christensen, M., Sleeman, M. W., Cowley, M. A., Grove, K. L., and Kievit, P. (2019) Reelin is modulated by diet-induced obesity and has direct actions on arcuate proopiomelanocortin neurons. *Mol. Metab. 26*, 18−29.
(18) Kim, M., Jeong, Y., and Chang, Y.-C. (2015) Extracellular matrix protein reelin regulate dendritic spine density through CaMKII*β*. *Neurosci. Lett. 599*, 97−101.
(19) Do, H. T., Bruelle, C., Tselykh, T., Jalonen, P., Korhonen, L., and Lindholm, D. (2013) Reciprocal regulation of very low density lipoprotein receptors (VLDLRs) in neurons by brain-derived neurotrophic factor (BDNF) and Reelin: involvement of the E3 ligase Mylip/Idol. *J. Biol. Chem. 288* (41), 29613−29620.
(20) Jossin, Y., Ignatova, N., Hiesberger, T., Herz, J., Lambert de Rouvroit, C., and Goffinet, A. M. (2004) The central fragment of Reelin, generated by proteolytic processing in vivo, is critical to its function during cortical plate development. *J. Neurosci. 24* (2), 514−521.
(21) Lambert de Rouvroit, C., de Bergeyck, V., Cortvrindt, C., Bar, I., Eeckhout, Y., and Goffinet, A. M. (1999) Reelin, the Extracellular Matrix Protein Deficient in Reeler Mutant Mice, Is Processed by a Metalloproteinase. *Exp. Neurol. 156* (1), 214−217.
(22) Lee, G. H., Chhangawala, Z., von Daake, S., Savas, J. N., Yates, J. R., 3rd, Comoletti, D., and D'Arcangelo, G. (2014) Reelin induces Erk1/2 signaling in cortical neurons through a non-canonical pathway. *J. Biol. Chem. 289* (29), 20307−20317.
(23) Lutz, D., Sharaf, A., Drexler, D., Kataria, H., Wolters-Eisfeld, G., Brunne, B., Kleene, R., Loers, G., Frotscher, M., and Schachner, M. (2017) Proteolytic cleavage of transmembrane cell adhesion molecule L1 by extracellular matrix molecule Reelin is important for mouse brain development. *Sci. Rep. 7* (1), 15268.
(24) Howell, B. W., Herrick, T. M., and Cooper, J. A. (1999) Reelin-induced tyrosine phosphorylation of disabled 1 during neuronal positioning. *Genes Dev. 13* (6), 643−648.
(25) Balmaceda, V., Cuchillo-Ibanez, I., Pujadas, L., Garcia-Ayllon, M. S., Saura, C. A., Nimpf, J., Soriano, E., and Saez-Valero, J. (2014) ApoER2 processing by presenilin-1 modulates reelin expression. *FASEB J. 28* (4), 1543−1554.
(26) Arnaud, L., Ballif, B. A., and Cooper, J. A. (2003) Regulation of protein tyrosine kinase signaling by substrate degradation during brain development. *Mol. Cell. Biol. 23* (24), 9293−9302.
(27) Pahle, J., Muhia, M., Wagener, R. J., Tippmann, A., Bock, H. H., Graw, J., Herz, J., Staiger, J. F., Drakew, A., Kneussel, M., Rune, G. M., Frotscher, M., and Brunne, B. (2020) Selective Inactivation of Reelin in Inhibitory Interneurons Leads to Subtle Changes in the Dentate Gyrus But Leaves Cortical Layering and Behavior Unaffected. *Cerebral Cortex 30* (3), 1688−1707.
(28) Del Rio, J. A., Heimrich, B., Borrell, V., Forster, E., Drakew, A., Alcantara, S., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., Derer, P., Frotscher, M., and Soriano, E. (1997) A role for Cajal-Retzius cells and reelin in the development of hippocampal connections. *Nature 385*, 70−74.
(29) Campo, C. G., Sinagra, M., Verrier, D., Manzoni, O. J., and Chavis, P. (2009) Reelin secreted by GABAergic neurons regulates glutamate receptor homeostasis. *PLoS One 4* (5), No. e5505.
(30) Pesold, C., Impagnatiello, F., Pisu, M. G., Uzunov, D. P., Costa, E., Guidotti, A., and Caruncho, H. J. (1998) Reelin is preferentially expressed in neurons synthesizing gamma-aminobutyric acid in cortex and hippocampus of adult rats. *Proc. Natl. Acad. Sci. U. S. A. 95* (6), 3221−3226.
(31) Tissir, F., and Goffinet, A. M. (2003) Reelin and brain development. *Nat. Rev. Neurosci. 4* (6), 496−505.
(32) Chen, Y., Sharma, R. P., Costa, R. H., Costa, E., and Grayson, D. R. (2002) On the epigenetic regulation of the human reelin promoter. *Nucleic acids research 30* (13), 2930−2939.
(33) Abdolmaleky, H. M., Cheng, K. H., Russo, A., Smith, C. L., Faraone, S. V., Wilcox, M., Shafa, R., Glatt, S. J., Nguyen, G., Ponte, J. F., Thiagalingam, S., and Tsuang, M. T. (2005) Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: a preliminary report. *Am. J. Med. Genet., Part B 134b* (1), 60−66.
(34) Lin, D. W., Chung, B. P., Huang, J. W., Wang, X., Huang, L., and Kaiser, P. (2019) Microhomology-based CRISPR tagging tools for protein tracking, purification, and depletion. *J. Biol. Chem. 294* (28), 10877−10885.

https://dx.doi.org/10.1021/acssynbio.0c00090
ACS Synth. Biol. 2020, 9, 1591−1598

(35) Bae, S., Park, J., and Kim, J. S. (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics 30* (10), 1473−1475.
(36) Park, J., Bae, S., and Kim, J.-S. (2015) Cas-Designer: a web-based tool for choice of CRISPR-Cas9 target sites. *Bioinformatics 31* (24), 4014−4016.
(37) Park, J., Lim, K., Kim, J. S., and Bae, S. (2017) Cas-analyzer: an online tool for assessing genome editing results using NGS data. *Bioinformatics 33* (2), 286−288.
(38) Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol. 26*, 1367−1372.
(39) Zhai, A., Zhu, X., Wang, X., Chen, R., and Wang, H. (2013) Secalonic acid A protects dopaminergic neurons from 1-methyl-4-phenylpyridinium (MPP(+))-induced cell death *via* the mitochondrial apoptotic pathway. *Eur. J. Pharmacol. 713* (1−3), 58−67.
(40) Ye, Q., Chen, C., Si, E., Cai, Y., Wang, J., Huang, W., Li, D., Wang, Y., and Chen, X. (2017) Mitochondrial Effects of PGC-1alpha Silencing in MPP(+) Treated Human SH-SY5Y Neuroblastoma Cells. *Front. Mol. Neurosci. 10*, 164.

https://dx.doi.org/10.1021/acssynbio.0c00090
ACS Synth. Biol. 2020, 9, 1591−1598